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EDITORIAL

This issue of the *Journal of the Serbian Chemical Society* contains selected articles from The Belgrade International Food Conference held in Belgrade, Serbia on 26–28 October, 2012. The conference was organized by the Center for Food Science and Molecular Biotechnology of the Faculty of Chemistry, University of Belgrade within an EU-funded project FCUB ERA "Reinforcement of the Faculty of Chemistry, University of Belgrade, towards becoming a center of excellence in food science and molecular biotechnology in the region of the western Balkan".

The main theme of the conference was: food, health and well being. The Conference was organized in eleven sessions and two poster sessions: Enzymes in food processing, Wastes and biomass valorization, Supplements, micronutrients and food additives, Food antioxidants, Nutrition science and bioactive compounds, New approaches to food analysis, Food allergens, Nutrition and immunology, Molecular biotechnology for the benefit of consumers, New functional foods, and Health effects of food.

The main goal of The Belgrade International Food Conference was to offer an inter-disciplinary arena of discussion and opinion-sharing for scientists dealing with different aspects of Food Science, to present a broad scope of multidisciplinary approaches to the field and to create a novel scientific network with a special focus on the promotion of young scientists. The scientific program included 20 lectures, 16 oral presentations and 57 poster presentations. In total, 120 participants from 17 countries attended the workshop representing 37 different institutions. The highest number of foreign participants came from Romania, Germany, Greece, France and Tunisia.

The Guest Editors wish to sincerely thanks all contributors to this special issue and the expert reviewers for their efforts to help compile a representative collection of contemporary topics in this multidisciplinary field; all the invited speakers, participants, and members of the Scientific and Organizing Committees for their contribution to the successful organization of the conference. The Guest Editors wish to express their profound gratitude to the Editor in Chief Branislav Nikolic, the Biochemistry and Biotechnology Sub Editor Olgica Nedic and the Technical Editors of the *Journal of the Serbian Chemical Society* for their support in the process of compiling this special issue devoted to the promotion of Food Science and Food Chemistry in particular.

Guest Editors
Tanja Ćirković Veličković
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Survey

SURVEY

Authentication of food allergens

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(Received 22 October 2012, revised 2 January 2013)

Abstract: Pure allergen batches are required for precise personalised diagnosis of food allergies. Furthermore, they can be used to develop sensitive allergen detection assays in foods and are valuable tools to develop novel immune-therapies. However, these reagents have to be well characterised and have to meet certain quality criteria. Within the EU-Project EuroPrevall, the concept of an allergen library comprising the most important food allergens from animal- and plant-derived foods was developed together with a catalogue of physico-chemical and immunological properties that had to be investigated. In close cooperation, partners from academia and the biotech industry applied well-established laboratory techniques as well as novel high throughput assays to analyse the most important features of the final protein batches. It is expected that this proof of concept will contribute to improved authentication of allergens for both routine application in allergy diagnosis and treatment and risk assessment in food production.

Keywords: food allergens; IgE; diagnosis.

CONTENTS

1. INTRODUCTION

Allergies are regarded as the epidemic of the present century affecting a steadily increasing number of people. It is usually harmless, non-toxic proteins that induce an immune response in predisposed individuals, evoking symptoms that range from mild and unpleasant local reactions to generalized and even life threatening conditions.

Up to now, no immunotherapy for food allergies is available; therefore avoidance of the incriminating food source is the method of choice.

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Great efforts have been undertaken to identify and characterise non-toxic food proteins that induce an IgE-mediated allergic response in patients. Many hundreds of allergens from animal- or plant-derived food have been identified in the last 2 decades. The information on these proteins, regarding their physicochemical properties and their allergenic relevance, including cross reactivities, has been entered into allergen databases. Of these, the IUIS allergen database (http://www.allergen.org/) provides the systematic nomenclature for allergens and their respective allergen designations. Entries for new allergens are only accepted upon approval of the allergen nomenclature subcommittee. Based on these datasets, it became clear that proteins with allergenic activity are restricted to a limited number of protein families and are not randomly distributed.[1] Despite this, it remains to be clarified what intrinsic factors contribute to the allergenic activity of certain proteins.

2. ALLERGEN PRODUCTION AND AUTHENTICATION

Purified food allergens are applied for *in vitro* diagnosis (component resolved diagnosis, CRD) and as reference materials for the quantification of the respective allergen in foods.[2] Furthermore, they are relevant tools to study the function–structure relationship of food allergens and the impact of food processing and the food matrix on the allergenicity of individual allergens. Finally, these well-characterised molecules are used to develop novel vaccination strategies for food allergies.

For each of these applications, a quality catalogue for purified food allergens has to be developed to harmonise the purification strategies and to enable a correct analysis of the obtained data.

Within the EU-funded project EuroPrevall, partners from academia and the biotech industry worked together to establish a food allergen library comprising the most important allergens from animal and plant derived food sources (Table I).[3] Purified allergens from cow's and goat's milk, hen's egg, fish, shrimp, fruits from the Rosaceae family (apple, peach), hazelnut, peanut and celery were included.[4] The majority of these are already in use for component resolved diagnosis and others are not yet included. These well-defined allergens were used to compare conventional *in vitro* diagnosis with CRD, to set up *in vitro* models of food processing and to develop novel allergen detection assays in foods.

The individual allergens were purified either from natural sources or produced as recombinant proteins. Prior to inclusion into the allergen library, a purified allergen had to meet several quality criteria. As the most important quality criteria, purity and identity, protein folding, post-translational modifications and immunochemical properties were identified.[5] The most important quality parameters and the analytical applied methods are summarised in Table II.

Individual allergens can occur as a single isoform or as a range of highly similar proteins with varying IgE binding activity. In addition, the abundance of a given allergen may influence the decision of whether to produce the protein in a heterologous expression system or to purify the single protein or a mixture of isoforms from natural sources. For both approaches, purity and verification of the correct sequence are prerequisites for further applications. As a routine method, 1D-gel-electrophoresis (SDS-PAGE) was applied and only protein batches with purity > 95 % were further analysed. Usually a combination of methods was used to assess purity, *i.e.*, HPLC and capillary electrophoresis. For natural allergens comprising a range of isoforms, 2D-gel electrophoresis was performed to identify the number of isoforms. To verify the correct *N*-terminal amino acid sequence, Edman degradation and mass spectrometry were performed to verify the correct mass data.

TABLE I. EuroPrevall Allergen library. The most important food allergens from animal and plant food sources were identified and either purified from natural sources or produced as recombinant proteins

Source		Protein family/functional properties	IUIS Allergen designation[a]	Origin
		Animal food allergens		
Cow's milk	*Bos domestica*	α-Lactalbumin	Bos d 4[b]	Cow's milk (raw)
		β-Lactoglobulin	Bos d 5[b]	Cow's milk (raw)
		Total casein	Bos d 8[b]	Cow's milk (raw)
Goat's milk	*Caprinus domesticus*	Total casein		Goat's milk (raw)
Hen's egg	*Gallus domesticus*	Ovomucoid	Gal d 1[b]	Hen's egg white
		Ovalbumin	Gal d 2[b]	Hen's egg white
		Ovotransferrin	Gal d 3[b]	Hen's egg white
		Lysozyme	Gal d 4	Hen's egg white
		Serum albumin	Gal d 5[b]	Hen's egg yolk
Carp	*Cyprinus carpio*	Parvalbumin	Cyp c 1[b]	Recombinant (*Escherichia coli*)
Codfish	*Gadus morhua*	Parvalbumin	Gad m 1[b]	Codfish muscle and recombinant (*E. coli*)
Shrimp	*Penaeus aztecus*	Tropomyosin	Pen a 1[b]	Recombinant (*E. coli*)
		Plant food allergens		
Apple	*Malus domestica*	Bet v 1 homologue, PR-10	Mal d 1[b]	Recombinant (*E. coli*)
		Thaumatin-like protein	Mal d 2	Apple fruit
		Non-specific lipid transfer protein	Mal d 3	Apple peel
		Profilin	Mal d 4	Recombinant (*E. coli*)

TABLE I. Continued

Source		Protein family/functional properties	IUIS Allergen designation[a]	Origin
		Plant food allergens		
Peach	*Prunus persica*	PR-10	Pru p 1[b]	Recombinant (*E. coli*)
		Non specific lipid transfer protein	Pru p 3[b]	Peach peel
Hazelnut	*Corylus avellana*	PR-10	Cor a 1[b]	Recombinant (*E. coli*)
Hazelnut	*Corylus avellana*	Profilin	Cor a 2	Recombinant (*E. coli*)
		Non-specific lipid transfer protein	Cor a 8[b]	Recombinant *(Pichia pastoris)*
		11S legumin, seed storage globulin	Cor a 9[b]	Hazelnuts
		7S vicillin, seed storage globulin	Cor a 11	Hazelnuts
Peanut	*Arachis hypogae*	7S seed storage globulin	Ara h 1[b]	Peanuts
		2 S albumins	Ara h 2, 6[b]	Peanuts
		11S seed storage globulin	Ara h 3[b]	Peanuts
		PR-10	Ara h 8[b]	Recombinant (*E. coli*)
Celeriac	*Apium graveolens*	PR-10	Api g 1[b]	Recombinant (*E. coli*)
		Profilin	Api g 4	Recombinant (*E. coli*)
		FAD-containing oxidase	Api g 5	Celeriac

[a]Allergen designations as listed in the IUIS Allergen Nomenclature database (www.allergen.org); [b]Allergens already used in routine component resolved diagnosis in either ImmunoCAP or ISAC chip format

TABLE II. Parameters and key methods used for the authentication of purified food allergens

Sequence verification
Matrix assisted laser adsorption MALDI (time of flight (TOF), quadrupole (Q)), *N*-terminal amino acid sequencing
Isoforms
Two dimensional polyacrylamide gel electrophoresis (2D-PAGE)
Folding/structure
Far-UV circular dichroism spectroscopy (CD), 1-D nuclear magnetic resonance spectroscopy (1D-NMR)
Purity/glycosylation/proteolysis
1-D Gel electrophoresis (SDS-PAGE), size exclusion chromatography, electrospray mass spectrometry (ES-MS), capillary electrophoresis
Biological function
Enzymatic activity, ligand binding assay
Immunological activity
IgE-Immunoblot, enzyme linked immunosorbent assay (ELISA), radioallergosorbent assay (RAST), inhibition assay, histamine release assay

Analysis of the 3D-structure of the final purified allergen is necessary, since it affects the immunochemical properties of individual allergens. For *in vitro* diagnosis application, the structural determinants of the purified allergen should be equivalent to the protein present in its natural matrix. Within the EuroPrevall project, far UV circular dichroism was routinely applied to assess the overall secondary structure, and the presence of an alpha helical, a beta-sheet and unordered structures. Furthermore, 1D-nuclear magnetic resonance spectroscopy of the allergens was applied to provide information on the tertiary structure of allergen, discriminating between a rigid 3D-structure, a mobile flexible structure and proteins with features of both, rigid and flexible mobile elements.[6]

Post-translational modifications of proteins may affect their overall allergenic activity. In this respect, glycosylation has an impact on IgE binding activity. For some proteins, glycan moieties contribute to the overall stability of the protein as has been shown for the celery allergen, Api g 5.[7] On the other hand, glycans account for increased recognition of specific IgE antibodies without clinical implications, as is known for some grass pollen allergens. Detection of glycans can be performed by specific staining for lectins. Enzymatic release of glycans with subsequent mass spectrometry analysis is direct proof of glycan moieties.

Finally, the immunological properties of the purified allergen batches have to be determined. The IgE binding properties of a protein are routinely tested in an immunoblot assay or in an ELISA format.[2] While the first approach provides additional qualitative information of the allergen, the second approach enables a semi-quantitative analysis. In addition, the levels of cross reactivity among related proteins can be assessed by inhibition assays in both formats. For *in vivo* approaches, skin prick tests with protein extracts and purified allergens have been performed, inducing a local allergic response in patients. Blood-derived IgE sensitized basophiles can be used for cellular tests. Upon addition of purified allergens, the basophiles are activated due to cross linking of the cell-bound IgE *via* the allergens. As readout of activation of the cells, surface markers such as CD63 or CD203c can be measured by flow cytometry.[8] Alternatively, released histamine is measured by fluorometric detection.

In conclusion, the proof of concept of an allergen library was established within the EuroPrevall framework. Selected allergens from the most important allergenic food sources were purified and characterised applying an array of physico-chemical and immunological methods. During the establishment of the library and collation of the allergen authentication protocols, it became evident, that there is a need for timely and efficient analytical methods for the authentication of proteins. It is expected that the data generated from the EuroPrevall allergen library will contribute to the attainment of important goals, such as the development of allergen standardisation and reference materials that in turn will

improve allergenic risk assessment of food production, diagnosis of food allergy and work towards immunotherapy.

ИЗВОД

УТВРЂИВАЊЕ АУТЕНТИЧНОСТИ АЛЕРГЕНА ХРАНЕ

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Чисти препарати алергена су потребни за тачну, личну дијагнозу алергије на храну. Они се, такође, могу користити за развој осетљивих тестова за детекцију алергена у храни и у имунотерапији. Ови препарати – реагенси морају бити добро окарактерисани и задовољити одређене критеријуме квалитета. У оквиру европског пројекта „EuroPrevall“ развијен је концепт библиотеке алергена у којој се налазе најважнији алергени хране животињског и биљног порекла, уз преглед њихових физичко–хемијских и имунолошких особина. Сарадници са универзитета и из биотехнолошке индустрије су заједнички применили познате лабораторијске технике, као и нове тестове, у анализи најважнијих карактеристика крајњих протеинских препарата. Очекује се да овакав концепт допринесе утврђивању аутентичности алергена, ради рутинске примене у дијагнози и терапији алергија, као и у процени ризика од алергије у производњи хране.

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Review

REVIEW

What makes peanuts so allergenic?

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(Received 5 October 2012, revised 24 January 2013)

Abstract: Peanut allergy belongs to one of the most severe food allergies. So far 12 peanut allergens have been registered by the IUIS Allergen Nomenclature Subcommittee. The different peanut allergens and factors that contribute to allergenicity are described herein. Peanut contains several class I food allergens (especially Ara h 1, 2, 3) that are stable against heat denaturation and proteolytic digestion and represent storage proteins. These allergens are often associated with severe allergic reactions. Additionally, peanut contains class II food allergens (Ara h 5 and 8), where the IgE reactivity is caused by cross reactions to inhalant allergens. These allergens are mostly associated with mild to moderate allergic reactions. However, the severity of the symptoms may change by involvement of additional factors. The peanut matrix consists to about 50 % of lipids, and allergen–lipid associations have been shown for several peanut allergens. Further factors influencing allergenicity depend on the peanut variety, geographical differences and alterations in food processing. Finally, the physiological function of allergens and the mechanisms by which they interact with the immune system are further modulating factors. Thus, the specific allergen structure, matrix, genetic variations, geographic alterations and further augmentation factors are important parameters that induce and influence allergenicity.

Keywords: allergenicity; augmentation factors; class I and II food allergens; epitope; IgE reactivity.

CONTENTS

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1. INTRODUCTION

Peanut is one of the most hazardous sources of food allergens that can cause severe, sometimes even fatal, anaphylactic reactions. About 100 µg of peanut protein can provoke symptoms in allergic individuals.[1] It is rather difficult to avoid peanuts, since they may occur as hidden allergens in exotic meals and as supplements, such as peanut oil in cosmetics. Due to their lipophilic matrix (peanuts contain about 50 % of lipids[2]), peanut proteins are considerably adhesive and may be resistant to degradation. Possible sensitization routes are the gastrointestinal tract, the respiratory tract, the skin, as well as breast feeding.

To answer the questions concerning peanut allergenicity, the complexity of peanut allergens, co-factors and interaction with the immune system at different interfaces have to be taken into account.

Three topics should be considered:

1. the structural characteristics of peanut allergens;
2. additional factors that can increase allergenicity;
3. mechanisms of sensitization by peanut allergens.

2. STRUCTURAL CHARACTERISTICS OF PEANUT ALLERGENS

The molecular structure is obviously related to allergenicity. The fact that allergens exist only in 1.5 % of the 9318 protein families[3] suggests a correlation between molecular structure and allergenicity. This was further confirmed by studies of da Costa Santiago *et al.*,[4] who demonstrated a correlation of allergenicity with structural uniqueness of proteins, while homologous proteins common in eukaryotic organisms and high amino acid conservation levels result in lower allergenicity or lead to immunological tolerance.

However, little is known about the typical structural characteristics of allergens.[5] Allergens are glycoproteins or proteins with a molecular weight of 10 to 70 kDa. They must carry at least two IgE-reactive epitopes to enable cross-linking of IgE antibodies on effector cells to release mediators. The allergenic molecules must be stable to reach the immune cells. Interestingly, low dosages cause allergy, since low concentrations increase Th2 cell activation. Usually allergens are readily soluble. The physiological functions of allergens also have to be considered as factors promoting allergenicity, although few publications exist on this particular subject: some allergens have been identified as proteases, protease inhibitors, transport proteins, regulatory proteins, storage proteins and defence proteins.

Subsequently, different peanut allergens and factors that contribute to allergenicity are described. Peanut allergens were identified by 2D immunoblotting

using sera from peanut allergic individuals. The protein spots were identified by tryptic mass fingerprinting and sequence homology was searched for in databases.[6] The complexity becomes evident when focusing on molecules of similar size but with different p*I* values. Many allergens consist of isoforms that differ from each other by exchange of one or a few amino acids and/or post-translational modifications.[7,8]

The peanut allergens as they are listed by the IUIS Allergen Nomenclature Subcommittee are summarized in Table I. Twelve different allergens from peanut *(Arachis hypogaea)* are known: Ara h 1–Ara h 13. The structure of the allergens varies from polypeptides (*e.g.*, oleosins), small molecules stabilized by disulfide bonds (*e.g.*, Ara h 2) to complex post-translation modified glycoproteins that oligomerize (*e.g.*, Ara h 1). The physiological function of peanut allergens range from storage proteins, protease inhibitors, pathogenesis-related proteins (PR proteins for defence) to transport proteins.

TABLE I. Overview of the peanut allergens (*Arachis hypogaea*). PTM, post-translational modifications; glyc, glycosylation; S–S, disulfide bond; PR, pathogenesis-related protein (Becker *et al.*,[9] modified)

Allergen	*MW* / kDa	p*I*	Structure	PTM	Function
Ara h 1	62–69	6.0–6.4	Cupin, vicilin type, 7S, trimer	glyc, S–S	Storage protein
Ara h 2	17–18	5.5	Conglutin type, 2S albumin	S–S	Storage protein, trypsin inhibitor
Ara h 3.01 Ara h 3.02 (Ara h 4)	56–59	5.4–5.6	Cupin, legumin type, 11S, hexamer	glyc, S–S, α- and β-subunits	Storage protein
Ara h 5	14	4.6	Profilin	–	Profilin
Ara h 6	14	5.0	Conglutin type, 2S albumin	S–S	–
Ara h 7	16–17	5.6–7.5	Conglutin type, 2S albumin	S–S	–
Ara h 8	16–17	5.0	Bet v 1 homologous protein	–	PR-10, transport
Ara h 9	9	9.3–9.5	Lipid transfer protein	S–S	PR-14, lipid transport
Ara h 10	16–18	9.4–9.6	Oleosin	–	Lipid transport
Ara h 11	14	9.1	Oleosin	–	Lipid transport
Ara h 12[18]	5	7.7	Defensin, dimer	S–S	PR-12, defence
Ara h 13[18]	5	8.2	Defensin, dimer	S–S	PR-12, defence

In contrast to pollen allergens that enter the organism without structural changes, peanuts are usually processed by roasting before consumption and subsequently undergo digestion under acidic conditions by pepsin in the stomach

and in basic conditions in duodenum by pancreatic enzymes, before fragments (single allergens) reach the immune system, a complex procedure, which influences their allergenicity.[10]

Food allergy is divided into two classes as described in Table II.[11] Class I food allergy is characterized by systemic and more severe reactions, which is associated with allergens resistant to heat, proteases and digestion, allowing these allergens to pass the gastrointestinal tract without structural changes. This category of allergens in peanut contains Ara h 1, 2 and 3, which are known to be responsible for severe anaphylactic reactions after peanut consumption.

TABLE II. Classification of food allergens into class I and II

Parameter	Class I	Class II
Sensitization route	Gastrointestinal	Inhalation
Characteristics	Thermally and proteolytically stable	Thermally and proteolytically unstable
Symptoms	Severe reactions Anaphylaxis	Often mild reactions OAS
Examples	Ara h 1, 2 and 3	Ara h 5 and 8

However, peanut also contains class II food allergens. In this case, the sensitization is usually raised against pollen allergens *via* the respiratory tract. Due to cross-reactive IgE epitopes in peanut allergens, such molecules can cause allergic reactions as well. Usually these allergens are thermally and proteolytically unstable, and often only mild symptoms are provoked.

The following description of the class I and II allergens of peanut is based on the review of Becker *et al.*[9]

2.1. Characteristics of class I food allergens of peanut

Some of the peanut allergens reveal many sequential IgE-binding epitopes, *e.g.*, Ara h 1 possesses 23 linear epitopes.[12] Even after partial cleavage during digestion, some IgE-binding epitopes remain on the fragments and probably cause histamine release.

Ara h 1 and Ara h 3 belong to the cupin superfamily with a molecular structure formed by a β-barrel core domain and two additional conserved sequence motifs. Ara h 1 is a 7S globulin and belongs to the vicilin type. Hydrophobic patches are located at the ends of the molecule; they are the binding edges for the formation of Ara h 1 trimers.[13] Ara h 3 tends to form hexamers. Its monomers are composed of an acidic and a basic subunit linked by a disulfide bond.[14] Ara h 3 is an 11S globulin and belongs to the legumin type.

Ara h 2, 6 and 7 are members of the prolamin superfamily. They are alcohol soluble proline and glutamine rich storage proteins that show a specific, highly conserved cysteine pattern (C–Xn–C–Xn–C–C–Xn–C–X–C–Xn–C–Xn–C),

whereby Ara h 6 contains 10 cysteines. Ara h 2, 6 and 7 belong to the 2S albumins. Ara h 2 is the most frequently recognized peanut allergen.[15] Studies by Maleki *et al.*[10] showed that the stability of this molecule is due to the four disulfide bonds. Roasting and denaturating conditions with 8 M urea did not alter the secondary structure, as demonstrated by CD spectroscopy. However, the conformational structure is destroyed by reducing procedures.

Ara h 9 also belongs to the prolamin superfamily, but to the subgroup of the non-specific lipid transfer proteins (LTP). The LTPs are largely distributed in fruits and vegetables and cause allergic reactions, especially in individuals of the Mediterranean region.[16,17] The underlying molecule is stabilized by four disulfide bonds and belongs to the pathogenesis-related proteins (PR12-protein).[11]

Two further families of peanut allergens are associated with lipids: the oleosins (Ara h 10 and 11) and the defensins (Ara h 12 and 13[18]). Oleosins are amphiphilic molecules that form a monolayer on oilbodies[19] and peanut defensins, isolated from lipophilic fractions, have recently been identified as IgE-reactive components.[18] Plant defensins are small, highly stable, cysteine-rich peptides that constitute a part of the innate immune system, primarily directed against fungal pathogens.[20]

2.2. Characteristics of class II food allergens of peanut

Class II food allergens in peanut are Ara h 5 and 8. Ara h 5 is a profilin.[21] Profilins are detectable in all eukaryotic cells and are involved in the formation of the cytoskeleton. Since they also exist in human cells, immune responses can only be elucidated to structurally and phylogenetically less related plant profilins.[22] Nevertheless, the structural differences to plant profilins are small. Clinical relevance seems to be negligible. The symptoms, if any occur, are usually only mild.

Ara h 8 is a Bet v 1-homologous protein (PR-10 protein)[23,24] and a class II food allergen, since the sensitization (especially in Northern Europe) is caused by the major birch pollen allergen Bet v 1. This plant family is rather large and consists of many members of fruits and vegetables from Rosaceae and Apiaceae, respectively.[25]

Even though the protein sequences can differ by 60 % among different members, the underlying 3D structure and surface identity (important for the antibody binding) are more similar. The characteristic 3D structure is formed by 7 antiparallel β-sheets and 2 short α-helices. These allergens are unstable to heat and digestion and, therefore, often cause only mild reactions, *e.g.*, the oral allergy syndrome (OAS).[24]

3. ADDITIONAL FACTORS INFLUENCING ALLERGENICITY

For allergy development, the specific structure of the allergen/s in the context of allergen exposure and the genetic background of the individual are of profound importance.

Allergenicity can vary considerably due to the variety of the allergen sources, the form of protein extraction and the further processing.

Comparing peanuts from different varieties and origins, a peanut species from Bali that showed nearly no Ara h 1 was evidenced by 2D PAGE and immunoblotting.[26] It might be speculated that this was a hypoallergenic peanut variant. However, when the allergenicity of the peanut extract was determined, it was found to be comparable to those other varieties, which is probably due to compensation with higher amounts of the other peanut allergens. This is a natural example that demonstrates that the elimination of a single allergen does not necessarily result in a lower overall allergenicity.

The extraction conditions also play an important role. Under alkaline conditions (carbonate buffer, pH 8), which mirrors the physiological conditions of peanut uptake in man, Ara h 1 and Ara h 3 are especially detectable, while under acidic conditions (acetate buffer, pH 5) oleosins and LTP (Ara h 9) appear more prominently.[27]

When an attempt was made to improve the isolation of Ara h 8 from an extract,[28] it was observed that lipophilic extraction by use of chloroform and methanol[29] produced a more efficient isolation, resulting in pure Ara h 8. It would not have been possible to identify the sera of Ara h 8-reactive patients in the usually isolated extract (carbonate buffer, pH 8). However, by application of the lipophilic extraction method, Ara h 8 could clearly be detected. This is a fact that has to be considered for diagnosis.

A further interesting phenomenon was observed in these experiments: extracts prepared from roasted peanuts revealed a stronger IgE reactivity when compared to the extract from unroasted peanuts (personal communication). This may be due to roasting as Maillard products are formed[30] by the reaction of carbohydrates and allergens, which was demonstrated for the peanut allergens Ara h 1 and 2.[10,13]

A lipophilic matrix can also have an important influence on the allergenicity of the molecules. Sancho *et al.*[31] demonstrated that phosphatidylcholine induced conformational changes in the apple allergen Mal d 1 and allowed this allergen to penetrate the phosphatidylcholine vesicle. By this, the digestion of the Mal d 1 was retarded, and the allergenicity was retained to a large extent. Since peanut consists of up to 50 % of lipids,[2] evidence was found that lipids also interfere with the IgE binding and digestibility of Ara h 8. After roasting, some covalent Ara h 8–lipid oxidation products might have been generated. Recombinant Ara h

8 without lipids was degraded rapidly, while Ara h 8 isolated from roasted peanuts was resistant in gastrointestinal digestion experiments.[28]

Not only Ara h 8, but also several other peanut allergens are associated with lipids. Thus, the lipid transfer protein Ara h 9, the oleosins, which float on the surface of oilbodies,[19] Ara h 10 and 11, and finally plant defensins were identified as by-product when Ara h 8 was isolated by lipophilic extraction.[18]

Besides the specific structure, the quantity of allergens can influence sensitization as well. Fox *et al.*[32] reported that high levels of environmental exposure to peanut during infancy caused higher rates of sensitizations. On the other hand, du Toit *et al.*[33] showed that early consumption of peanuts in infancy seems to be associated with a low prevalence of peanut allergy. They determined a 10-fold lower prevalence for peanut allergy in Jewish children in Israel than in Jewish children in the U.K. Israeli children consume peanuts in high quantities in the first year of life, while U.K. infants avoid peanuts. Other probable factors arising by atopy, social class, genetic background and peanut allergenicity were ruled out in the study.[33] This indicates that a window of opportunity might exist in early life that causes tolerance induction.[34]

Furthermore, the route of the allergen exposure seems to be decisive. In peanut allergic patients, the proliferation of peanut-specific memory T-cells with the skin-homing factor CLA+ (cutaneous lymphocyte antigen) predominates, while peanut-tolerant groups have a mixed skin- and gastrointestinal-homing factor composition CLA+/$\alpha 4\beta 7$+.[35] These results further support the hypothesis that the initial route of allergen exposure in early life is crucial: allergic sensitization might occur *via* the skin, while passage *via* the gut induces tolerance.[35]

Geographical parameters and environmental factors can also influence the occurrence of allergic reactions to specific allergens. Concerning peanut allergens, a clear distribution of the North European population exposed mainly to birch pollen results in reactivity to the Bet v 1-homologous Ara h 8, while in the Mediterranean countries, the reactions are especially based on the lipid transfer proteins. This is probably caused by exposure to the marker allergen Pru p 3 from peach, which cross-reacts with Ara h 9.[17,36]

Food processing habits can also influence sensitization. It is interesting to note that peanuts are usually cooked in China, while they are mainly roasted in other countries.[37] Although peanut consumption is high in China, peanut allergy does not seem to be as prevalent as it is in the U.S.A. The reason might be due to the food processing. By cooking peanuts, some allergens may be washed out, while roasting can generate new stable allergenic epitopes, *e.g.*, Maillard products.[38]

Last but not least, factors such as alcoholic drinks, non-steroidal anti-inflammatory drugs (NSAIDs), exercise and menses can increase food allergic symptoms.[39,40]

4. WHAT ARE THE MECHANISMS OF THE SENSITIZATION BY PEANUT ALLERGENS?

To understand the effect of allergenicity, it is important to investigate how specific allergens interact with the epithelium and the immune response-triggering cells.

Several food allergens have been identified as proteases, *e.g.*, actinidin of kiwi (Act d 1), and Cavic *et al.*[41] were able to demonstrate similar morphological changes and desquamation of epithelial cells after exposure to Act d 1 and with the house dust mite allergen Der p 1.[42] Although no allergenic protease have hitherto been identified in peanut, it might be speculated that, for example, subtilisin, a serine protease in peanut kernels,[43] disrupts the tight junctions, thereby enabling peanut allergens passage through the epithelial barrier.

The allergens Ara h 2, 6 and 7 show molecular similarities to the soybean trypsin inhibitor.[10] This might result in incomplete digestion of allergens, which can consequently elucidate allergic reactions. Shreffler *et al.*[44] demonstrated a binding of the glycoprotein Ara h 1 *via* its carbohydrate moiety (β-glucan) to CD209 on dendritic cells, the DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3 grabbing non-integrin). As a consequence, a shift towards Th2 activation occurs, as shown in human *in vitro* experiments. The results correspond to those obtained in studies of Royer *et al.*,[45] which showed that several native allergens with a carbohydrate moiety (among them Ara h 1) can bind to the mannose receptor on dendritic cells (C-type lectin) causing Th2 cell polarization.

Khodoun and co-workers[46] proved that components from peanut extract can activate complement in mice *in vivo* and *in vitro* and they also demonstrated this effect *in vitro* in man. C3a and to a lower degree Fcγreceptors were identified as contributing to peanut extract-induced anaphylactic shock. This was shown to occur without the involvement of the adaptive immune system. To date, the elucidating components from peanut extract have not been identified.

Furthermore, the association of proteins with lipids and lipophilic molecules can have structural and functional effects. Karp[47] hypothesized that intrinsic adjuvant activity may be provided for by the association of the proteins with their lipid cargo and that this underlies their immunogenicity and/or allergenicity. In this context, Ara h 8 and other lipophilic allergens may support or enhance the binding to receptors of the innate immune system, such as the Toll-like receptors TLR2 and TLR4 that bind lipopeptides and lipopolysaccharides (LPS), respectively. Dependent on the LPS concentration, the immune responses can vary considerably. In an animal model with ovalbumin, a dose of <1 ng LPS led to tolerance, a dose of 100 ng LPS to sensitization with a Th2 immune response and a dose of 100 µg to Th1 inflammation.[48]

For the Bet v 1-homologous peanut allergen Ara h 8, a binding to lipid rafts and allergen uptake *via* caveolae was demonstrated. Interestingly, this binding was only detected in birch pollen allergic individuals, but not in healthy controls.[49] The receptor, however, has not yet been identified.

5. CONCLUSIONS

The presented data provide evidence for a clear relationship between molecular structure and allergenicity, although many further influencing factors may be involved. Besides the allergen structure, the variety of the source of allergens, the matrix, the manner of processing, and with regards to the consumer, the genetic background, the qualitative and quantitative exposure, the route of exposure (gastrointestinal tract, respiratory tract, skin), and host-associated additional factors. Preliminary investigations have been performed to better understand these interactions. Since the development of an allergic phenotype depends on many factors, it is important to dissect and study the different factors.

Furthermore, the clinical relevance of the sensitization should be determined, since sensitization does not necessarily result in clinical symptoms. Clinical problems arise from inter- and intra-individual variability of the level triggering allergic reactions.

A first step is to switch from prick test with mixtures from allergenic sources, which can only differentiate between sensitized or non-sensitized, to a component-resolved diagnosis, which allows discrimination between the different allergens and, hopefully, to find marker allergens (*e.g.*, Ara h 2) that allow severe symptoms to be predicted.[50]

ИЗВОД

ШТА КИКИРИКИ ЧИНИ ЈАКИМ АЛЕРГЕНОМ?

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Алергија на кикирики је једна од најјачих алергија на храну. До сада је регистровано 12 алергена кикирикија од стране надлежног тела (IUIS Allergen Nomenclature Subcommittee). У овом раду описујемо различите алергене кикирикија и факторе који доприносе алергености. Кикирики садржи неколико алергена класе I (посебно Ara h 1, 2 и 3), који одолевају топлотном и протеолитичким разлагању и чине депо протеине. Они често изазивају јаке алергијске реакције. Кикирики садржи и алергене класе II (Ara h 5 и 8), који индукују IgE реактивност која има унакрсно препознавање инхалаторних алергена. Ови алергени изазивају благу до средње јаку алергеност. На јачину симптома могу утицати и додатни фактори. Кикирики се састоји од 50 % липида и за неколико алергена је констатовано асосовање са липидима. На алергеност утиче врста кикирикија, географско порекло и начин обраде за исхрану. Физиолошки пут алергена, као и механизми интеракције са имуним системом су, такође, модулаторни фактори. Према томе, специфична структура алергена, конзистенција, генетске варијације, географско по-

рекло и други појачавајући фактори су важни параметри за индукцију и даљи ток алергијске реакције.

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REVIEW

Kiwifruit as a food allergen source

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Abstract: Since its first appearance on the market, kiwifruit has become very popular in the human diet due to its pleasant taste, low caloric value and high content of vitamin C. However, kiwifruit allergy has become a frequent cause of type I hypersensitivity in the western society. The molecular basis for kiwifruit allergy has been ascribed to up-to-now 11 identified IgE reactive molecules. They are proteins and glycoproteins with a molecular mass between 10 and 50 kDa. The major kiwifruit allergen is a cysteine protease denoted as Act d 1, which represents 50 % of the soluble protein extract. Due to differences in the abundance of the protein components and biological activity, the quality of kiwifruit extracts intended for allergy diagnosis can vary in content and amount of IgE reactive molecules. In addition, the quality of allergen extracts for allergy diagnosis depends on the fruit ripening stage and storage conditions. In terms of clinical reactivity, it has become evident that kiwifruit allergy is not a homogeneous disorder. Different patterns of IgE reactivity accompany several clinical subgroups that have been identified in different geographical regions. In the last decade, enormous progress has been made in the isolation and characterization of kiwifruit allergens. This paper presents an overview of the structural features of kiwifruit allergens.

Keywords: allergy; kiwifruit; food allergens; IgE reactivity.

CONTENTS

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1. INTRODUCTION

Since its first appearance on the fruit market, kiwifruit has become very popular in the human diet due to its pleasant taste, low caloric value and high content of vitamin C; and numerous investigations on health-promoting properties of this fruit have been reported.[1,2] Kiwifruit has also found extensive application in the food industry as a meat tenderizer,[3] milk coagulant[4] and fruit ingredient in jams and jellies, syrups, confectionery, *etc*.[5]

However, simultaneously, kiwifruit is a good example of the possible consequences encountered by the introduction of a novel item into the human diet. In 30 years, from the first report of allergic reaction in 1981,[6] kiwifruit has become one of the top ten sources of food allergy, as shown in recent studies from Finland, Sweden and France.[7]

Kiwifruit was formally described for the first time in 1847 and given the Latin name *Actinidia chinensis.* The plant is native to the Yangtze valley of southeast China and has been a favorite of the local population for centuries.[8] The transformation of a small, hard, and wild Chinese berry into the fleshier, tastier kiwifruit began in 1904 when a school teacher from New Zealand arrived back from a short visit to China with seeds of what was then called Chinese or Ichang gooseberry, and from these a local nurseryman produced two plants from which almost all today's cultivars outside of China descend. Kiwifruit benefited from the warm, humid climate and volcanic soil of New Zealand's north island and soon became popular in the area, in the beginning valued more as a decorative vine with snowy white flowers than for its fruit. Over the next three decades, gardeners developed superior kiwifruit vines through careful selection, pruning, and grafting. During World War II, American soldiers stationed in New Zealand developed a liking for the taste of this fruit and in 1962, when New Zealand began with the export of this fruit to the USA, as part of a marketing campaign, they renamed it from Chinese gooseberry to kiwifruit, after the national bird of New Zealand.[9,10]

Kiwifruit belongs to the large genus of *Actinidia* and was first known under the botanical name *A. chinensis.* However, this species was discovered to be

polymorphic comprising several varieties (var. *chinensis*, var. *hispida* and var. *setosa*). In 1986, it was concluded that enough differences between the varieties exist to reclassify *A. chinensis* var. *chinensis* and *A. chinensis* var. *hispida* into two different species. Kiwifruit that is commercially grown and readily available worldwide is now known under the botanical name *A. deliciosa* (A. Chev) C.F. Liang & A.R. Ferguson var. *deliciosa*.[11] Until 1999, the world kiwifruit production was mostly based on the green-fleshed cultivar *Actinidia deliciosa* cv Hayward, when the yellow-fleshed cultivar *A. chinensis* cv Hort16A became available on the international market under the commercial name Zespri Gold. These two varieties not only differ in taste, but also in their allergenicity.

2. CLINICAL MANIFESTATIONS OF KIWIFRUIT ALLERGY

Since it was first described in 1981,[6] kiwifruit allergy has been observed with increasing frequency in western populations.[12] Symptoms of kiwifruit allergy vary from mild symptoms localized to the oral mucosa to severe systemic reactions, particularly in young children.[12] Kiwifruit allergy can be the result of cross-reactivity with pollen and latex, the most frequent association being with birch and grass pollinosis;[13] however, monosensitizations have also been reported. Symptoms severity and sensitization patterns of kiwifruit allergy are geographically dependent, with different allergen sensitization patterns seen across Europe. More severe symptoms to kiwifruit allergy in allergic patients were recorded in the birch-free Mediterranean area than in central Europe.[14] Aleman *et al*. provided evidence that kiwifruit allergy is not a homogeneous disorder because several clinical subgroups can be established.[15]

Food allergy is a consequence of primary sensitization of the immune system to food allergens or from primary sensitization to inhalant allergens (pollens or latexes in allergies to plant foods) in atopic persons. It has been postulated that direct sensitization by food allergens *via* the oral route is only possible when the allergens possess resistance to proteolysis in the digestive tract. This feature is regarded as decisive for the potential of food allergens to induce severe systemic reactions mediated by immunoglobulin E antibodies.

Structural similarities of proteins from different sources provide an explanation for a number of clinically observed cross-reactivity cases.[13] Due to cross-reactivity between plant and pollen allergens, patients with allergy to pollens often report oral allergy syndrome (OAS) after ingestion of certain fruits, nuts and vegetables. Pollen–food cross-reactive IgE antibodies are usually implicated in mild symptoms of allergy, such as OAS. The major birch pollen allergen Bet v 1 and the pollen profilins, the major source of cross-reactive IgE binding epitopes, are sensitive to pepsin digestion, and therefore the clinical symptoms of allergy are restricted to the oral cavity.

3. MOLECULAR BASIS OF KIWIFRUIT ALLERGY

The molecular basis of kiwifruit allergy is attributed to eleven International Union of Immunological Societies (IUIS) nominated kiwifruit allergens (www.allergen.org) (Table I).

TABLE I. Kiwifruit allergens

Allergen	Biochemical name	*MW* / kDa (SDS–PAGE)
Act d 1	Cysteine protease (actinidin)	30
Act d 2	Thaumatin-like protein	24
Act d 3	–	40
Act d 4	Phytocystatin	11
Act d 5	Kiwellin	26
Act d 6	Pectin methylesterase inhibitor	18
Act d 7	Pectin methylesterase	50
Act d 8	Pathogenesis-related protein PR-10	17
Act d 9	Profilin	14
Act d 10	Lipid transfer protein 1	10
Act d 11	Major latex protein/ripening-related protein family	17

3.1. Act d 1

Actinidin (EC 3.4.22.14) is a cysteine protease from the papain superfamily and is abundant in kiwifruit.[16] Beginning from the 1970s, this enzyme has been studied in detail and its 3-dimensional structure, amino acid and nucleotide sequences are known.[17–19] The mature form of actinidin is comprised of a single polypeptide chain organized into two domains with 3 disulphide bridges and has a molecular weight of 23.8 kDa and a p*I* value of 3.5.[17,20,21] Nieuwenhuizen *et al.* identified more than 10 different mRNA molecules encoding actinidin isoforms with predicted p*I* values ranging from acidic (3.9) to basic (9.3).[22] Actinidin has a wide pH activity range (4–10) and wide substrate specificity, preferentially hydrolyzing the amide and ester bonds at the carboxyl side of lysine residues.[23,24]

Actinidin represents more than 50 % of total soluble protein content in the green-fleshed kiwifruit cultivar (*A. deliciosa* cv. Hayward), but this protein appears to be at a very low level in Hort16A, the most important commercial cultivar of gold kiwifruit (*A. chinensis*).[16,22] Immunolocalization showed that actinidin was present inside the vacuole of the small cells of the outer pericarp of mature *A. deliciosa* fruit at harvest.[22] Several other cysteine proteases, which are actinidin homologues from the papain family, are also accumulated at high levels in specific tissues or cell types, including papain from papaya, bromelain from pineapple and ficin from fig.

The physiological role of these enzymes is unknown, but it has been proposed that they function as storage proteins or are part of a defense mechanism against insects, plant diseases and other forms of stress.[25,26]

Actinidin (Act d 1) is a kiwifruit allergen and is considered a marker of monosensitization to this allergen food source, since no cross-reactivity with birch or grass pollen has been observed.[15,20,27,28] In most studies, actinidin was identified as a major allergen in kiwifruit allergic patients.[7,25,27–29] An exception to this was a study performed in 2007 by Lucas *et al*. on kiwifruit allergic patients in the United Kingdom in which none of the 30 patients showed IgE binding to purified actinidin in Western blot.[30] Actinidin retains allergenicity after thermal treatment, a significant feature in terms of the possible development of allergic reactions after the consumption of food products containing processed kiwifruit.[21]

Although some studies indicate that actinidin is susceptible to degradation upon passage through the gastrointestinal tract,[31] others showed that actinidin retains its enzymatic activity under conditions of gastric and intestinal digestion and aids in the digestion of food proteins.[32,33] In a 2012 study by Čavić *et al.*, the proteolytical activity of actinidin, which leads to changes in the morphology and adhesion of intestinal epithelial cells, was proposed as a possible route for oral sensitization to this allergen.[34]

3.2. Act d 2

The second IgE binding molecule isolated from *A. deliciosa* was a thaumatin-like protein (TLP) and it was denominated Act d 2.[13] Act d 2, as well as other TL proteins, belongs to the pathogenesis related (PR) 5 family of proteins.[35] It is a protein of 225 amino acids, with the first 24 representing a signal peptide that designates this protein to the apoplast. It has basic p*I* values of 9.4 and 9.5.[13,14] A potential *N*-glycosylation site is located on $N^{189}FS$ and glycosylation was suggested to exist based on its ability to bind to concanavalin A lectin.[13] Kiwi TLP, similar to other members of the PR 5 family, possesses antifungal activity against *Botrytis cynerea*, *Mycosphaerella arachidicola*[36] and *Saccharomyces carlsbergensis.*[13] The anomalous migration of kiwifruit TLP in SDS–PAGE is due to an unusually high number of S–S bridges, a peculiarity common for TLPs.[13] Namely, it has 8 disulphide bridges between cysteines Cys^{33}–Cys^{224}, Cys^{74}–Cys^{84}, Cys^{89}–Cys^{95}, Cys^{140}–Cys^{213}, Cys^{146}–Cys^{196}, Cys^{154}–Cys^{164}, Cys^{168}–Cys^{177} and Cys^{178}–Cys^{183}.

There are opposing studies concerning TLP stability under simulated gastrointestinal conditions.[13,31] The first obtained results suggested that purified TLP was digested in simulated gastric fluid (SGF) in only 1 min, while TLP in crude extract resisted digestion for 8 min.[13] However, later studies suggested that the stability of TLP during simulated gastrointestinal digestion largely depended on the enzyme:substrate ratio employed in the experimental setting, as well as whether reducing or non-reducing conditions were employed for SDS–PAGE analysis of the reaction mixture. Under non-reducing conditions, 25 % of the allergen re-

mained intact following *in vitro* gastric digestion and during duodenal digestion residual intact Act d 2 was still present when analyzed by SDS–PAGE. Under reducing conditions, the allergen promptly disappeared.[31] The observed differences stem from the different digestion protocols used in the experiments, *i.e.*, different amounts of pepsin used for the simulated *in vitro* digestion. However, it can be assumed, as suggested by a later study[31] and by similar results obtained for milk[37] and wheat[38] allergens, that disulfide bonds are important in the resistance of this and other allergens to digestion with proteases commonly en-countered in the gastrointestinal tract.

Thermal stability experiments suggest that Act d 2 aggregates following heating at pH 7, but not at pH 2.[31] These results show that cross-linking of Act d 2 molecules at pH 7 by intermolecular disulfide bonds was induced by heating. The cleavage of disulfide bonds at neutral pH and high temperature was previously observed for thaumatin,[39] but also in unrelated proteins including ovalbumin and transferrin.[40] The most probable mechanism of this reaction is β-elimination, in which a base-catalyzed subtraction of a β-proton from a cysteine results in cleavage of disulfide bonds. This creates intermolecular disulfide linkages and induces aggregation. In contrast, thermal denaturation at acidic conditions (such as those found in juice) reversibly unfolded the protein.[31]

Members of the TLP family have a role as allergens in a wide panel of plant foods and in several pollens, although there is little experimental evidence of plant foods and/or pollen cross-reactivity. Gavrović-Jankuović *et al.*[13] showed positive skin prick tests (SPT) in 80 % of the tested polysensitized patients (4 out of 5) and IgE reactivity in Western blot (7 out of 7 in crude extract). In a study by Palacin *et al.*,[14] *in vitro* (specific IgE detected in 64 % and 88 % of individual sera by ELISA and immunodetection assays, respectively) and *in vivo* (52 % of positive SPT responses) reactivity pointed to Act d 2 as the second major allergen in the analyzed kiwi-sensitized population of Spain. Immunoblotting experiments by Bublin *et al.*, showed that in extracts prepared from kiwifruit jam and whey, Act d 2 was present and its allergenic activity was not decreased by technological treatments.[31]

3.3. Act d 3

Act d 3 is a strongly glycosylated 40 kDa protein present in two genetic variants, and is homologous to hypothetical hydrolases from castor bean and other plant species.[14,41] The high sequence identity with the putative *Ricinus communis* protease suggests a functional role for Act d 3 in kiwifruits that could explain the presence in preparations of the purified allergen of possible self-degradation products that retain IgE-binding potency.[14]

Act d 3.02 seems to be a minor component of kiwifruit extracts, yet, in contrast, it represents a major kiwi allergen based on its high specific IgE prevalence

(62 %) in sera from kiwi-sensitized patients. The potential clinical relevance of Act d 3 was further supported by the statistical correlation between IgE levels to this allergen and anaphylactic symptoms. All these data are in agreement with those observed previously for Act d 3.01, both *in vitro* (66 % of sera with specific IgE) and *in vivo* (13 out of 15 patients with positive SPT responses).[41] Moreover, Act d 3 could probably correspond to the 38-kDa protein described by Lucas *et al*. as the major kiwifruit allergen in the United Kingdom.[30] Thirty six percent of the 22 patients with combined kiwifruit/pollen or kiwifruit/pollen/latex allergy were sensitized to Act d 3, as reported by Bublin *et al*.[29] Moreover, these authors claimed that IgE binding to highly cross-reactive allergens (rAct d 8, rAct d 9) or Act d 3 was not a clinically specific marker for kiwifruit allergy in the presence of pollen or pollen/latex sensitization.[29]

The complex glycans (cross-reactive carbohydrate determinants) attached to Act d 3 can be a source of cross-reactivity between kiwifruit and other plant foods and pollens, the actual clinical relevance of which remains to be explored.[14]

3.4. Act d 4

Act d 4 is a 11 kDa allergen first reported as an IgE binding component of kiwifruit extract.[28] It belongs to the family of cysteine proteinase inhibitors (CPI) named phytocystatins.[42] There are 3 isoforms of phytocystatins present in kiwifruit, with isoform 1 being the most abundant.[42] Act d 4 was identified and isolated as isoform 1.[43]

Act d 4 is a type I plant cystatin synthesized as a pre-protein of 116 amino acids, with the first 26 amino acids representing a signal sequence that is cleaved off from the mature protein.[42,43] Immuno-tissue print results indicated that CPI is most abundant in the outer layer of the pericarp, near the peel and the innermost part of the pulp – sites where it could act as a natural barrier against pathogens entering the fruit.[44] It is a glycoprotein with a p*I* of 6.9 which binds Con A lectin, mannose-specific banana lectin and fucose-specific *Aleuria aurantia* lectin.[43] The molecular masses of the mature protein were determined by MALDI to be 10902.5 Da and 11055.2 Da.[43] These different masses observed could probably be attributed to the presence of different glycosylation isoforms present in kiwifruit.

Act d 4, similar to other type I plant cystatins, showed antifungal activity against two phytopathogenic fungi (*Alternaria radicina* and *B. cinerea*), by inhibiting fungal spore germination. *In vivo*, Act d 4 was able to prevent artificial infection of apple and carrot with spore suspensions of *B. cinerea* and *A. radicina*, respectively. It also exerted activity on both intracellular and fermentation fluid proteinases.[44] Act d 4 influenced the growth of phytopathogenic bacteria *Agrobacterium tumefaciens* (76.2 % growth inhibition using 15 μM CPI), *Burkholderia cepacia* (75.6 % growth inhibition) and, to a lesser extent, *Erwinia ca-*

rotovora (44.4 % growth inhibition) by inhibiting proteinases that are excreted by these bacteria.[45]

Act d 4 was first identified as an IgE binding component of kiwifruit extract.[28] However, a positive skin prick reactivity with Act d 4 was induced in three kiwifruit allergic patients, as well as the upregulation of CD63 and CD203c molecules in the basophile activation assay. IgE reactivity was detected in dot blot analysis and subsequent negative Western blot analysis using sera from six kiwifruit patients, which suggested the presence of conformational IgE epitopes on the Act d 4 molecule. As an activator of effector cells in type I hypersensitivity Act d 4 is a functional allergen contributing to the clinical symptoms of kiwifruit allergy.[43]

3.5. Act d 5

The fifth allergen detected in kiwifruit extract was named kiwellin and was designated as Act d 5.[46] Kiwellin was first identified in green kiwifruit and described as an allergen and one of the major protein components of this fruit.[46] It was also identified in the gold kiwifruit species, where it appears as the most abundant protein component.[47] Anomalous behavior of Act d 5 was observed in SDS-PAGE (traveling as a 20 kDa band in non-reducing and a 28 kDa band in reducing conditions), which, as observed for TLP, is a consequence of disulphide bridges, as it contains 14 cysteine residues. It contains 189 amino acid residues and sequence heterogeneity was found at position 61, where His replaced Tyr in approximately 40 % of the protein molecules.

Kiwellin is cleaved into the following four peptides: kissper, KiTH1, KiTH2 and KiTH 3.[47] Kissper is a 39-residue peptide isolated in good yield from the edible part of kiwifruit; its amino acid sequence showed 100 % identity with the first 39 residues of the *N*-terminal region of kiwellin.[48] Kissper is derived from the processing of the precursor kiwellin through the cleavage of the peptide bond between Thr39 and Thr40. The capacity of kissper to permeabilize synthetic membranes was tested, and while kissper showed anion selectivity,[48] it was at concentration values generally lower than those reported for several pore-forming peptides, such as defensins, thionins, cecropins, cryptidin, duramycin, *etc*.[49,50] The high amount of kissper found in ripe kiwi fruit and its strong resistance to proteolysis suggest that it could very likely affect the gastrointestinal physiology.[48]

KiTH was detected on SDS–PAGE as a 20-kDa band. Elucidation and analysis of the primary structure of purified KiTH revealed 100 % amino acid sequence identity with the C-terminal region (residues 40–189) of kiwellin. KiTH and kissper were isolated from green kiwifruit in approximately stoichiometric amounts, which suggested that both were produced following a proteolytic cleavage of kiwellin.[47] KiTH and kissper were not detected in gold kiwifruit extract, but it cannot be excluded that they were present in very low, undetectable

amounts.[47] The observation that their presence in green kiwifruit was correlated with a high amount of actinidin suggested a possible involvement of this protease in their generation. Two KiTH forms found in the green kiwifruit extracts derived from *in vivo* cleavage between Thr^{39} and Thr^{40} (site 1) and between His^{41} and Ser^{42} (site 2) of kiwellin, were also identified as products of *in vitro* digestion by actinidin. A third form of KiTH, showing two additional residues at the N terminus, was obtained after *in vitro* enzymatic cleavage of kiwellin, suggesting a significant effects of the environmental conditions on the specificity of the proteolytic action. The experimental data demonstrated that KiTH and kissper are produced following *in vitro* proteolytic processing by actinidin and that environmental conditions may affect the ratio of the digestion products.[47]

Serological tests and Western blot analysis showed that kiwellin is specifically recognized by IgE of patients allergic to kiwifruit.[46] Similar to kiwellin from green kiwifruit,[46] the homologous protein from gold kiwifruit displays IgE-binding capacity. Both proteins were detected by the same sera, and even the levels of the signal on Western blot were comparable, thus suggesting conservation of the IgE-binding epitopes. KiTH was also detected as an IgE binding molecule.[47] The obtained results suggested that (i) kiwellin may have a hidden IgE-binding epitope that becomes available in KiTH, following the removal of kissper, and (ii) kissper might be an IgE-binding epitope by itself.[47]

On testing a population of subjects allergic to kiwifruit using the standard protocol for SPT, eight out of 29 (28 %) had a positive reaction to Act d 5. The observation that some subjects had a positive reaction either at neutral or acidic pH values suggests that this allergen, depending on the experimental conditions, may expose different epitopes. CD measurements under different experimental conditions indicated that the three-dimensional structure of Act d 5 is modulated by the solvent pH and polarity. Therefore, it may be hypothesized that, depending on the environments encountered, this allergen may undergo *in vivo* conformational changes and expose different epitopes, inducing the synthesis/interaction of different specific IgEs.[51]

3.6. Act d 6

A protein acting as a powerful inhibitor of plant pectin methylesterase (PMEI) was isolated from kiwifruit and denoted as Act d 6.[52] This protein is comprised of 152 amino-acid residues, accounting for a molecular mass of 16277 Da, with a predominant alpha-helix conformation in the secondary structure.[52] The protein has five cysteine residues but neither tryptophan nor methionine.[52] Analysis of fragments obtained after digestion of the protein alkylated without previous reduction identified two disulfide bridges connecting cysteines Cys^{9}–Cys^{18}, and Cys^{74}–Cys^{114}, while Cys^{140} bears a free thiol group. A database search indicated a similarity between PMEI and plant invertase inhibitors. In

particular, the four Cys residues, which in PMEI are involved in the disulfide bridges, are conserved. A comparison of the sequence of these inhibitors confirms the existence of a novel class of proteins with significant sequence conservation, comprising plant proteins acting as inhibitors of sugar metabolism enzymes, and probably involved in various steps of plant development.[52]

Considering that PMEI interacts with pectin methylesterase (PME), which is localized in the cell wall, it is probable that a signal sequence is required to direct the protein toward its final cellular localization. In fact, preliminary experiments of tissue immunological staining indicate that Act d 6 is concentrated in a layer close to the cell membrane.[52]

The relative expression levels of the PMEI genes in kiwifruit, analyzed by competitive PCR, increased with progression of fruit maturation. Given that the PME activity also showed its highest level at the fully ripened stage of maturation, the increase in PMEI expression may not indicate direct inhibitory effects on the PME activity and fruit maturation process.[53]

3.7. Act d 7

Act d 7 belongs to the family of pectin methylesterases (PME), a class of proteins involved in pectin metabolism during different physiological and pathological processes, such as fruit ripening[54] and response to pathogen attack.[55] PME was purified from a salt extract of kiwifruit cell wall by a combination of ion exchange and affinity chromatography (on pectin methylesterase inhibitor PMEI).[56] PME consists of two forms (PME_1 and PME_2). Both isoforms have a neutral p*I* of 7.3.[56] The molecular weight of purified kiwi PME, determined by both gel filtration and SDS–PAGE, was 50 kDa.[56] Act d 7 is a monomeric protein.[56] Kiwi PME was reported to be glycosylated with a molecular mass of 57 kDa,[57] however in this case, glycosylation may approximately account for 30 % of the total molecular mass of the protein.[58] The two isoforms of PME differ in their degree of glycosylation as shown by different retention times on concanavalin A–sepharose with respect to a glucose gradient.[57]

Kiwi PME showed two activity optima at pH 6.5 and 8.0–8.5, both in the presence and in the absence of 100 mM NaCl. The enzyme has a pH and salt dependent activity. It shows lowered activity at the lower pH optimum in the absence of salt, which may be due to the adverse effect of specific charged groups on the enzyme/substrate interaction.[58] The two isoforms also differ in thermostability, with PME_1 being more stable than PME_2.[57]

Binding experiments, performed by surface plasmon resonance, showed that PMEI strongly interacts with immobilized kiwi PME, as indicated by the extremely low dissociation rates observed at pH values ranging from 3.5 to 8.0.[58] The observation that only extreme pH conditions can dissociate the complex kiwi PME–PMEI may address the hypothesis that, *in vivo*, kiwi PME is irreversibly

inactivated by PMEI when it has no further physiological function at the end of the ripening process.[58]

In Western blot analysis, 13 % of patients had a positive IgE directed to purified allergen. Patients positive to Act d 7 were also positive to Api g 5, Ana c 2 and Hev b 4, which are known as allergens containing cross-reactive carbohydrate determinants (CCDs).[59] This can be explained by the high cross reactivity of CCDs from different allergen sources.[7]

3.8. Act d 8

Act d 8 is a homologue to the major birch pollen allergen Bet v 1.[60] Act d 8 (from *A. deliciosa*) and Act c 8 (from *A. chinensis*) are encoded by open reading frames (ORFs) of 471 and 474 nucleotides, corresponding to 157 and 158 amino acid residues, respectively.[61] There are thirteen ORF sequences corresponding to Act d 8 isoforms detected in green kiwi extract, while six were detected in gold kiwi extract.[61] The amino acid sequence identity between Act d 8 and Act c 8 was 70 % and to Bet v 1 (CAA54696) 53 and 54 %, respectively.[61] The predicted molecular masses of Act d 8 and Act c 8 are 16922 Da and 17387 Da, and their calculated p*I* values are 5.36 and 5.82, respectively.[61]

Transcripts for Act c 8 and Act d 8 are considerably less abundant, with Act c 8 appearing to be more highly expressed than Act d 8 in both green and gold kiwifruit.[61]

The secondary structures of recombinantly produced Act d 8 and Act c 8 were determined by CD spectroscopy and the spectra were similar to CD spectra obtained with rBet v 1.0101.[61]

IgE binding of purified rAct d 8 and rAct c 8 was confirmed in both ELISA and immunoblot experiments on the sera from eight kiwifruit/birch pollen allergic patients. Both purified proteins were able to bind IgE from patient sera in both ELISA and immunoblot. The cross-reactivity of rBet v 1.0101, rAct d 8, and rAct c 8 was assayed in an IgE ELISA inhibition assay. Pretreatment of the sera from individual kiwifruit/birch pollen allergic patients with rAct d 8 or rAct c 8 as inhibitor resulted in reduced IgE binding to rBet v 1.0101, while IgE binding to rAct d 8 and rAct c 8 was completely inhibited by pre-incubation with rBet v 1.0101.[61]

The results of the localization studies show that a Bet v 1-related protein was recognized in the peripheral pulp by a polyclonal anti-Bet v 1 antibody in green and gold kiwifruit.[61]

Allergic symptoms elicited by a member of the Bet v 1 family are usually confined to the oral mucosa or angioedema of the lips. Such mild symptoms have been described in Bet v 1-mediated fruit allergy to apple[62] and cherry.[63] These findings could be explained by heat liability and low resistance to digestion of the Bet v 1 homologous proteins.

Birch pollen-related food allergy is highly prevalent and often perennial. Recent study has shown that high food allergen-specific IgG_4/IgE ratios seem associated with food tolerance, potentially because specific IgG_4 blocks IgE binding to food allergens. Thus, the presence of food allergen-specific IgG_4 antibodies is no diagnostic marker for birch pollen-related food allergy.[64]

3.9. Act d 9

Act d 9 or profilin is an actin-binding protein involved in the dynamic turnover and restructuring of the actin cytoskeleton.[65] It is found in all eukaryotic organisms in most cells. Profilin is important for spatially and temporally controlled growth of actin microfilaments.[65]

Profilin binds sequences rich in the amino acid proline in diverse proteins. While most of profilin in a cell is bound to actin, profilins have over 50 different binding partners. Many of these are related to actin regulation, but profilin also seems to be involved in activities in the nucleus, such as mRNA splicing.[66] Profilin binds some variants of membrane phospholipids. The function of this interaction is the sequestration of profilin in an "inactive" form, from where it can be released by action of the enzyme phospholipase C. IgE reactive profilins are present in birch,[67] grass[68] and other pollen.[69–71]

A primary structure comparison of Act d 9 and profilins from other allergen sources revealed amino acid sequence identity with Cap a 2 from bell pepper (93 %), Hev b 8 from latex (90 %) and Bet v 2 from birch pollen (75 %).

rAct d 9 demonstrated IgE binding reactivity *in vitro* in 36 % patients with combined kiwifruit/pollen or kiwifruit/pollen/latex allergy.[29]

3.10. Act d 10

Act d 10 belongs to the family of plant lipid transfer proteins or LTPs.[72] Plant LTPs are widely distributed, structurally related, small proteins involved in defense mechanisms. Although their lipid binding ability has been well reported, the biological function of LTPs is still largely unknown. The plant LTP family includes two subfamilies according to their molecular masses: the 9-kDa LTP1 and the 7-kDa LTP2. Although LTP1 and LTP2 share a common compact fold consisting of four α-helices stabilized by four disulfide bridges, the pairing partners of cysteines are not completely conserved between the two subfamilies, that also display a low overall sequence similarity (about 30 % identity).[73] To date, 63 LTPs have been characterized as allergens, 46 of them expressed in edible parts of plants, almost all of them belonging to the LTP1 protein subfamily, and just two to the LTP2 subfamily (www.allergome.org).

LTP was detected and purified in both gold and green kiwifruit as Act c 10 and Act d 10, respectively. Both proteins are present in seed extract, but not in pulp extract.[72] Direct protein sequencing of the purified protein allowed the

identification of two different isoforms of Act d 10. Possible additional isoforms, suggested by elution profiles obtained during purification, were not identified probably because of the low yield.[72]

MALDI–TOF mass spectrometry of purified nAct d 10 provided two mass values, 9.464 and 9.484 kDa, which are in good agreement with the values deduced from the amino acid sequence of the two isoforms having alanine (9.458 kDa) or threonine (9.488 kDa) as N-terminal residues, respectively.[72]

Analysis by SDS–PAGE and RP-HPLC of LTP samples subjected to digestion in SGF showed that nAct c 10 and nAct d 10, similarly to nPru p 3, are resistant to gastric digestion. Moreover, similar to the results reported by Cavatorta *et al.*,[74] Pru p 3 was partially digested by trypsin, whereas nAct d 10 and nAct c 10 appeared to be resistant.[72]

A homology search in Uniprot protein database realized using the BLAST algorithm (www.expasy.org) showed the sequence identity between Act d 10 and other already known allergenic LTPs to be not very high, ranging between 55 % with Ara h 9 (isoform Ara h 9.0201) and 35 % with Par j 2. The identities among the full-length amino acid sequence of the six allergenic LTPs: Act d 10, Ara h 9, Art v 3, Cor a 8, Mor n 3 and Pru p 3 are in the range from 42 to 70 %. The sequence identity values between Act d 10 and other allergenic LTPs, such as Api g 2 (celery stalk), Cit s 3 (orange), Fra a 3 (strawberry), Lac s 1 (lettuce), Len c 3 (lentil), Lyc e 3 (tomato), Mal d 3 (apple), Ory s 14 (rice), Pla or 3 (plane tree pollen), Pru du 3 (almond), Pyr c 3 (pear), Sin a 3 (mustard), Tri a 14 (wheat), Vit v 1 (grape), Zea m 14 (maize) are found in the narrow range of 40–55 %.[72]

3.11. Act d 11

Act d 11 is a 17-kDa protein found in variable amounts in extracts of green kiwifruit. This is a ripening-related protein, the amount of which is influenced by natural ripening and post harvesting treatments, including exposure to the plant hormone ethylene.[75] Act d 11 displays the highest sequence identity with members of the major latex protein/ripening-related protein (MLP/RRP) family, which belongs to the Bet v 1 superfamily.[76] A lower sequence identity is shared with members of the PR-10 protein family, including Bet v 1.[75]

Several antigenic regions of the surface of Bet v 1 and of co-recognized allergens were described following studies based on the mapping of conserved residues,[77,78] phage-displayed allergen mimotope technology[79] and X-ray crystallography.[80] Most of the amino-acid residues, which belong to B-cell or T-cell epitopes in Bet v 1 or in Bet v 1-related allergens,[77,81] are conserved in Act d 11 and cluster mainly in the regions comprising the p-loop motif and the protein C-terminal domain, where the local sequence identity is significantly high.[75]

The residue Glu[45] was reported to be critically important in Bet v 1 for IgG and IgE binding.[81] Like several MLP/RRPs, Act d 11 shares E45 with Bet v 1 and with most of the homologous allergens. The capacity of Act d 11 to inhibit, at least partially, IgE binding to Bet v 1 and to homologues, such as Cor a 1, Dau c 1 and Mal d 1, suggests epitope-sharing regions higher than that inferable from the low overall sequence identity.[75]

Act d 11 showed IgE reactivity in SPT, double blind placebo-controlled food challenge and immunoblot. Act d 11 was able to inhibit partially Bet v 1, Mal d 1 and Cor a 1 IgE binding. Soluble Bet v 1 achieved 100 % IgE inhibition of almost all Act d 11 positive sera.[75]

4. CROSS-REACTIVITY BETWEEN KIWIFRUIT AND LATEX AND/OR POLLEN ALLERGENS

Cross-reactivity (CR) occurs when an adaptive immune response to a particular antigen causes reactivity to other antigens that are structurally related to the inducer.[82] The World Health Organization guidelines for the prediction of allergenicity specify that a protein can be considered to cross-react with an allergen if they share at least 35 % sequence similarity in a fragment of 80 amino acids or complete identity with a peptide of 6–8 amino acids from an allergen. Latex-fruit syndrome is the association of latex allergy and allergy to plant foods, which affects up to 50 % of latex-allergic patients.[83] The foods most frequently involved are banana (28 %), avocado (28 %), chestnut (24 %) and kiwi (20 %). With these foods, clinical symptoms are often severe, as is the case with other foods less frequently related to latex (fig, papaya and tomato). Allergy to latex usually precedes food allergy, although this is not always the case. Frequently, the spectrum of food allergies increases with time.[83]

Patients allergic to pollen from birch and other Fagales show symptoms of allergy to plant foods. Pollinosis precedes the symptoms induced by the foods. These tend to be slight, characteristically OAS, and occur following ingestion of the raw food. The main culprit allergen, which is involved in more than 90 % of patients with allergy to plant foods associated with allergy to birch pollen, is Bet v 1,[84] a PR-10, which gives rise to cross-reactivity with its homologues in these foods. In kiwifruit, the two Bet v 1 homologues identified so far in green and gold specimens are Act d 8 and Act c 8,[61] but also Act d 11.[75]

Profilins are structural proteins that are both ubiquitous and very well conserved during evolution. They are considered incomplete allergens, capable of inducing sensitization by inhalation, but not by ingestion, due to their lability against peptic digestion. Thus, whilst in the north of Europe, it is associated with allergy to birch pollen,[85] while in Spain, it is more frequent and associated mainly with pollinoses due to grasses.[86] The clinical manifestation of this food allergy is OAS induced by the raw food. Several foods could be involved, given

that many allergenic profilins have been described in plant foods that are eaten raw. Profilin present in green kiwifruit extract was designated Act d 9.[29]

In the study of Gavrović-Jankulović *et al*., a molecular basis of IgE cross-reactivity between meadow fescue pollen and kiwifruit has been found between Fes p 4, a 36-kDa meadow fescue allergen and a 24-kDa kiwifruit protein.[13]

5. DIAGNOSIS OF KIWIFRUIT ALLERGY

At present, the diagnosis of kiwifruit allergy is unsatisfactory. *In vitro* and *in vivo* tests based on commercially available kiwifruit extracts frequently fail to detect specific IgE, resulting in a low sensitivity.[29] Diagnosis performed by skin prick testing with kiwifruit extract has a sensitivity of only 40 to 50 %, while the measurement of food specific serum IgE has a sensitivity of 17 to 60 %.[87] Due to the poor correlation between a suggestive case history and a skin prick test (SPT) performed with commercial kiwifruit protein extracts, improved *in vivo* diagnosis of kiwifruit allergy necessitates the use of fresh fruit, such as in the prick–prick technique. However, although prick-to-prick tests with fresh kiwifruit have a higher sensitivity (83 to 100 %), they also have a low specificity (31 %) and are difficult to standardize.[12,15] The low diagnostic sensitivity of *in vitro* and *in vivo* tests is related to the low level or absence of kiwifruit proteins from commercially available kiwifruit extracts. Preparation of higher quality extracts that would give reproducible results is limited by the natural variability of the plant source material.[29] It was also shown that the natural ripening stage, cold storage, and ethylene treatment influence the protein composition and IgE-binding profiles of both green and gold kiwi fruit extracts.[88,89]

Two recent studies showed that the use of individual kiwifruit allergens increased the diagnostic sensitivity compared with use of commercial extracts. In a study by Bublin *et al*., the authors evaluated the use of individual allergens for component-resolved *in vitro* diagnosis of kiwifruit allergy. The study was performed on thirty patients with a positive double-blind placebo-controlled food challenge (DBCFC) to kiwifruit. Specific IgE to 7 individual allergens and allergen extracts was measured by ImmunoCAP. The use of individual allergens raised the diagnosis sensitivity from the 17 % obtained with commercial extract to 77 %, but the diagnostic specificity was lowered from 100 to 30 %. Using only kiwi allergens Act d 1, Act d 2, Act d 4, and Act d 5 gave a diagnostic sensitivity of 40 %, whereas diagnostic specificity remained high (90 %).[29] The performance of a component-based allergen micro-array for the diagnosis of kiwifruit allergy was evaluated by Bublin *et al*. The specific IgE and IgG4 levels to a panel of nine kiwifruit allergens where measured in sera of 237 individuals with kiwifruit allergy. The panel of kiwifruit allergens showed a diagnostic sensitivity of 66 %, a specificity of 56 % and a positive predictive value of 73 %.[7]

Molecular basis of kiwifruit allergy has been extensively investigated in the last decade and huge progress has been made in the isolation and characterization of kiwifruit allergens. However, kiwifruit allergen extracts are still employed in clinical settings for *in vivo* allergy diagnosis by the skin prick test. In order to improve performance of allergy diagnosis, it seems that the component-resolved concept with a selected panel of natural and/or recombinant kiwifruit allergens will replace allergen extracts in future allergy testing.

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И З В О Д

ПЛОД КИВИЈА КАО ИЗВОР АЛЕРГЕНА ХРАНЕ

МИЛИЦА ПОПОВИЋ, МИЛИЦА ГРОЗДАНОВИЋ и МАРИЈА ГАВРОВИЋ-ЈАНКУЛОВИЋ

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Од првог појављивања на тржишту плод кивија је постао изузетно популаран састојак хумане исхране услед пријатног укуса, ниске калоријске вредности и високог садржаја витамина С. Међутим, алергија на киви је постала учестали узрок преосетљивости типа I у западном друштву. До сада је откривено 11 IgE везујућих молекула који чине молекулску основу алергије на киви. То су протеини и гликопротеини молекулских маса између 10 и 50 kDa. Главни алерген кивија је цистеин-протеаза означена као Act d 1, која сачињава 50 % растворних протеина плода кивија. Услед разлике у заступљености протеинских компоненти и биолошкој активности, квалитет протеинских екстраката кивија који се употребљавају у дијагностификовању алергије може варирати у садржају и количини IgE реактивних молекула. Такође, квалитет алергених екстраката зависи од степена зрелости воћа приликом брања, као и од услова складиштења воћа након брања. По питању клиничке реактивности постало је очигледно да алергија на плод кивија не представља хомогени поремећај. Различити обрасци IgE реактивности уочени су код неколицине клиничких подгрупа које су идентификоване у различитим географским регијама. Током последње деценије начињен је велики напредак у изоловању и карактеризацији IgE везујућих протеина кивија. У оквиру овог рада даћемо преглед структурних особина алергених протеина кивија.

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REVIEW

Vitamin D and allergies

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Abstract: An increasing amount of evidence has established that the biologically active form of vitamin D, 1,25-dihydroxyvitamin D3, possesses immunoregulatory properties. Vitamin D exerts its effects through binding to the nuclear vitamin D receptor (VDR), which is expressed by cells of the immune system. Most of the immunological effects mediated by vitamin D–VDR are regulatory, inhibiting adaptive immune responses. It has become apparent that the incidence of vitamin D insufficiency is surprisingly high in the general population. A link between low vitamin D serum levels and the increased prevalence of allergic diseases has been proposed. This possible connection was investigated in numerous studies on associations between vitamin D serum concentrations and different allergic conditions, as well as studies on the effect of vitamin D supplementation. Although there is some evidence for a protective role of vitamin D in asthma, no consensus on the role of vitamin D in allergic disease has yet been reached. Still, treatment strategies involving vitamin D supplementation to risk groups, combinatorial corticosteroid and vitamin D treatment in asthma and vitamin D as an immunomodulator in allergen-specific immunotherapy show promise for the future.

Keywords: vitamin D; allergy; immunomodulator.

CONTENTS

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1. BIOSYNTHESIS AND MODE OF ACTION

Vitamin D is generated in the human body during exposure to sunlight. Only a minor proportion is normally obtained through diet, *e.g.*, from oily fish species and vitamin D supplemented food products.[1] UVB radiation converts 7-dehydrocholesterol in the skin into pre-vitamin D3, which after isomerization is converted to cholecalciferol. After transport to the liver, cholecalciferol is hydroxylated by 25-hydroxylase to form 25-hydroxyvitamin D3 (calcidiol), which is the main circulating form of vitamin D. To generate the biologically active form of vitamin D, *i.e.*, 1,25-dihydroxy vitamin D3 or calcitriol, 25-hydroxyvitamin D3 is subjected to hydroxylation by 1α-hydroxylase (CYP27B1).[2,3] This second hydroxylation step mainly occurs in the kidney, but CYP27B1 is also expressed in a number of other tissues (Fig. 1). Both vitamin D2 (ergocalciferol) and vitamin D3 are used as vitamin D supplements.[4] Assays analyzing serum vitamin D levels measure 25-hydroxyvitamin D, including 25-hydroxyvitamin D2 and 25--hydroxyvitamin D3.[4] Hereafter, the term "vitamin D" will be used for both biologically active calcitriol and when referring to supplementation or serum levels of vitamin D.

The biological effect of vitamin D is mediated by the nuclear vitamin D receptor (VDR). The VDR belongs to the nuclear hormone receptor superfamily and is expressed in most tissues of the body. When vitamin D binds to VDR, the receptor dimerizes with the retinoic X receptor (RXR) and the complex activates transcription of genes through interaction with VDR-responsive elements (VDRE) in the promoter regions of these genes.[2,5,6] A couple of thousand target genes have been found to respond to vitamin D/VDR activation.[7] Vitamin D plays a well-established role in calcium-phosphate homeostasis and bone metabolism, not least evident by the skeletal symptoms seen in rickets and osteomalacia caused by vitamin D deficiency. However, as indicated by the widespread expression of both CYP27B1 and VDR, vitamin D has several "non-classical" effects in addition to its role in calcium metabolism. These non-classical functions of vitamin D include regulation of hormone secretion, cellular proliferation and cell differentiation.[3] Vitamin D also exerts immunomodulatory effects.[5,8,9]

2. VITAMIN D AND THE IMMUNE SYSTEM

One clear indication that vitamin D might play a role in the regulation of immune responses comes from the observations that cells of the immune system express VDR and respond to vitamin D. Thus, *e.g.*, monocytes,[10] dendritic cells (DC)[11] and T-cells[12,13] express the VDR, and VDREs are found in genes important for immune regulation, *e.g.*, FOXP3,[14] IL-10,[15] and CD14.[16] Another observation indicating a possible role of vitamin D in immune regulation is the increased prevalence of cancer, allergic and autoimmune diseases coinciding with an increasing prevalence of life style related vitamin D insufficiency.[17] Moreover,

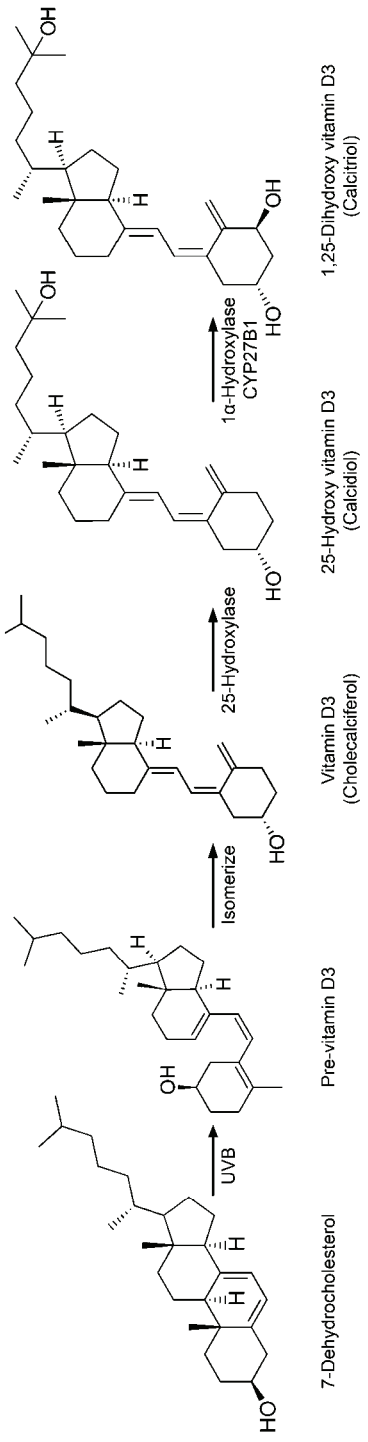

Fig. 1. Metabolic activation of vitamin D. Conversion of 7-dehydrocholesterol to vitamin D3 occurs in the skin, conversion from vitamin D3 to 25-hydroxyvitamin D3 occurs in the liver and the biologically active 1,25-dihydroxyvitamin D3 is produced in the kidney and other tissues, including cells of the immune system.

genetic polymorphisms in the VDR gene have been linked to immune and inflammatory conditions such as Crohn's disease,[18] tuberculosis[19] and asthma.[20,21]

Vitamin D can affect both innate and adaptive immunity.[22] Cells of importance for the innate response to pathogens, *e.g.*, macrophages and epithelial cells, are able to produce locally active vitamin D as they express CYP27B, and to respond to vitamin D through their expression of VDR.[23–25]. Furthermore, vitamin D promotes the synthesis of the antimicrobial peptide cathelicidin, which is part of the innate response to pathogens,[26,27] and induces autophagy in macrophages, important for the clearance of intracellular pathogens.[28,29] Innate responses may be negatively regulated by vitamin D through the down-regulation of pathogen pattern-recognition receptors, such as Toll-like receptors (TLR), and suppression of TLR-mediated inflammation.[30] If vitamin D seems to have both activating and regulatory effects on the innate immune response, the effect on adaptive immunity is mainly regulatory. Vitamin D may limit adaptive immune responses through its suppressive action on DCs. Several studies showed that vitamin D inhibits the differentiation and maturation of DCs by down-regulation of co-stimulatory molecules and reduces the production of pro-inflammatory cytokines.[31–33] When cultured with vitamin D *in vitro*, tolerogenic DCs are generated, *i.e.*, the DCs adopt an immature phenotype and produce lower levels of IL-12 and enhanced levels of IL-10.[34,35] Such tolerogenic DCs are able to promote regulatory T-cell (Treg) responses.[31,36,37] It was also shown that vitamin D alone or in combination with dexamethasone induces Tregs.[38–41] Although the promoting effect of vitamin D on regulatory T cell responses is well established, the data on vitamin D's effects on Th2 responses are contradictory. A Th2 promoting effect was suggested by data showing that both Th1 and Th17 responses are inhibited by vitamin D.[42–46] Moreover, vitamin D was shown to enhance the development of Th2 cells from naïve T-cells in mice.[47] On the other hand, by its ability to induce Tregs, as discussed above, vitamin D can inhibit Th2 responses. A mechanism involving decreased expression of OX40 and increased expression of TGF-β by DCs that reduces the Th2 response has been described.[48] Taken together, the pronounced effect of vitamin D on immune regulation suggests that vitamin D insufficiency may play a role in allergic disease.

3. VITAMIN D IN ALLERGIC DISEASE

Allergic disorders are caused by an imbalanced immune response elicited against allergens, which results in a Th2 skewed response and allergic inflammation. Depending on the target tissue for allergen exposure, the allergy may manifest as rhinoconjunctivitis or allergic asthma to inhalant allergens, eczema to food- or contact allergens, gastrointestinal/systemic symptoms to ingested food allergens and systemic reactions to injected allergens. The prevalence of allergic diseases has increased considerably during the last decades.[49] One explanatory

model for this increase is the hygiene hypothesis, stating that a reduced microbial burden due to improved hygiene in modern society leads to a hypersensitive immune system and increased risk of developing allergy.[50] The modern Westernized life style also implies changed diet and less time spent outdoors, factors that influence the vitamin D status. Indeed, it has been reported that a large proportion of the population in modern societies has vitamin D serum levels corresponding to deficiency or insufficiency. In a study on a US pediatric population, 9 % of the examined children and adolescents were vitamin D deficient (defined as <15 ng ml^{-1}) and as many as 61 % were considered vitamin D insufficient (15–29 ng ml^{-1}).[51] In an adult British population, approximately 15 % had vitamin D serum levels <25 nmol L^{-1} (considered to correspond to vitamin D deficiency) during the winter–spring season.[17] There is no consensus regarding cut-off levels for vitamin D deficiency and insufficiency but recent US guidelines have proposed vitamin D levels above 20 ng mL^{-1} to be considered as vitamin D sufficiency.[52] It should be noted that guidelines for vitamin D serum levels are based on bone health and that consistent clinical/epidemiologic data on optimal levels for beneficial effects on non-musculoskeletal health are lacking. Still, it was suggested already in 1999 that there may be a link between the nutritional intake of vitamin D and allergies.[53] Since then many studies have investigated the relationship between vitamin D and allergy. In a review from 2012, Reinholz *et al.* presented a table of clinical studies on vitamin D in allergic disease.[54] Eighteen of the listed studies suggested a protective role of vitamin D, six a deleterious role and two studies reported no role for vitamin D in allergic disease. These studies represent populations of various ages and geographic locations, patients with different allergic manifestations as well as subjects with no allergic diagnosis, different means of determining vitamin D status and heterogenic study designs. Taken together, these factors illustrate the difficulties encountered to reach a consensus regarding the role of vitamin D in allergy. The complexity is exemplified below, where some of the studies are discussed.

One may divide studies investigating the role of vitamin D in allergy into two main categories. In association studies, vitamin D serum levels are linked to the incidence of allergic disease. Supplementation studies investigate the effect of vitamin D supplementation, given prenatal (maternal supplementation) or postnatal, on allergic status later in childhood or adult life. In 45 years old subjects from a British cohort, low (<25 nmol L^{-1}) and high levels (>135 nmol L^{-1}) of serum vitamin D correlated with higher IgE serum concentrations compared to "normal" vitamin D levels.[55] No correlation was found between vitamin D serum levels and total IgE levels in a Chinese population with recently diagnosed asthma. However, it was shown that vitamin D insufficiency was prevalent in the same population and that vitamin D status correlated with lung function.[56] The relationship between lung function and vitamin D serum levels was examined in

14,091 subjects, 20 years or older, participating in a cross-sectional study conducted in the US. The subjects underwent spirometry and serum vitamin D concentrations were determined. In this study, a strong positive relationship was found between serum vitamin D levels and lung function measured as forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC).[57] In contrast, in another case-controlled study on adult patients with asthma and controls performed in the UK, no association between vitamin D and asthma severity or lung function could be found.[58] These studies were all conducted on adults and vitamin D status may be of more importance at an early age during the development of the immune system. Camargo *et al.* analyzed maternal intake of vitamin D during pregnancy and correlated it to recurrent wheeze at three-year age in a prospective pre-birth cohort study in the US.[59] In this study, a higher maternal intake of vitamin D resulted in lower prevalence of recurrent wheeze, which is a predictor of asthma. A study performed on children in Costa Rica showed that 28 % of asthmatic children exhibited vitamin D deficiency or insufficiency (< 30 ng mL^{-1}) despite living at a latitude with high sun exposure. Several disease markers, such as total serum IgE, eosinophil count, hospitalization for asthma, anti-inflammatory medication and airway hyper-responsiveness, inversely correlated with vitamin D serum concentrations in this study population.[60]

Vitamin D supplementation has been examined in relation to allergic disease. A couple of studies on children who received oral vitamin D supplementation reported an increased risk for the development of allergic disease in childhood or later in life.[61–63] Vitamin D supplementation may nonetheless be beneficial by reducing the risk of respiratory infections and, consequently, lowering the risk of asthma exacerbations. This was recently shown in a randomized placebo-controlled trial on school children in Japan.[64] Children receiving vitamin D supplementation during December–March had a reduced incidence of influenza A infections compared to those receiving placebo. In a subgroup of children with asthma, exacerbations were less common in the vitamin D supplemented group.

4. VITAMIN D IN EXPERIMENTAL MODELS OF ALLERGY AND ASTHMA

To understand better the mechanisms of how vitamin D may affect allergy, murine experimental models have been employed. In these models, mice are sensitized with a model allergen to evoke an allergic Th2 skewed immune response, followed by allergen challenge in the airways. The allergic immune response, allergic inflammation and airway hyper-reactivity are then investigated. Data obtained from VDR knockout mice (VDR KO) indicated that vitamin D might play a deleterious role in allergy and asthma. The VDR KO mice failed to develop airway inflammation and airway hyper-reactivity after chicken albumin (OVA) sensitization, despite high IgE levels and Th2 cytokine production.[65,66] It

should be noted that in this experimental model, vitamin D treatment did not affect airway inflammation or hyper-reactivity in sensitized wild type mice. In contrast, Topilski *et al.* applied a mouse model for OVA-induced experimental asthma, in which they could show that pretreatment with intraperitoneally (i.p.) administered vitamin D reduced the eosinophilic airway inflammation and IL-4 levels in the bronchoalveolar lavage fluid (BALF) of sensitized mice. Treatment after sensitization but before airway challenge with OVA also significantly reduced the airway inflammation, but to less extent compared to vitamin D given prior to sensitization.[67] Another study using an OVA model demonstrated an increased allergen induced Th2 profile but a decreased inflammatory response detected in the BALF of mice treated with vitamin D before and during the sensitization and allergen challenge protocol.[68] These results from experimental models suggest that vitamin D may have dual effects on the allergen specific Th2 response and allergen-induced airway inflammation.

5. VITAMIN D AS A POSSIBLE THERAPEUTIC TOOL IN ALLERGY AND ASTHMA

The results reported by Topilski *et al.* indicate that it is possible to decrease the allergic airway inflammation by vitamin D treatment in previously sensitized mice with an established Th2 skewed allergen specific immune response.[67] The concept to use vitamin D as an immunomodulator in allergy treatment was further explored by Taher *et al.*[69] Mice sensitized to OVA were treated with OVA alone or in combination with vitamin D prior to airway challenge with OVA. Co-administration of vitamin D with OVA significantly inhibited the airway hyper-responsiveness and potentiated the OVA treatment effect by increasing the reduction of serum OVA-specific IgE levels, airway eosinophilia and Th2-related cytokines. The treatment-potentiating effect of vitamin D seemed to be mediated by the immunoregulatory cytokines IL-10 and TGF-β, since the levels of these cytokines were elevated in the vitamin D-treated mice and the treatment effect was abrogated in the presence of antibodies to these cytokines.[69] A similar concept was tested in a mouse model for cat allergy.[70] In this study, the major cat allergen Fel d 1 was covalently linked to vitamin D and treatment of Fel d 1 sensitized mice with the Fel d 1-vitamin D vaccine was compared to treatment with Fel d 1 alone. Both treatments decreased allergen-specific IgE, Th2 cytokines in the BALF, airway eosinophilia and airway hyper-responsiveness, and in addition generated Fel d 1–specific IgG. The Fel d 1–vitamin D vaccine was more potent than Fel d 1 alone in inhibiting the allergen-challenge induced airway symptoms, especially the eosinophilic inflammation.[70] Both these studies present promising strategies for improving the current allergen specific immunotherapy by combining an allergy vaccine with vitamin D. The concept of covalently linking vitamin D directly or indirectly to an allergen is particularly interesting as it might enhance the efficacy of the immune modulation,

but it is a challenge to produce stable vaccine formulations with preserved vitamin D biological activity.

Vitamin D was demonstrated to enhance dexamethasone stimulated IL-10 synthesis in human CD4+ T-cells.[38] Interestingly, vitamin D was able to restore the lost ability of T-cells from patients with steroid-refractory asthma to respond to dexamethasone by IL-10 production *ex vivo*.[39] Three patients with steroid-refractory asthma were given oral vitamin D and peripheral blood cells were collected before and after treatment. *In vitro* stimulation of the cells in the presence of dexamethasone showed that vitamin D treatment enhanced steroid responsiveness and led to increased production of IL-10.[39] The data suggest that vitamin D could potentially restore the therapeutic effect of glucocorticoids in patients with steroid-resistant asthma.

6. CONCLUDING REMARKS

An increasing amount of evidence points to an important regulatory role for vitamin D in innate and adaptive immunity. The current data suggest that vitamin D may have a beneficial effect on asthma, while the influence on the allergic immune response remains unclear. Carefully designed long-term interventional studies with vitamin D supplementation are required to elucidate further the role of vitamin D in allergy. Still, treatment strategies involving vitamin D supplementation to risk groups, combinatorial corticosteroid and vitamin D treatment in asthma and vitamin D as an immunomodulator in allergen specific immunotherapy show promise for the future.

И З В О Д

ВИТАМИН Д И АЛЕРГИЈЕ

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Све је више података да биолошки активна форма витамина Д, 1,25-дихидрокси--витамин D3, има имунорегулаторне особине. Витамин D свој ефекат испољава након везивања за рецептор за витамин D у једру (VDR), који је експримиран у ћелијама имуног система. Већина имунолошких ефеката посредованих витамином D–VDR су регулаторни, инхибирајући адаптивни имуни одговор. Међу становништвом је изненађујуће велика заступљеност недостатка витамина D. Има показатеља да постоји веза између мале концентрације витамина D у серуму и алергијских болести. У бројним студијама је испитивана могућа веза између серумске концентрације витамина D и различитих алергијских стања, као и ефекат узимања витамина D. Иако постоје подаци о заштитној улози витамина D у астми, нема општег става о улози витамина D у алергијским болестима. Употреба витамина D код ризичних група, комбинована терапија кортикостероидима и витамином D код астме, као и употреба витамина D као имуномодулатора у алерген-специфичној имунотерапији, обећавајући су терапеутски правци.

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Review

REVIEW

Ameliorating effects of antioxidative compounds from four plant extracts in experimental models of diabetes

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Abstract: Given that oxidative stress plays a major role in pancreatic β-cell dysfunction and ultimate destruction, as well as in different complications of diabetes, therapy with antioxidants has assumed an important place in the management of diabetes. The relatively limited effects of established antioxidant compounds have stimulated efforts to develop new therapeutic strategies, *e.g.* to increase the endogenous antioxidant defences through pharmacological modulation of key antioxidant enzymes. Plant extracts are gaining popularity in treating diabetes because many substances synthesized by higher plants and fungi possess antioxidant activities and can prevent or protect tissues against the damaging effects of free radicals. This review summarizes experimental models of diabetes and possible mechanisms that lie behind the antioxidative effects of α-lipoic acid (LA), a powerful antioxidant and compound that stimulates cellular glucose uptake, as well as of plant extracts from sweet chestnut (*Castanea sativa*), edible mushroom (*Lactarius deterrimus*) and natural products containing β-glucans in the treatment of diabetes. Their roles in preventing pancreatic β-cell death and in ameliorating the effects of severe diabetic complications are discussed.

Keywords: diabetes; oxidative stress; lipoic acid; plant antioxidants.

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1. INTRODUCTION

Diabetes mellitus is a chronic metabolic disease with an aetiology linked to both genetic and environmental factors. Diabetes has become a global health problem due to its high incidence and latent harmful and lethal effects. According to the World Health Organization (WHO),[1] 347 million people worldwide have diabetes, which has greatly increased the cost of treating both the disease and its numerous devastating complications. According to the International Diabetes Federation (IDF), 4.6 million people die each year from the consequences of diabetes,[2] with more than 80 % of diabetes-related deaths occurring in low and middle income countries.[1] According to The Public Health Institute of Serbia "Dr Milan Jovanović Batut",[3] 630,000 people or 8.2 % of the Serbian population suffer from diabetes and about 3,000 diabetics die each year. Type 1 diabetes (T1D) is characterized by destruction of pancreatic *β*-cells and thereby loss of insulin secretion. Type 2 diabetes (T2D) is associated with progressive insulin resistance and *β*-cell dysfunction. Deficiency of insulin secretion or action in diabetes causes prolonged hyperglycaemia that in turn leads to severe diabetic complications, such as retinopathy, neuropathy, nephropathy, cardiovascular problems, liver disease and limb amputation. Diabetes treatment includes insulin injection in combination with application of hypoglycaemic drugs. However, current control of diabetes-associated complications and mortality is not satisfactory.

Limitations in diabetes treatment have stimulated efforts to develop new therapeutic strategies. Growing evidence in both experimental and clinical studies suggests that oxidative stress plays an important role in pancreatic *β*-cell destruction/dysfunction and subsequent complications of diabetes. Therefore, strategies for diabetes management include antioxidant protection. However, established antioxidant compounds, such as vitamins C and E, have yielded limited effects, indicating the necessity for examination of other antioxidant compounds. Given that antioxidant enzyme expression and function is deregulated in diabetes, pharmacological modulation of key enzymes that are responsible for reducing the oxygen radical load is a potentially more effective approach than the use of systemic antioxidants.[4,5] It is therefore imperative to continuously identify new products with antioxidant activities for use in "causal" therapy of diabetes.[6]

Plant extracts are gaining popularity in diabetes treatment because of their efficacy, low incidence of side effects, their accessibility and low cost. Identifying new agents from plants with hypoglycaemic and antioxidative activities is of great importance. In this review, a summary will be given of the ameliorating effects and potential mechanisms of the actions of the known antioxidant compound α-lipoic acid (LA), extracts of the sweet chestnut (*Castanea sativa*), edible mushroom (*Lactarius deterrimus*) and their combination (MIX Cs/Ld), and of a β-glucan-enriched extract in the treatment of diabetes, *i.e.*, in the prevention of pancreatic β-cell death and amelioration of the severe complications in diabetes. Experimental models of diabetes will also be discussed.

2. OXIDATIVE STRESS IN THE DEVELOPMENT OF DIABETES AND ITS COMPLICATIONS

Oxidative stress is generally defined as a persistent imbalance between the concentrations of generated highly reactive free radical reactive oxygen species (ROS) and reactive nitrogen species (RNS) on the one hand and the antioxidant defence of the organism on the other. Hyperglycaemia promotes the formation of elevated levels of free radicals, especially ROS, *via* different routes of activation: glucose autoxidation,[7] non-enzymatic protein glycation,[8] increased metabolism of glucose through the hexosamine pathway,[9] excessive activation of the polyol pathway by unused glucose,[10] and by advanced glycation end-products (AGE) formed by the interaction of glucose with proteins.[11] One of the main sources of free radicals in diabetes is glucose autoxidation.[12] In a transition-metal dependent reaction, the enediol form of glucose is oxidized to an enediol radical anion that is converted into reactive ketoaldehydes and to superoxide anion radicals ($O_2^{\bullet-}$). The superoxide anion radicals undergo dismutation to hydrogen peroxide (H_2O_2), which, if not degraded by catalase (CAT) or glutathione peroxidase (GSH-Px), in the presence of transition metals can lead to the production of extremely reactive hydroxyl radicals (OH·).[13] Superoxide anion radicals can also react with nitric oxide (NO) to form reactive peroxynitrite. Peroxynitrite is chemically unstable under physiological conditions and reacts with all major classes of biomolecules, mediating cytotoxicity,[14] AGE signalling, through the receptor for AGE (RAGE), and inactivation of enzymes by altering their structure,[15] thereby supporting additional free radical accumulation.[16] Excess levels of free radicals damage cellular proteins, lipids and nucleic acids, leading to cell death in various tissues (the cardiovascular system, retina, kidneys, liver, peripheral nerves and skin), thus contributing to diabetes complications. The harmful effects of ROS and RNS are neutralized by the endogenously expressed antioxidant enzymes (superoxide dismutases (SODs), CAT, GSH-Px) and non-enzymatic antioxidants (endogenous reduced glutathione (GSH) and exogenous vitamins C and E).[7]

3. EXPERIMENTAL MODELS OF DIABETES MELLITUS

Experimental models play an important role in understanding diabetes, as well as in evaluating the pharmacological actions of different agents. Isolated rat, mouse and human pancreatic islets are used for investigating the mechanisms involved in *β*-cell dysfunction and destruction in diabetes.[17–19] The use of primary *β*-cells in research is limited because their purification and maintenance of native characteristics is technically demanding. To overcome these limitations, investigators have produced immortalized *β*-cell lines.[20] The most widely used insulin-secreting cell lines are rat insulinoma cells (RIN and INS-1), hamster pancreatic *β*-cells (HIT) and mouse insulinoma cells (MIN and *β*TC). Although the properties of the cell lines differ slightly from those of primary *β*-cells, they are extremely valuable tools for the study of molecular events underlying *β*-cell function, including the testing of the effects of potential drugs in diabetes management.

Several animal models have been developed for *in vivo* studies of diabetes and anti-diabetic agents. Genetic models of diabetes include the spontaneous development of diabetes in rats[21,22] and genetically engineered diabetic mice to either overexpress (transgenic) or underexpress (knockout) proteins thought to play a key role in glucose metabolism.[23,24] Surgical model of diabetes include the complete removal of the pancreas (pancreatectomy) or partial pancreatectomy (more than 80 % resection in rats). These models allow for the evaluation of the effect of natural products in an animal without the interference of side effects induced by chemical drugs used to induce experimental diabetes.[25,26] However, these models are rarely used because of the highly specialized technical skills required and the high percentage of animal mortalities.

Chemical induction is the most popular procedure for inducing diabetes in experimental animals and has been proven repeatedly to be useful for the study of multiple aspects of the disease. Streptozotocin (STZ), a naturally occurring glucosamine–nitrosourea compound is the most frequently used drug for experimental diabetes induction in laboratory rats. As a glucose analogue, STZ selectively accumulates in *β*-cells *via* the glucose transporter (GLUT2). As an alkylating agent, STZ fragments DNA.[27] DNA damage induces activation of the DNA repair process that leads to enhanced ATP dephosphorylation, which supplies a substrate for xanthine oxidase, resulting in ROS formation. The diabetogenic effect of STZ also relies on its ability to liberate NO which participates in DNA damage. STZ is capable of inducing T1D either by direct *β*-cell destruction after administration of a single large dose of STZ (65–150 mg kg^{-1}),[28] or *via* an immune cell-mediated mechanism using multiple low doses of STZ (40 mg kg^{-1}).[29] A single high dose of STZ causes extensive non-physiological *β*-cell necrosis, whereas multiple low doses of STZ induce limited apoptosis, which elicits an autoimmune reaction that eliminates the remaining cells.[30] There is a general

consensus that the experimental model of multiple low-dose STZ-induced diabetes resembles more closely the *in vivo* state of insulinaemia, reflecting its autoimmune nature and resulting onset of diabetes.

4. NUMEROUS ANTIOXIDANT COMPOUNDS AND PLANT EXTRACTS IN DIABETES MANAGEMENT

A number of studies have demonstrated that treatment with antioxidants reduces oxidative stress and alleviates diabetic complications in diabetic subjects and animals.[31] Vitamins C and E, and LA are the most studied antioxidants. Vitamin C is the strongest physiological antioxidant. It regenerates vitamin E through redox cycling and increases intracellular GSH levels.[32] Small clinical trials showed that vitamin E, as well as a combination of vitamin E and C, exerted beneficial effects on the cardiovascular system in T1D patients[33,34] and improved renal function in T2D patients.[35] However, in large-scale clinical trials, *i.e.*, Heart Outcomes Prevention Evaluation (HOPE),[36] Secondary Prevention with Antioxidants of Cardiovascular Disease in End Stage Renal Disease (SPACE),[37] the Primary Prevention Project (PPP)[38] and the Study to Evaluate Carotid Ultrasound Changes in Patients Treated With Ramipril and Vitamin E (SECURE),[39] vitamin E treatment failed to provide any benefit in cardiovascular disorders or nephropathy. A multifactorial approach is more efficient than conventional therapy for the prevention of oxidative stress-induced vascular complications in diabetes.[40] Daily supplementation of vitamin C (250 mg), vitamin E (100 mg), folic acid (400 mg) and chromium picolinate (100 mg) in combination with multifactorial intensive therapy resulted in an almost 50 % decrease in cardiovascular incidents.

LA stimulates cellular glucose uptake and possesses direct radical-scavenging and metal-chelating properties, and has the ability to regenerate other antioxidants.[41] Naturally occurring LA is present in low amounts in vegetables and animal tissues where it functions as a coenzyme in pyruvate dehydrogenase and α-ketoglutarate dehydrogenase mitochondrial reactions. The most abundant plant sources of LA are spinach, followed by broccoli and tomatoes. Synthetic LA has a relatively long history of use as a nutritional supplement in European countries and the United States, and as a therapeutic agent in the treatment of diabetic neuropathy and retinopathy.[42] Studies with LA, *i.e.*, Alpha Lipoic Acid in Diabetic Neuropathy (ALADIN) I, II and III,[43–45] and Deutsche Kardiale Autonome Neuropathie (DEKAN),[46] investigated the effect of LA treatment on sensory symptoms of diabetic polyneuropathy as assessed by the Total Symptom Score (SYDNEY)[47] and meta-analysis[48] led to its approval for the treatment of diabetic neuropathy, and initial results are more promising than those obtained with vitamin E. Parallel with LA use, questions of its safety and effectiveness have been raised. A daily oral dose of 600 mg provides an optimum risk-to-benefit ratio in

human diabetics.[42] LA supplementation at higher doses causes a few serious side effects, such as gastrointestinal disorders and allergic reactions. Selection of the appropriate dose of LA for application in diabetes is critical.[49] As cholestatic hepatitis was probably caused by LA (600 mg day^{-1}) treatment of symptomatic diabetic neuropathy,[50] the authors suggest liver enzyme levels be monitored during LA treatment.

Phytochemicals, the bioactive non-nutrient plant compounds in fruit, vegetables, grains and other plant foods, have been linked to reductions in the risk of major chronic diseases. It is estimated that more than 5,000 phytochemicals have been identified, but that a large percentage remains unknown.[51] Phytochemicals with antioxidative effects include a variety of phytosterols, terpenes and especially polyphenols, such as flavonoids, tannins and phenylpropanoids. A direct correlation between the total phenolic content and antioxidant capacity was established and explained through a number of different mechanisms, such as free radical scavenging, metal ion chelation and hydrogen donation.[52–54]

There is a growing interest for the use of plant extracts because purified bioavailable phenolic compounds are difficult to obtain, and because extracts sometimes have better antioxidant activities than the pure molecules.[55] Taken alone, the individual antioxidants studied in clinical trials do not appear to have consistent diabetes-preventive effects. Studies of different fruit combinations showed greater total antioxidant activity because of their additive and synergistic relationships.[51] An isolated pure compound can lose its bioactivity or may not exhibit it in the same way as when present in whole foods. This partially explains why no single antioxidant can replace a combination of natural phytochemicals contained in plants in accomplishing health benefits. Although plants are rich in antioxidants, individual “antioxidant” molecules cannot just be extracted, packed in pills in high doses and expected to provide high levels of protection.[5] Pills or tablets cannot mimic the balanced natural combination of phytochemicals present in plants. Phytochemicals differ in molecular size, polarity, and solubility, and these differences may affect the bioavailability and distribution of each phytochemical in different macromolecules, sub-cellular organelles, cells, organs, and tissues. These observations have lead to the concept that antioxidants are better implemented through whole food consumption than as expensive dietary supplements. Further research on the health benefits of phytochemicals in whole foods is of essential interest.[56]

Many investigations have studied the effects of antioxidant components of plants on diabetes and its complications. Antioxidant and antihyperglycemic properties of *Allium cepa* L., *Anoectochilus formosanus*, *Lycium barbarum*, *Cassia fistula* L., *Aloe vera*, *Vitis aestivalis* and *Coffea arabica* in chemical models of diabetes have been demonstrated.[57–63] In addition, *Centaurium erythrea* and *Aegle marmelos* extracts and quercetin, a flavonoid antioxidant present in many

plants, alleviate STZ-induced cell damage and oxidative stress in rat pancreas.[65–66] Medicinal plants with proven antidiabetic and related beneficial effects in diabetes treatment also include: *Allium sativum*, *Eugenia jambolana*, *Momordica charantia*, *Ocimum sanctum*, *Phyllanthus amarus*, *Pterocarpus marsupium*, *Tinospora cordifolia*, *Trigonella foenum graecum* and *Withania somnifera*.[67]

5. NOVEL MECHANISM OF ANTIOXIDATIVE EFFECT OF LA IN DIABETES

Numerous studies indicate that LA exerts its antioxidant effect by increasing the endogenous defence response of cells through enhanced synthesis of endogenous low molecular weight antioxidants and antioxidant enzymes.[42] The levels of antioxidant enzymes are regulated by gene expression, as well as by post-translational modifications.[68] Emerging data indicates that the post-translational addition of *β*-*N*-acetylglucosamine (*O*-GlcNAc) to proteins has a role in the aetiology of diabetes.[69] *O*-linked glycosylation of certain proteins is increased in hyperglycaemia because of activation of the hexosamine pathway, which produces uridine-diphospho-*N*-acetylglucosamine (UDP-GlcNAc), a substrate for the glycosylation reaction. This modification is dynamic and may disturb the normal dynamic balance between *O*-GlcNAcylation and *O*-phosphorylation that controls enzyme activity, DNA binding, protein–protein interactions, the half-life of proteins and their sub-cellular localization. Elucidation of the specific roles of *O*-GlcNAc in transcription, cell signalling, glucose toxicity and insulin resistance should lead to new avenues for the diagnosis and treatment of diabetes.[69]

A novel mechanism of the antioxidant effect of LA in diabetes progression through decreased *O*-GlcNAcylation of the key proteins that are involved in redox signalling pathways was hypothesized.[70,71] In these experiments, LA was applied at a dose of 10 mg kg^{-1} i.p., which corresponds to 600 mg LA day^{-1} in humans for 4 weeks, starting from the last day of STZ administration (40 mg kg^{-1} i.p. for 5 consecutive days). These studies focused on the antioxidant defence system of red blood cells (RBC) and kidneys. RBC are exposed to some of the highest levels of oxidative stress in the body because they continuously transport oxygen and are the first cellular structures to respond to increased ROS presence. RBC damage is a reflection of the general state of oxidative stress in the whole organism.[72] In the hyperglycaemic environment, RBCs are subjected to compositional changes and are affected at the functional level.[73] In agreement with other reports, enzymatic silencing of CuZnSOD and CAT in RBC under diabetic conditions were observed,[74,75] which was associated with increased levels of *O*-GlcNAc-modification of CuZnSOD and CAT, and of the heat shock proteins HSP70 and HSP90.[70] *In vitro* studies showed that glycosylation causes a 40 % lowering of CuZnSOD activity in RBC.[76] It was shown that LA administration to diabetic rats preserved the structural and functional integrity of RBC by adjusting the redox disturbance and by decreasing the *O*-GlcNAcylation of SOD and CAT.

It was hypothesized that the induction of HSP90 and the lowering of the levels of *O*-GlcNAc-modification of HSP70 and HSP90 as a result of the LA treatment is an important defence mechanism in RBC, since HSPs monitor, protect and maintain the structure and stability of erythrocyte proteins. These results are valuable because functional and healthy RBC could delay or inhibit further diabetic complications, especially neuropathy.

The renal-protective effect of LA is associated with a reduction of oxidative stress.[77] Recently performed work revealed that LA administration activates a coordinated cytoprotective response against diabetes-induced oxidative injury in kidneys through an *O*-GlcNAc-dependent mechanism, which influences the expression and activities of CuZnSOD and CAT. The observed upregulation of the antioxidant enzyme genes during LA treatment in diabetic kidney was accompanied by nuclear translocation of the nuclear factor-erythroid-2-related factor (Nrf2), enhanced expression of HSPs and by a reduction of *O*-GlcNAcylation of HSP90 and HSP70, and of the extra-cellular regulated kinase (ERK) and p38. Under unstressed conditions, Nrf2 resides in the cytoplasm as an inactive complex bound to a repressor molecule known as Keap1 (Kelch-like ECH-associated protein 1) that facilitates its ubiquitination.[78] Upon activation, Nrf2 translocates to the nucleus where it heterodimerizes with specific cofactors and coordinates the upregulation of cytoprotective genes through the initiation of transcription at an antioxidant response element (ARE).[79] In addition, it was reported that HSP90 interaction with Keap1 can mediate Nrf2 activation.[80] LA can oxidize critical thiols on the Keap1 dimer to halt Nrf2 degradation and to prevent Keap1 from binding newly synthesized Nrf2. LA also activates the protein kinase signalling pathways that lead to the phosphorylation of Ser40 on Nrf2, allowing it to dissociate from Keap1 and to translocate to the nucleus.[42] Inhibition of nitrogen-activated kinases (MAPKs), ERK and p38 prevents the accumulation of Nrf2 in the nucleus independently of its phosphorylation.[81] It was suggested that MAPK-mediated phosphorylation of molecular chaperones or some other type of accessory protein is required for Nrf2 nuclear translocation.[82] Based on our obtained results and existing literature data, a model that illustrates the potential mechanisms by which LA ameliorates kidney damage in diabetes by inducing SOD and CAT expression is presented in Fig. 1.

6. POSITIVE EFFECTS OF *L. deterrimus* (Ld) AND *C. sativa* (Cs) EXTRACTS AND THEIR COMBINATION (MIX Ld/Cs) ON *β*-CELL SURVIVAL

Examination of compounds and factors that can regulate *β*-cell survival, growth and functioning is of great interest in the context of the prevention of diabetes development and its progress. The antioxidant properties and beneficial effects of extracts obtained from the edible mushroom *Lactarius deterrimus* (Ld), the sweet chestnut *Castanea sativa* (Cs) and their combination (MIX Ld/Cs) on STZ-induced rat pancreatic *β*-cell (Rin-5F cells) death have been described.[83,84]

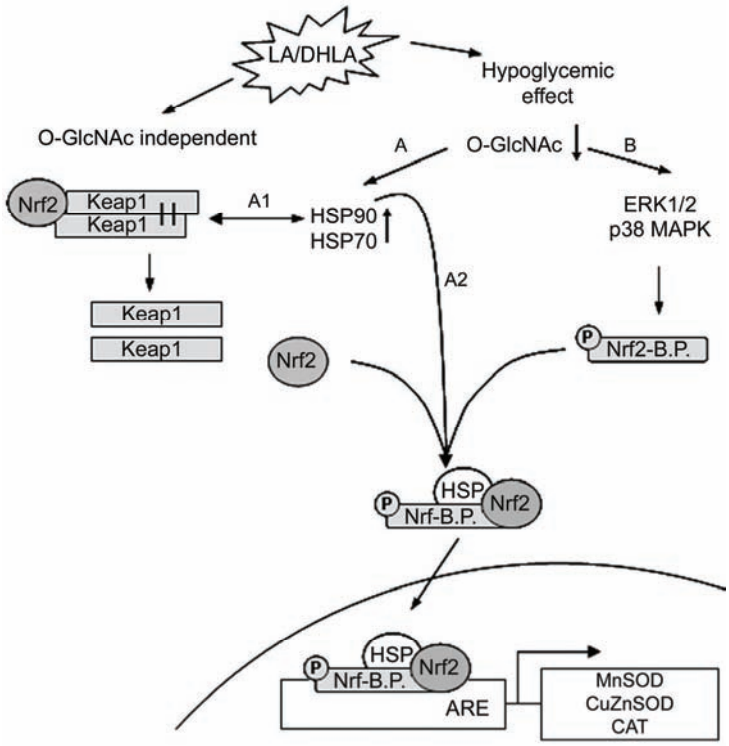

Fig. 1. Potential mechanisms of LA-regulated SOD and CAT gene expression in kidneys of diabetic rats. Pathway **A** illustrates LA-induced protein expression of HSPs and their decreased *O*-GlcNAc modifications that could influence their interaction with Keap1, causing a subsequent release of Nrf2 (**A1**) and/or formation of HSP-Nrf2 heterocomplex that translocates to the nucleus and binds ARE (**A2**), promoting the transcription of genes for MnSOD, CuZnSOD and CAT. In a LA-orchestrated *O*-GlcNAc-dependent mechanism (pathway **B**), reduced *O*-GlcNAc modification of ERK and p38 could enhance their activity. Activated ERK and p38 could phosphorylate a certain Nrf2-binding protein (Nrf2-B.P.) that could assist the nuclear translocation of Nrf2 released from Keap1.

The Cs extract exhibited a remarkably high level of antioxidant activity *in vitro*, while the Ld extract displayed good H_2O_2 and NO scavenging activities. MIX Ld/Cs demonstrated strong antioxidant effects *in vitro*, and astonishingly, a very effective Fe^{2+} chelating effect, despite the very low individual chelating activities of the Ld and Cs extracts. This is in correlation with the concept that no single antioxidant can replace the health benefits of a combination of natural phytochemicals because of their additive and synergistic effects.[51] Each extract and especially their combination increased Rin-5F cell viability after the STZ treatment as a result of a significant reduction in DNA damage and improved redox status. It is suggested that different mechanisms underlie the antioxidant effects of Cs, Ld and MIX Ld/Cs (Fig. 2). The antioxidant property of the Cs extract probably re-

lies on its ROS scavenging activity.[55,83,85,86] It directly correlates with the extremely high content of phenolic compounds, especially of hydrolysable tannins (ellagic and gallic acids and their derivatives). The beneficial biological effects of these compounds *in vivo* are related to the high free radical-scavenging activity they exhibit *in vitro*.[87] The antioxidant properties of the Ld can be explained by a strong NO scavenging activity. The low phenolic content of the Ls extract suggests that some other non-phenolic compounds or secondary metabolites[88] were responsible for its beneficial effect, such as the essential trace elements Se and Zn. Se functions as a cofactor of some antioxidant enzymes,[89] while Zn protects enzyme sulfhydryls from oxidation and reduces the formation of the hydroxyl radical from H_2O_2 by competing with redox-active transition metals.[90] It is suggested that the MIX Ld/Cs displayed the most beneficial effect on cell survival through the additive and synergistic effects of the different antioxidant activities contained in Cs and Ld extracts.[56] These results provide compelling evidence that mixtures of extracts acquire new qualities with respect to the individual extracts and individual components. This feature explains the improved antioxidant and beneficial effects that were exerted on *β*-cells.

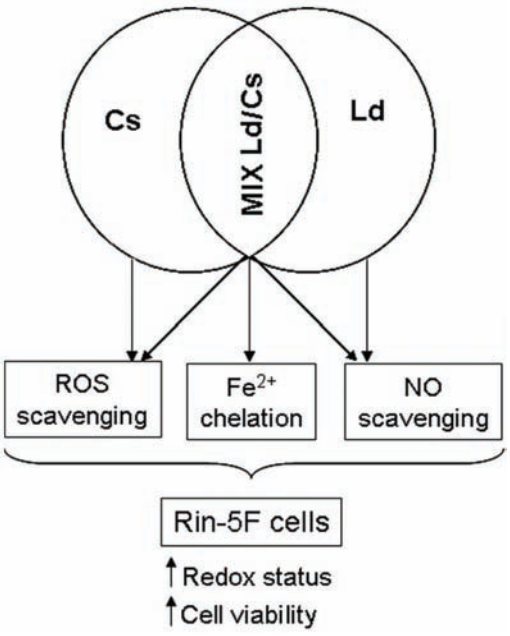

Fig. 2. Potential mechanism of Cs, Ld and MIX Ld/Cs action on the improvement of Rin-5F cell redox status and survival.

The antioxidant properties of the mushroom and chestnut extracts need to be confirmed *in vivo* on a rat model of STZ-induced diabetes (work in progress).

Duly aware of the limitations of the *in vitro* model system, it is proposed that the MIX Ld/Cs can reduce oxidative stress in β-cells and that it could thereby potentially attenuate the process that underlies the development and progression of diabetes.

7. EFFECTS OF β-GLUCANS ON DIABETES AND THE ASSOCIATED COMPLICATIONS

Natural products containing β-glucans as active components have been proposed to improve general health.[91–93] β-Glucans belong to a group of polysaccharides that are characterized by their location in the cell wall. Some microorganisms, mushrooms and cereals, such as barley and oats, are rich in β-glucans.[94] The macromolecular structure of β-glucans depends on both the source and method of isolation. The biological activities of β-glucans are determined by their primary structure, solubility, degree of branching, molecular weight, the charge on their polymers and structure in aqueous media.[95] On reviewing the literature, it became obvious that the observed effects of β-glucans and glucan-containing products are controversial.[96] While there are reports that emphasize the immune stimulatory[97,98] and pro-inflammatory effects of β-glucans,[99] as well as increased generation of ROS,[100] other studies described their free radical scavenging activities,[101] and anti-inflammatory[102] and antioxidative effects.[103] Among several mechanisms proposed for the protective effects of β-glucan, a major one is related to its antioxidant activity.[104]

β-Glucans have shown great potential in the treatment of diabetes.[92] They are effective in lowering blood glucose concentrations and decreasing hyperlipidaemia and hypertension. In addition, β-glucans also promote wound healing and alleviate ischemic heart injury. Foods containing β-glucans have been used in clinical trials in the treatment of diabetes.[105,106] Our preliminary results are based on observations obtained after treating STZ-induced diabetic rats with a comercially available β-glucan-enriched extract (80 mg kg^{-1} for four weeks, starting from the last day of STZ treatment). Treating diabetic rats with β-glucan promoted a systemic improvement that could be expected to increase the resistance of the organism to the onset of diabetic complications. The beneficial effect of the β-glucan-enriched extract against diabetes-associated liver and kidney injury was mediated through its hyperglycaemia lowering, anti-inflammatory and antioxidant actions. It was speculated that the observed properties of the applied commercial β-glucan-enriched extract could be attributed to the effects of β-glucan and other components of the preparation. Mechanisms underlying its effect on diabetes and associated complications need to be investigated using pure β-glucan.

8. CONCLUSIONS

Despite numerous strategies designed to improve different diabetes-related symptoms, the current control of diabetes-associated complications and mortality

is not satisfactory. Targeting of oxidative stress in the management of diabetes using synthetic antioxidants, such as vitamins A, C and E, yielded limited results and the presence of side effects. This has renewed interest in the therapeutic potential of bioavailable compounds, extracts and complex mixtures. Due to the relatively lower number of side effects and lower cost, naturally derived substances provide a useful source of potential novel anti-diabetogenic pharmaceutical entities and dietary supplements to existing therapies. Considering that phytochemicals can reduce the major risk factors in diabetes, such as hyperglycaemia, hyperlipidaemia and oxidative stress, the use of medicinal plants represents a promising approach for treating diabetes. It is therefore imperative to continuously identify naturally occurring products with antioxidant activities for use in the "causal" therapy of diabetes.

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И З В О Д

ПОЗИТИВНО ДЕЈСТВО АНТИОКСИДАТИВНИХ ЈЕДИЊЕЊА ИЗ БИЉНИХ ЕКСТРАКАТА У ЕКСПЕРИМЕНТАЛНОМ МОДЕЛУ ДИЈАБЕТЕСА

СВЕТЛАНА ДИНИЋ, АЛЕКСАНДРА УСКОКОВИЋ, МИРЈАНА МИХАИЛОВИЋ, НЕВЕНА ГРДОВИЋ, ЈЕЛЕНА АРАМБАШИЋ, ЈЕЛЕНА МАРКОВИЋ, ГОРАН ПОЗНАНОВИЋ И МЕЛИТА ВИДАКОВИЋ

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Терапија антиоксидансима заузима значајно место у лечењу дијабетеса с обзиром да оксидативни стрес у великој мери доприноси нарушавању функције и структуре *β*-ћелија панкреаса као и развоју компликација у дијабетесу. Због ограниченог дејства постојећих антиоксидативних једињења трага се за новим терапијским решењима у третману дијабетеса, као што је повећање ендогене антиоксидативне заштите организма путем фармаколошке модулације кључних антиоксидативних ензима. Примена биљних екстраката у лечењу дијабетеса постаје све популарнија. Многе супстанце које се налазе у саставу виших биљака и гљива поседују антиоксидативна својства која могу да заштите ткива од штетних утицаја слободних радикала. У овом ревијалном раду описани су експериментални модели дијабетеса као и могући механизми који леже у основи антиоксидативног дејства α-липонске киселине (LA), снажног антиоксиданса и једињења које стимулише ћелијску апсорпцију глукозе, као и биљних екстраката изолованих из слатког кестена (*Castanea sativa*), јестивих печурака (*Lactarius deterrimus*) и природних производа који садрже *β*-глукан у лечењу дијабетеса. Описани су њихова улога у спречавању смрти *β*-ћелија панкреаса као и благотворно дејство на компликације у дијабетесу.

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Strain differences in the toxicity of the vitamin K antagonist warfarin in rats

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*Abstrac*t: Warfarin (3-(α-acetonylbenzyl)-4-hydroxy coumarin) is a vitamin K (VK) antagonist that inhibits vitamin K-dependent (VKD) processes, such as blood coagulation. It also exerts an influence on some non-VKD-related activities. In this study, the effect of sub-acute (30-day) oral warfarin (2 and 1 mg L^{-1}) intake on hematological parameters was examined in two rat strains, Albino Oxford (AO) and Dark Agouti (DA), that differ in their sensitivity to certain chemicals. Greater susceptibility to the anticoagulant effect of 2 mg L^{-1} of warfarin was observed in AO rats and was associated with an increase in the relevant hematological parameters in this strain. Although both strains responded to 2 mg L^{-1} of warfarin with quantitative changes in the peripheral blood leukocytes, differential bone marrow and lung responses were observed. Strain-related differences in the pro-inflammatory activity of peripheral blood granulocytes and in mononuclear cell IFN-γ production were observed. Recognition of differences in quantitative and qualitative effects of oral warfarin on processes other than hemostasis might be of relevance for those humans who are on warfarin therapy.

Keywords: warfarin; rats; anticoagulant effect; hematology; peripheral blood leukocytes.

INTRODUCTION

Warfarin (3-(α-acetonylbenzyl)-4-hydroxy coumarin) and other coumarin analogs are antagonists of vitamin K that inhibit the vitamin K-dependent (VKD) step in the synthesis of several factors required for normal blood coagulation.[1]

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Interference with the synthesis of biologically active coagulation factors results in increases in the clotting time up to the point where no clotting occurs. Anticoagulants of the 4-hydroxy-coumarin type are used in prophylactic medicine to prevent thromboembolic diseases and as pesticides for rodents (anticoagulant rodenticides).[2,3] Warfarin inhibits vitamin K epoxide reductase (VKOR), giving rise to depletion of hydroquinone, a co-factor for the γ-glutamyl carboxylase, an enzyme that mediates carboxylation of glutamyl (Gla) residues on precursors of several proteins involved in coagulation, including II (FII, prothrombin), VII (FVII), IX (FIX), and X (FX) factors.[2] Inhibition of VKOR by warfarin also affects the catalytic rate of other VKD proteins, such as proteins required for the regulation of bone growth and calcification (bone Gla protein, BGP/osteocalcin and matrix Gla protein, MGP) as well as those involved in the growth of vascular smooth muscle cells and mesangial cells.[4–7] Moreover, warfarin exerts influence on some non-VKD-related activities, including anti-tumor and immunomodulating activities.[8,9] As regards the immune system, both stimulatory and suppressive effects of warfarin (and other coumarin congeners) were observed.[10–15] Some clinical complications of warfarin therapy and adverse reactions associated with inflammatory cells attendance in affected tissues implies the pro-inflammatory potential of this chemical.[16–21]

It was previously demonstrated that acute (intraperitoneal or epicutaneous route) or sub-acute oral administration of warfarin at doses resulting in anticoagulation exert systemic pro-inflammatory effects in rats and suggested that peripheral blood neutrophils are the target of this agent.[22–25] The impact of sub-acute oral warfarin intake on other hematological parameters besides peripheral blood leukocyte numbers and mononuclear cell activity was explored in this study in order to see whether there were broader effects of warfarin at the systemic level. Bearing in mind the well-known differences in the response of humans to anticoagulation by warfarin,[26] these effects were examined comparatively in two rat strains known for their differences in immune responses to chemical insult, *i.e.*, Albino Oxford (AO) and Dark Agouti (DA) rats.[27,28]

EXPERIMENTAL

Chemicals

Warfarin sodium (Serva Feinbiochemica, Heidelberg, Germany), dextran 500,000, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phorbol-12-myristate-13-acetate (PMA), hexadecyltrimethylammonium bromide (HTAB), *o*-dianisidine dihydrochloride and myeloperoxidase (MPO) (all from Sigma Chemical Co., St. Louis, MO, USA), nitroblue tetrazolium (NBT) (ICN Pharmaceutical, Costa Mesa, CA, USA), concanavalin-A (ConA) (Pharmacia, Uppsala, Sweden) were used in experiments. The culture medium RPMI-1640 (PAA laboratories, Austria) supplemented with 2 mM glutamine, 20 μg mL^{-1} gentamycin (Galenika a.d., Serbia) and 5 % (v/v) heat-deactivated fetal calf serum (PAA Laboratories, Austria), *i.e.*, complete medium, was used in the cell culture experiments.

Animals and warfarin treatment

Twelve-week old, female DA (weighing 200–232 g) and AO rats (weighing 295–325 g), conventionally housed at the Institute for Biological Research "Siniša Stanković" (Belgrade, Serbia) were used in the experiments. The treatments were performed in adherence with the Guidelines of the Ethical Committee of the Institute. In the pilot experiments, rats were given 4 mg L^{-1} of warfarin solution in drinking water, which (at consumption volume of 20–30 mL of solution daily) corresponded to 0.367 (±0.047) mg of warfarin per kg daily. However, massive hemorrhage was observed in AO rats during the last week of warfarin intake, with subsequent death of more than 80 % of individuals, while less than 15 % of the DA rats died. No fatal outcome was observed in either rat strain at two (2 mg L^{-1}) and four times (1 mg L^{-1}) lower warfarin doses. Thus, the consequences of the intake of these two lower non-lethal warfarin doses were analyzed further. At least two independent experiments were conducted with four to six animals assigned to each treatment group. Rats were given warfarin solution in drinking water *ad libitum* for 30 days. Control rats were given drinking water only.

Prothrombin time

The prothrombin time (*PT*) was determined in samples of blood withdrawn from the abdominal artery diluted in citrate buffer (blood to citrate ratio 5:1) by a one-stage method using citrate plasma and Thromborel S reagents (Behring Diagnostics GmbH, Marburg, Germany) with Siemens BCS-XP Dade Behring equipment (Marburg, Germany).

Hematology

Complete blood tests analyses were conducted automatically using a Siemens ADVIA 120 flow cytometer (Terytown, N.Y., USA). In this way, the white blood cell count (*WBC*) and percentage of leukocytes, red blood cell count (*RBC*), hemoglobin concentration (*Hb*), hematocrit (*HCT*), mean corpuscle volume (*MCV*), platelet number (*PLT*) and mean platelet volume (*MPV*) were measured.

Bone marrow leukocyte counts

Total bone marrow counts were determined using an improved Neubauer hemocytometer following cell staining with Türck solution. Differential cell counts were determined by differentiating at least 1000 cells of bone marrow smears stained according to the May–Grünwald–Giemsa protocol.

Lung histology

Lungs were excised, cut and immediately fixed in 4 % formaldehyde (pH 6.9). After processing, the tissue was embedded in paraffin wax for sectioning at 5 μm. Hematoxylin and eosin (H&E)-stained histology slides were subsequently analyzed in a blinded manner by experienced pathologist using a Coolscope digital light microscope (Nikon Co., Tokyo, Japan).

Peripheral blood granulocyte and mononuclear cell isolation

Peripheral blood leukocytes were isolated from the heparinized blood by dextran sedimentation and centrifugation (700 *g*, 20 min at room temperature) on an OptiPrep (Nycomed AS, Norway) density gradient. Polymorphonuclear cells (granulocytes) were obtained from the pellet fraction, following the lysis of erythrocytes with the isotonic NH_4Cl solution. The mononuclear cells were harvested from the band at the interface of plasma and OptiPrep density medium.

MTT assay for leukocyte viability

Granulocyte and mononuclear viability was determined in freshly isolated cells by a quantitative colorimetric assay for metabolic viability described for humans,[29] which is based on the reduction of the tetrazolium salt MTT to a colored end-product, formazan, by several mitochondrial dehydrogenases in viable cells. The formazan produced was dissolved in an acidified sodium dodecyl sulfate solution (10 % SDS–0.01 M HCl) and the optical density (*OD*) was measured using a microplate spectrophotometer (GRD, Rome, Italy) at 540 nm.

Peripheral blood granulocyte and mononuclear cell activity

Granulocyte activation was evaluated by a cytochemical nitroblue tetrazolium (NBT) reduction assay for the respiratory burst based on the capacity of granulocytes to reduce NBT *via* respiratory burst oxidase.[30] Briefly, NBT (10 μL, 5 mg mL^{-1}) was added to a granulocyte suspension (5×10^5 cells $well^{-1}$ of a 96-well plate, in 100 μL) and incubated for 30 min with 100 ng mL^{-1} PMA (stimulated NBT reduction) or solely in medium (spontaneous NBT reduction). The absorbance of the produced formazan was measured as described above.

The granulocyte myeloperoxidase (MPO) activity was assessed based on the oxidation of *o*-dianisidine dihydrochloride by cells.[31] To 966 μL of substrate solution (0.167 mg mL^{-1} *o*-dianisidine dihydrochloride and 0.0005 % H_2O_2 in 50 mM potassium phosphate buffer, pH 6.0), 33 μL of granulocyte lysate, obtained by repeated freeze–thaw, was added. The absorbance was read at 450 nm (at three-minute intervals up to ten minutes) against an MPO standard. The values are expressed as MPO units per 10^6 cells.

To examine interferon-γ (IFN-γ) production, mononuclear cells were cultured at 5×10^5 cells $well^{-1}$ in a 96-well plate for 48 h in culture medium solely (spontaneous production) or in the presence of 1 μg mL^{-1} of ConA (ConA-stimulated production). The concentration of IFN-γ produced was measured by enzyme-linked immunosorbent assays (ELISA) for rat IFN-γ (R&D systems, Minneapolis, USA). The cytokine titer was calculated using a standard curve constructed with known amounts of recombinant IFN-γ.

Data display and statistical analysis

The results were obtained from two independent experiments and are expressed as means ± standard deviation (*SD*). Statistical analysis was performed using Statistica 6.0 (StatSoft Inc., Tulsa, Oklahoma, USA). The statistical significance was defined by the Mann–Whitney U test. Values of p less than 0.05 were considered significant.

RESULTS

Anticoagulant effects of warfarin

Rats were given 1 and 2 mg L^{-1} of warfarin in their drink water. Every individual consumed 20–30 mL of warfarin solution daily, in this way consuming 0.090±0.009 (0.078–0.107) and 0.191±0.016 (0.164–0.215) mg kg^{-1} day^{-1} at 1 and 2 mg L^{-1}, respectively. Prolongation of the mean prothrombin time was observed in the AO rats following consumption of 2 mg L^{-1} of warfarin only (Fig. 1). No changes in *PT* were observed at the lower (1 mg L^{-1}) warfarin dose.

Hematology

Thirty-day consumption of 2 mg L^{-1} of warfarin resulted in significantly lower total leukocyte counts and in a tendency (p = 0.07) of decreasing in the AO and DA rats, respectively (Table I). A decrease in lymphocyte with unchanged

neutrophil concentrations was observed in the individuals of both strains. Besides leukocytes, several other hematological parameters were affected in AO rats, including an increase in red blood cell counts, hemoglobin concentration, hematocrit, mean corpuscle volume and platelet counts. No changes in these parameters were observed in DA rats, although the highly variable platelet counts resulted in a numerical increase at this warfarin dose. Consumption of 1 mg L^{-1} was without effect, except for an increase in HCT in AO rats.

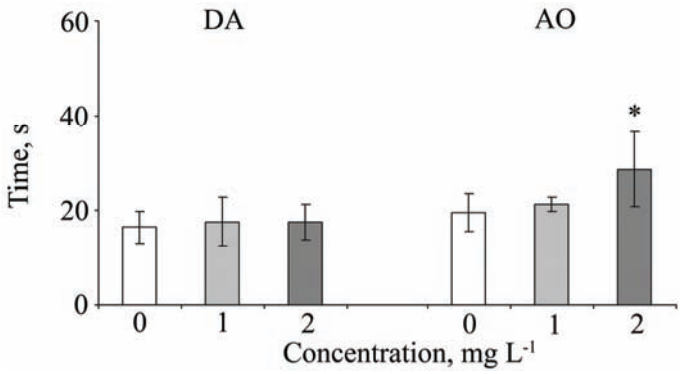

Fig. 1. Anticoagulant effect of warfarin intake in AO and DA rats expressed through the prothrombin time (*PT*). The results are expressed as mean values ± *SD* from two independent experiments with six animals per each animal group. Significance at * $p < 0.05$ *vs*. controls (0 mg L^{-1} of warfarin) of the respective strain.

Bone marrow leukocyte counts

Given the quantitative changes in the peripheral blood leukocytes at 2 mg L^{-1} of warfarin, an analysis of bone marrow leukocytes was conducted in rats that consumed this warfarin dose. Significant increase in number of immature neutrophils (metamyelocytes) and a tendency ($p = 0.060$) of an increase in the mature granulocyte pool were observed only in DA rats (Table II). No changes in the number of lymphocytes or monocytes were observed in either strain.

Lung histology

As lungs harbor an intravascular reservoir of leukocytes, predominantly neutrophils, called marginated pool, which constantly exchanges with the circulating cells,[31] histological analysis of lungs from rats that consumed 2 mg L^{-1} of warfarin was conducted next (Fig. 2). It revealed neutrophil attendance in AO rats (neutrophil lung infiltration noted in three out of four animals), as compared to DA rats, where neutrophils were observed in the lungs of one out of four individuals that had consumed warfarin.

TABLE I. Hematological parameters following consumption of warfarin; abbreviations: *WBC*, white blood cell count; *NE* / %, percent of neutrophils; *LY* / %, percent of lymphocytes, *MO* / %, percent of monocytes; *EO* / %, percent of eosinophils; *BA* / %, percent of basophils; *RBC*, red blood cell count; *Hb*, hemoglobin concentration; *HCT*, hematocrit; *MCV*, mean corpuscular volume; *MHC*, mean corpuscular hemoglobin; *MCHC*, mean corpuscular hemoglobin concentration; *PLT*, platelet count; *MPV*, mean platelet volume; data represent the mean values ±*SD* from two independent experiments with four to six animals per group

Parameter		DA warfarin dose, mg L^{-1}			AO warfarin dose, mg L^{-1}		
		0	1	2	0	1	2
WBC	/ 10^9 L^{-1}	9.91±1.70	7.88±2.02	7.32±1.07	5.88±2.06	4.13±1.88	3.22±0.68[a]
NE	/ 10^9 L^{-1}	4.61±0.56	3.07± 1.17	4.66±0.54	2.24±0.93	1.55±0.74	2.12±0.50
	/ %	45.05±5.15	38.20±4.76	64.03±3.61	37.13±5.85	37.54±4.58	65.61±7.05
LY	/ 10^9 L^{-1}	4.88±1.10	4.39±0.80	2.37±0.50[a]	3.35±0.76	2.36±1.14	0.91 ±0.25[b]
	/ %	48.82±4.67	56.33±4.04	32.17±3.19	57.83±4.37	56.80±4.76	28.57 ±7.46
MO	/ 10^9 L^{-1}	0.29±0.11	0.34±0.07	0.22±0.10	0.21±0.07	0.12±0.07	0.14±0.04
	/ %	2.84±0.79	4.33±0.58	2.83±1.17	3.75±1.50	3.20±2.17	4.23±0.95
EO	/10^9 L^{-1}	0.11±0.05	0.06±0.01	0.06±0.04	0.05±0.01	0.08±0.02	0.06±0.02
	/ %	1.16±0.44	0.77±0.21	0.73±0.51	0.93±0.19	2.08±0.92	1.73±0.72
BA	/ 10^9 L^{-1}	0.03±0.01	0.03±0.002	0.02±0.01[a]	0.02±0.01	0.02±0.01	0.01±0.01[a]
	/ %	0.36±0.15	0.37±0.06	0.23±0.15	0.38±0.10	0.36±0.17	0.20±0.12
RBC	/ 10^{12} L^{-1}	7.06±0.59	7.91±0.11	7.52±0.37	7.55±0.61	7.95±0.34	8.51±0.22[b]
Hb	/ g L^{-1}	124.60 ±10.43	127.00±1.73	136.50±8.53	139.50±11.45	138.80±4.09	160.71±7.02[a]
HCT	/ %	0.36±0.02	0.40±0.01	0.41±0.02	0.39±0.02	0.42±0.01[a]	0.46±0.02 [b]
MCV	/ fl	51.52±4.18	50.83±1.98	54.47±1.91	51.28±2.62	53.28±1.25	54.71±1.19[a]
MCH	/ pg	17.67±0.85	16.25±0.23	18.16±0.98	18.54±1.80	17.54±0.30	18.90±0.78
MCHC	/ g L^{-1}	343.58 ±38.77	320.26±9.10	334.12±10.00	361.25±38.54	327.27±4.11	347.21±8.99
PLT	/ 10^9 L^{-1}	87.60 ±145.40	694.33 ±19.01	759.67 ±112.61	620.50±61.07	712.80±70.96	724.71 ±55.50[a]
MPV		7.44±0.81	8.53±0.12	8.47±0.27	7.75±0.81	8.12±0.04	8.23±0.46

[a]Significantly different from control animals of the respective strain (0 mg L^{-1}) at $p < 0.05$; [b]significantly different from control animals of the respective strain (0 mg L^{-1}) at $p < 0.01$

TABLE II. Total and differential bone marrow leukocyte counts; data represent the mean values ±*SD* from two independent experiments with four to six animals per group

Cell number × 10^{-6} per femur	DA Warfarin dose, mg L^{-1}		AO Warfarin dose, mg L^{-1}	
Parameter	0	2	0	2
Total	72.4±10.7	74.9±6.6	72.7±7.1	70.7±1.2
Metamyelocyte	8.70±2.1	11.77±2.3[a]	6.48±0.9	6.36±0.4
Granulocyte	31.02±4.9	34.16±3.2	24.92±3.5	24.21±0.8
Lymphocyte	31.73±5.7	29.82±9.0	40.42±4.1	39.19±1.5
Monocyte	0.94±0.3	1.13±0.5	0.83±0.2	0.88±0.1

[a]Significantly different from control animals of the respective strain (0 mg L^{-1}) at $p < 0.05$

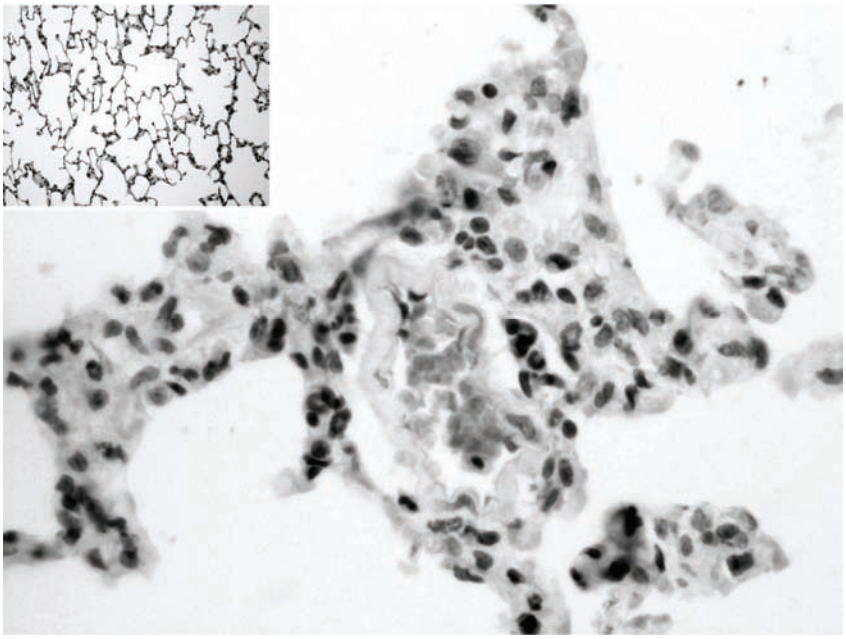

Fig. 2. Histology of lungs of an AO rat following oral intake of 2 mg L^{-1} of warfarin. Perivascular neutrophil attendance and interstitial edema. Inset: lung histology of a control animal (0 mg L^{-1} of warfarin).

Peripheral blood granulocyte viability and activity

To determine whether there were qualitative changes in the peripheral blood leukocytes, some of the basic aspects of activity (metabolically-based viability, activation/priming for respiratory burst and myeloperoxidase activity) were examined in neutrophils taken from animals that had consumed the higher warfarin dose (2 mg L^{-1}). As shown in Fig. 3, an increase in the MTT reduction by freshly isolated peripheral blood granulocytes of DA rats that had consumed warfarin was observed, while a similar capacity of reduction was observed in the control

and warfarin-exposed AO rats (Fig. 3A). No difference in level of spontaneous NBT reduction could be observed between the control and rats that had consumed warfarin (Fig. 3B). Stimulation with PMA resulted in an increase in the NBT reduction by cells from the control and treated animals of both strains. Although PMA-stimulated NBT reduction capacity was more pronounced in AO compared to DA rats ($p < 0.05$), no difference was noted between control and the warfarin-treated AO rats, while the levels of reduction attained in DA rats that had consumed warfarin were higher than those from the controls.

Measurements of the intracellular MPO content in neutrophils revealed lower values in the control DA (*vs.* AO) rats, but it increased significantly after warfarin consumption in the DA rats (Fig. 3C), while it remained unchanged in the AO rats.

Peripheral blood mononuclear cell viability and activity

As a drop in the peripheral blood lymphocyte counts was observed in rats that had consumed 2 mg L^{-1} of warfarin, the viability of the blood mononuclear cells was next determined. No difference in MTT reduction was observed between the control and the warfarin-treated rats of either strain (Fig. 4A). To determine whether warfarin exerted an influence on the mononuclear cell activity, the production of IFN-γ was determined (Fig. 4B). While there was no effect on the spontaneous IFN-γ production by mononuclear cells from DA rats, consumption of warfarin resulted in an increase in the AO rats. Stimulation with ConA, however, resulted in significantly higher production of this cytokine in DA rats that had consumed warfarin, while it was similar in the control and warfarin-treated AO rats.

DISCUSSION

In this study, the effects of sub-acute (30-day) oral intake of warfarin on hematological parameters and on peripheral blood granulocyte and mononuclear cell activity were examined comparatively in two rat strains known to differ in their susceptibility to chemical insult. The lower susceptibility to the anticoagulant effect of warfarin, the increase in some of the hematological parameters (RBC and PLT counts, Hb concentration and HCT values) as well as the lack of qualitative effects (peripheral blood granulocyte) or different pattern of mononuclear cell activity in DA *vs.* AO rats demonstrated the differential responsiveness to warfarin treatment in the two rat strains. The differential susceptibility to hemorrhage might have accounted for differences in mortality at the highest warfarin dose, in line with data showing that impaired coagulation, along with the hemorrhage generally resulted in the death of rodents.[3] The increase in prothrombin time, which reflects the basic biological activity of warfarin, at a dose that resulted in no changes in this parameter in DA rats, also depicts the higher responsiveness of AO rats to warfarin anticoagulation. Strain-related differences in the metabolism of warfarin shown in laboratory rat strains[32,33] might be responsible for the differences observed in the prothrombin time in AO *vs.* DA rats.

Differences in cytochrome P450 (CYP) enzymes as well as vitamin K epoxide reductase complex subunit 1 (VKORC1) might have accounted for these differences, as polymorphism in these enzymes affects the anticoagulant action of warfarin in humans.[34]

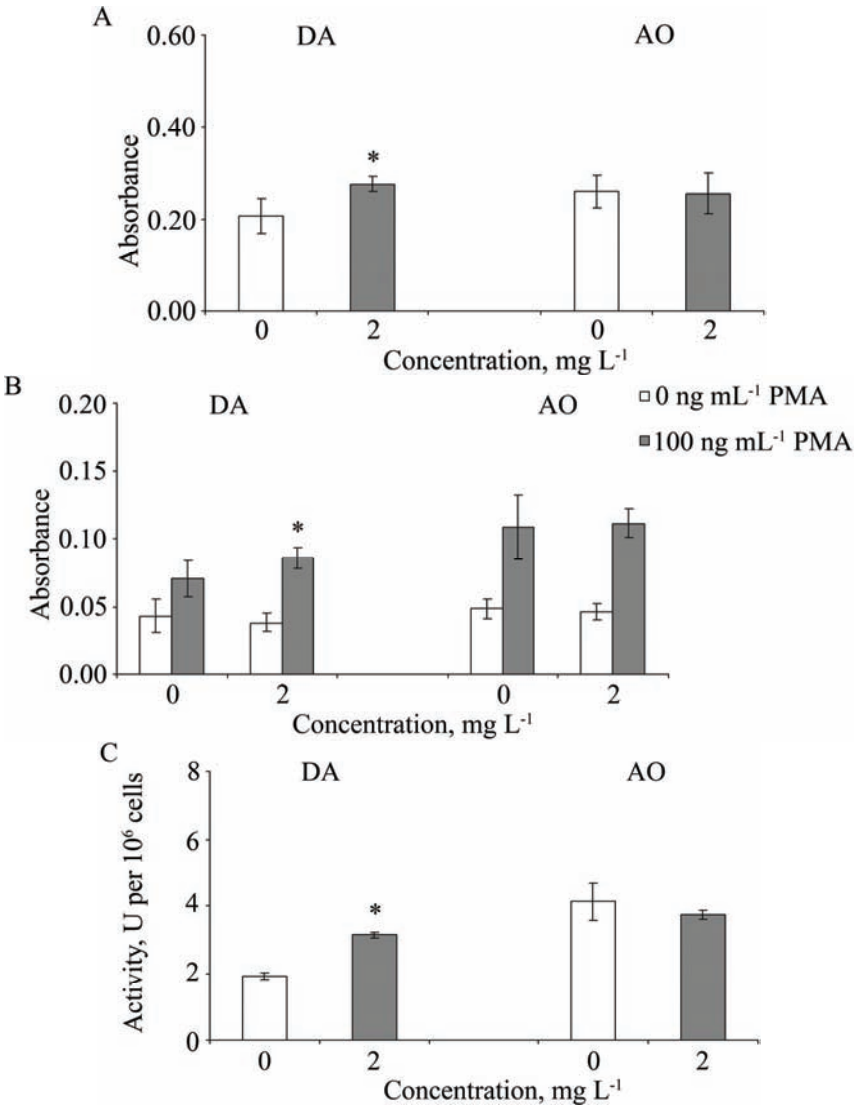

Fig. 3. The effect of warfarin intake on peripheral blood granulocyte viability and activity. A. Reduction of MTT. B. The spontaneous and PMA-stimulated reduction of NBT. C. Intracellular MPO activity. The results are expressed as mean values ± *SD* from two independent experiments with four to six animals per group. Significance at * $p < 0.05$ *vs.* controls (0 mg L^{-1} of warfarin) of the respective strain.

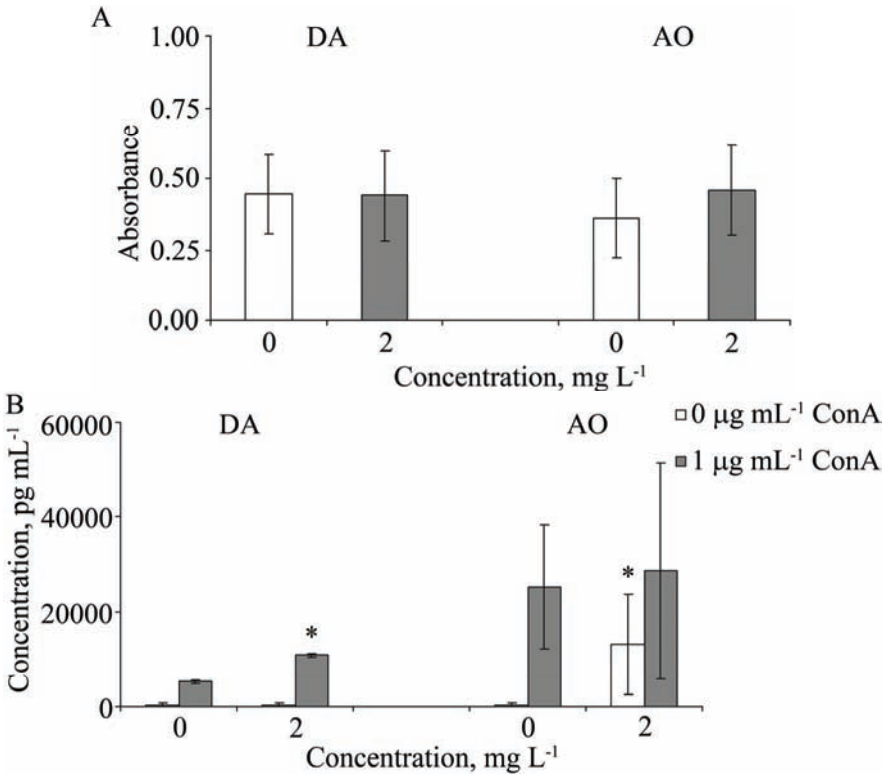

Fig. 4. The effect of warfarin intake on peripheral blood mononuclear cell viability and activity. A. Reduction of MTT. B. IFN-γ production. The results are expressed as mean values ± *SD* from two independent experiments with four to six animals per group. Significance at $*p < 0.05$ *vs.* controls (0 mg L^{-1} of warfarin) of the respective strain.

On the other hand, the differential effects of warfarin on some hematological parameters and peripheral blood leukocytes in the two strains imply that the effect of warfarin might depend on the parameter/activity examined. Indeed, the differential sensitivity of individuals of these two strains to warfarin was demonstrated by the hemoconcentration in AO rats (resulting from an increase in the number of red blood cells) following the consumption of the higher warfarin dose.

A decrease in the lymphocyte counts in the peripheral blood of rats that had consumed warfarin accounted for a drop in the leukocyte counts in both rat strains. This could not be ascribed to warfarin cytotoxicity, as there was a lack of changes in the viability of freshly isolated mononuclear cells. The underlying mechanisms of leuko/lymphopenia are presently unknown, but leukocyte migration to the focuses of hemorrhage in peripheral tissues might be responsible. Increases in immature bone marrow neutrophils imply that there is a need for these cells in the periphery and that this might be an underlying mechanism to uphold neutrophil

numbers in DA rats. Neutrophil attendance in lungs (pronounced in AO rats) implies the exchange of neutrophils from circulation with the lung's intravascular reservoir of leukocytes (marginated pool).[31]

Differential effects of warfarin consumption on leukocyte functional activity were observed in these strains. While no effects on granulocyte viability were observed in AO rats, an increase in the MTT reducing capacity by cells from warfarin-treated DA rats was observed. As the MTT assay depends on mitochondrial activity in viable cells and is influenced by their metabolic activity, it actually reflects the overall functional state of granulocytes.[29,35] A cytochemical assay for the respiratory burst, which is a measure of cellular capacity to reduce NBT *via* tetrazolium reducing respiratory burst phagocyte oxidase, is often employed as an *in vitro* measure of peripheral blood granulocyte activation.[36,37] Similar levels of spontaneous NBT reduction by granulocytes between controls and rats that had consumed warfarin imply the lack of the capacity of this chemical to activate granulocytes. However, significantly higher NBT reduction provoked by PMA in the granulocytes from warfarin-treated DA rats compared to the controls indicated to a primed state of these cells, *i.e.*, a state of increased responsiveness to exogenous stimulation.[38] An increase in intracellular MPO content, which along with phagocyte oxidase, is a source of oxidant activity in phagocytes,[39] emphasises the effect of warfarin intake on the oxidative activities of granulocyte in peripheral blood. The priming of granulocytes of DA rats for the respiratory burst and the higher intracellular MPO content imply the higher responsiveness of these rats to warfarin, compared to AO rats.

The lack of an effect of warfarin intake on the peripheral blood mononuclear cell production of IFN-γ (the main mononuclear cell cytokine under conditions of systemic inflammation) in DA when compared to AO rats implies the differential susceptibility of these cells to warfarin. In addition, the lack of priming of mononuclears from warfarin-treated AO rats (similar levels of IFN-γ production in response to ConA stimulation) in contrast to the increased levels of cytokine produced in DA rats shows refractoriness of the mononuclear cells to exogenous stimulation. These data also suggest that not only peripheral blood neutrophils, but also mononuclear cells present targets for warfarin. The underlying mechanisms responsible for differential granulocyte and mononuclear cell responsiveness to oral warfarin might be ascribed to differences in signaling pathways and/or sensitivity to chemical signals, such as pro-inflammatory cytokines, known to modulate leukocyte activities. These assumptions warrant future attention.

CONCLUSIONS

The present study has demonstrated strain-related differential responsiveness to oral warfarin intake in rats not only in terms of anticoagulation, but in certain hematological parameters and in inflammation–relevant peripheral blood gra-

nulocyte and mononuclear cell activity as well. Recognition of differences in quantitative and qualitative effects of oral warfarin on processes other than hemostasis might be of relevance for those humans who are on warfarin therapy.

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ИЗВОД

СОЈНЕ РАЗЛИКЕ У ТОКСИЧНОСТИ АНТАГОНИСТЕ ВИТАМИНА К ВАРФАРИНА КОД ПАЦОВА

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Варфарин (3-α-ацетонилбензил)-4–хидроксикумарин) је антагонист витамина К (ВК) који инхибира процесе зависне од овог витамина, укључујући коагулацију крви. Осим тога, он испољава и активности које не зависе од витамина К као што су антитуморска и имуномодулаторна активност. У овом раду је испитан ефекат субакутног (30 дана) оралног уноса варфарина на хематолошке параметре и активност леукоцита периферне крви код два соја пацова *Albino Oxford* (АО) и *Dark Agouti* (DA) који се разликују у осетљивости на исте хемијске агенсе. Код јединки АО соја запажена је већа смртност након конзумирања дозе од 4 mg L^{-1} као и већа осетљивост на антикоагулантно дејство варфарина при нижим дозама (2 mg L^{-1}) које је праћено повећањем неких хематолошких параметара. Иако код јединки оба соја долази до повећања броја неутрофилних леукоцита периферне крви при дози од 2 mg L^{-1}, промене у основним проинфламаторним активностима ових ћелија су запажене само код јединки DA соја. Промене у броју неутрофилних леукоцита у крви DA јединки су праћене повећањем броја гранулоцитних прекурсора у коштаној сржи, док присуство неутрофила у плућима АО јединки указује на размену ћелија између периферне крви и плућног интраваскуларног пула ћелија. Диференцијалне сојно–зависне промене у активности мононуклеарних ћелија периферне крви су такође запажене. Разлике у ефекту орално унетог варфарина могу да имају импликације за особе на оралној варфаринској терапији.

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JSCS–4424 *Review*

REVIEW

Health aspects of *Spirulina* (*Arthrospira*) microalga food supplement

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Abstract: *Spirulina*, now named *Arthrospira*, is a microscopic and filamentous cyanobacterium that has a long history of use as a safe food, lacking toxicity. It is commercially produced in large outdoor ponds under controlled conditions. The aim of this review article is to summarize the recent available information concerning the human clinical potential and applications of *Spirulina*, as well as clinical data related to the safety and side effects of *Spirulina*. Potential health benefits of *Spirulina* are mainly due to its chemical composition, which includes proteins (the highest protein content of any natural food, 55–70 %), carbohydrates, essential amino acids, minerals (especially iron), essential fatty acids, vitamins and pigments. In this respect, three major bioactive components of *Spirulina*, the protein phycocyanin (a biliprotein pigment), sulfated polysaccharides and gamma linolenic acid seem to play significant roles in imparting improved human body functions. Furthermore, new experimental evidence supports the immunomodulation and antiviral effects of *Spirulina* supplementation. According to the Dietary Supplements Information Expert Committee of the United States Pharmacopeial Convention, the available clinical evidence does not indicate a serious risk to health or other public health concerns due to *Spirulina*. However, a few cases of severe side effects have been reported.

Keywords: cyanobacterium; phycocyanin; phycocyanobilin; sulfated polysaccharides.

CONTENTS

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1. INTRODUCTION

Agriculture is faced with multiple challenges in the 21st century: *i*) increase in food production for a growing world population, which is expected to increase by about 2.3 billion people over the next 40 years, mostly in developing countries, *ii*) insufficient fresh water supply together with land degradation, which causes losses in agricultural productivity, *iii*) increase of the production of feedstock for bioenergy, *iv*) adoption of more efficient and sustainable production methods and *v*) adaptation to climate change.[1,2]. Furthermore, it is presently accepted that malnutrition is a silent massacre. According to United Nations sources, millions of children every year die either from malnutrition or are victims of malnutrition and micronutrient deficiency – the lack of key vitamins and minerals – with severe consequences on their physical and intellectual development.[3,4] Moreover, both overweight and underweight people may suffer either from a deficiency or an excess of the intake of nutrients needed for healthy living.[5]

Taking into account the above challenges, a future increase of the global production of food and food protein and a combat of malnutrition could be addressed through the development of non-traditional farm products by biotechnological intervention. Such a solution seems today economically viable by supporting the mass cultivation of microalgae rich in protein, vitamins and other functional nutrients known to benefit health. In this respect, *Spirulina* microalga seems to offer the perfect solution.

Spirulina, now named *Arthrospira*, are microscopic photosynthetic and filamentous cyanobacteria (blue–green algae) that have a long history of use as food. Cyanobacteria are believed to have evolved 3.5 billion years ago and they are the first group of bacteria that evolved that could fix atmospheric carbon dioxide into organic carbon compounds using water with the simultaneous evolution of oxygen. Before Columbus, Mexicans (Aztecs) exploited this microorganism as human food, while presently the African Kanembou tribe in the Lake Chad area (Republic of Chad) employs it for the same purpose. The name *Spirulina* derives from the spiral or helical nature of its filaments. *Arthrospira* is the scientific name of a cyanobacteria genus comprising a whole group of edible cyanobacteria sold under the name *Spirulina*. Among the various *Arthrospira* species, *A. platensis* and *A. maxima* are the most important. *Arthrospira* trichomes (filaments), which contain cylindrical cells aligned together in spirals or in straight lines. These filaments have variable length (usually 100–200 μm) and a diameter close to 6–12 μm, but the cell dimensions, degree of coiling and length of filaments

vary with species. The cell organization of *Spirulina* is typical of a prokaryote gram-negative bacterium with a lack of membrane-bound organelles. The cell wall constitutes a weak envelope that is composed of a number of layers, mostly of a peptidoglycan and lipopolysaccharide nature. The *Spirulina* cells have a number of inclusions, such as thylacoid membranes with phycobilisomes, carboxysomes, ribosomes, DNA fibrils and gas vacuoles, as well as polyglycan, polyphosphate and cyanophycin granules.[6–10]

Spirulina grows naturally in alkaline lakes but is commercially produced in large outdoor or greenhouse ponds under controlled conditions. Microalgal cultivation is based on a photosynthetic process using sunlight, nutrition elements and CO_2 contained in a fresh water culture medium under a relatively high temperature (optimum temperature: 35–38 °C). Commercial culture of *Spirulina* is followed by harvesting the biomass, drying and packaging.[9,11]

World market evolution of *Spirulina* involves mainly dried *Spirulina* whole biomass used as a human health food supplement, assuming that its consumption may benefit, prevent, help, or cure common diseases and malnutrition. Other commercial products containing *Spirulina* biomass or *Spirulina* extracts or active ingredients include protein supplements in animal feed, products for the improvement of pet health, natural colors for foods and cosmetics and purified biomolecules for medicine and biotechnology.[10] The objectives of this paper are to review recent literature on various health and safety aspects of *Spirulina* food supplements and their possible side effects.

2. CHEMICAL COMPOSITION

Chemical analysis of *Spirulina* showed that it is an excellent source of proteins, vitamins, dietary minerals and pigments. The biochemical composition depends upon the specific *Arthrospira* source, culture conditions and season of production.[9,10,12–14]

The protein content of *Spirulina* (50–70 % of the dry weight) exceeds that of meat, dried milk, eggs, soybeans or grains. *Spirulina* proteins are complete, since all the essential amino acids are present. The highest values for the essential amino acids are those for leucine, valine and isoleucine. When compared to standard alimentary proteins (from meat, eggs or milk), it is somewhat deficient in methionine, cysteine, and lysine, but is superior to all plant proteins including proteins from legumes.[6,10,13]

Spirulina proteins with significant health effects are the phycobiliproteins phycocyanin C and allophycocyanin at an approximately 10:1 ratio, which are proteins with linear tetrapyrrole prosthetic groups (phycocyanobilin) that in their functional state are covalently linked to specific cysteine residues of the proteins and they form light-harvesting antenna complexes of the cyanobacteria. *Spirulina*

is the only food containing phycocyanins, which represent about 15–25 % of the dry biomass of the microalga.[15,16]

Half of the total *Spirulina* lipids are fatty acids.[12,14] A detailed analysis of *Spirulina* fatty acids showed the presence of essential fatty acids (mostly ω-6). The rare polyunsaturated fatty acid γ-linolenic acid (GLA) with putative medicinal properties represents 10–20 % of the fatty acids in *A. maxima*, compared to 49 % in *A. platensis* and can be considered one of the best known source of GLA after human milk and some little used vegetable oils such as evening primrose, borage, blackcurrant seed and hemp oil. 10 g of *Spirulina* provide over 100 mg of GLA (which corresponds to more than two capsules of evening primrose oil).[9] Other major fatty acids present are the unsaturated oleic and linoleic acids as well as the saturated palmitic acid, which forms more than 60 % of lipids in *A. maxima*. Monogalactosyl- and sulfoquinovosyl-diacylglycerol as well as phosphatidylglycerol are the major *Spirulina* lipids (20–25 % each).[17] It is important that sulfolipids from cyanobacteria are active against the AIDS virus.[10,12]

Virtually all the assimilable *Spirulina* carbohydrates consist of polymers containing glucose. The major polymeric component in *A. platensis* is a branched polysaccharide, structurally similar to glycogen.[6] High molecular weight anionic polysaccharides with antiviral and immunomodulating activities (see below) have been isolated from *Spirulina*.[18] A sulfated polysaccharide fraction with antiviral action (calcium spirulan) was extensively purified and shown to be composed of rhamnose, 3-*O*-methylrhamnose (acofriose), 2,3-di-*O*-methylrhamnose, 3-*O*-methylxylose, uronic acids and sulfates.[19,20] Recently, an acidic polysaccharide fraction has also been isolated from *A. platensis*, which induces the synthesis of TNF-α in RAW macrophages.[18]

Spirulina is claimed to be the richest whole-food source of provitamin A (β-carotene), with 20 g of *Spirulina* also fulfilling the significant body requirements of vitamins B1 (thiamine), B2 (riboflavin) and B3 (niacin).[10,12,13,21] Its mineral content varies depending on the culture medium. The most interesting minerals in *Spirulina* are iron, calcium, phosphorus and potassium.[10,12] The analytical data for the chemical composition of *Spirulina* are presented in Table I.

Whole-genome sequences of several *Spirulina* strains have already appeared in the literature. *A. platensis* NIES-39 genome structure is estimated to be a single circular chromosome of 6.8 Mb, yielding 6,630 protein-coding genes, two sets of rRNA genes and 40 tRNA genes.[22] Whole-genome sequencing of the *Arthrospira* PCC 8005 strain, which was selected by the European Space Agency (ESA) as a nutritional product and an oxygen producer of the Micro-Ecological Life Support System Alternative (MELiSSA) for long-term manned space missions, showed the presence of 6,279,260 bases with an average G+C content of 44.7 %, 5,856 protein-coding sequences and 176 genes encoding RNA were also predicted.[23] Recently, the draft whole-genome shotgun sequencing of *A. maxima*

was obtained. The draft genome was approximately 6.0 Mb in total, with 5,690 protein-coding sequences.[24]

TABLE I. Analytical composition of *Spirulina* (*Arthrospira*)

Component	Relative dry weight, %	Reference
Proteins	50–70	12
Carbohydrates	15–25	10,13
Lipids	6–13	14
Nucleic acids	4.2–6	12
Iron	0.058–0.18	12
Calcium	0.13–1.4	12
Phosphorus	0.67–0.9	12
Potassium	0.64–1.54	12
Carotenoids	0.37–0.59	10,18
Chlorophyll α	0.66–1.2	10,13,18
Ash	3–11	9
Moisture	4–9	9

3. HEALTH ASPECTS

Although historically *Spirulina* was used as a food component, it has been thoroughly investigated using *in vitro* and *in vivo* experiments, including cell and tissue culture, animal testing as well as human clinical trials, for its role in human health management. A huge number of publications in peer reviewed scientific journals and book chapters covering health aspects of *Spirulina* have appeared during the last three decades. These articles described experimental approaches involving whole cell *Spirulina* preparations, various cell extracts and purified biomolecules, aiming at elucidating the potential health benefits of the consumption of this microalga, so far with exciting results. Potential health effects included: immunomodulation, antioxidant, anticancer, antiviral and antibacterial activities, as well as positive effects against malnutrition, hyperlipidemia, diabetes, obesity, inflammatory allergic reactions, heavy metal/chemical-induced toxicity, radiation damage and anemias.[10,12,25–30] In this respect, the most promising active *Spirulina* constituents appeared to be the protein phycocyanin,[30] sulfated polysaccharide fractions,[18] GLA[31] and certain sulfolipids.[32] While these medicinal claims may be based on experimental observations, more research is needed, especially with larger scale randomized studies in humans, in order to rate the effectiveness of *Spirulina* as a nutraceutical and source of potential pharmaceuticals, and to understand the mechanisms of action of specific *Spirulina* biomolecules, their short-term and long-term effects, and the safety of their use in functional foods.

Available new information or data not covered by previous review articles concerning human clinical potential of *Spirulina*, and data related to the safety and side effects of *Spirulina* are summarized below.

3.1. Immunomodulation

Forty volunteers of both sexes with an age of 50 years or older took a *Spirulina* supplement (3 g per day) for 12 weeks. A steady increase in the average values of the mean corpuscular hemoglobin in subjects of both sexes was recorded. An increase of indoleamine 2,3-dioxygenase enzyme activity (a sign of immune function) and white blood cell count were also observed for the majority of subjects.[33]

In a recent clinical trial involving two studies, a pilot study with 11 individuals and a double-blind placebo controlled study with 12 individuals, healthy volunteers supplemented their diet with 200 or 400 mg day^{-1}, respectively, for seven days with Immulina® (a commercial extract of *A. platensis*, which is known to activate THP-1 monocytes and CD+ T cells *in vitro* and enhance immunological functions in mice). An enhancement of natural killer cell activity following administration of Immulina® was observed. Evidence was presented that Braun-type lipoproteins of the *Spirulina* commercial extract were responsible for the major portion of the *in vitro* monocyte activation.[34]

3.2. HIV-infected and undernourished patients

In a randomized study to compare the effect of *A. platensis vs*. soybean as food supplements on insulin-resistant HIV-infected patients, 33 patients received 19 g of supplement (*Spirulina* or soybean) daily for 8 weeks. It was concluded that the insulin sensitivity in HIV patients improved more when *Spirulina* rather than soybean was used as nutritional supplement.[35] Furthermore, when HIV-infected or HIV-negative undernourished children and HIV-infected adults were treated with *Spirulina* supplementation, clinical improvement was always observed, including weight increase, improvement of hematological parameters and decrease in the HIV viral load.[36–39]

3.3. Clinical potential of phycocyanobilin

The chromophore phycocyanobilin (PCB) of *Spirulina*, was found to strongly inhibit NADPH oxidase activity, since in mammalian cells it is reduced to phycocyanorubin, a close homolog of bilirubin, which shows a potent inhibitory activity of this enzyme complex (observed in nanomolar intracellular concentrations). Due to the central roles of NADPH oxidase activation in pathology, PCB supplementation may induce prevention and therapy of various diseases in part mediated by NADPH oxidase overactivity in affected tissues. Medical conditions associated with or linked to NADPH oxidase activity include among others: cardiovascular diseases, metabolic syndrome, diabetic complications, Parkinson's disease, Alzheimer’s disease, rheumatoid arthritis, allergic reactions and cancer. Administration of PCB may be achieved by any desirable route including ingestion of whole *Spirulina*, phycocyanin protein or isolated tetrapyrrole chromo-

phore. PCB represents about 4.7 % of the mass of phycocyanin. Thus, about 0.66 % of the dry mass of *Spirulina* is PCB, or about 15 g of *Spirulina* can be expected to provide about 100 mg of PCB.[40,41]

3.4. Safety aspects

Spirulina is regulated as a food and as a dietary supplement.[13] The Food and Drug Administration (FDA) of the USA has categorized several *Arthrospira* dried biomass products as "generally recognized as safe" (GRAS) for human consumption.[42] *Spirulina* has typically been studied in daily doses of 1 to 10 g and a recommended dosage for adults is usually in the range of 3–10 g day^{-1}.[9,10,12]

The amount of iodine contained in 10 g of dried *Spirulina* biomass is only 3 μg[44] or less (not detected).[44] Since the upper safe level for total daily intake of iodine (for a 60 kg bodyweight adult), established by the Scientific Committee on Food (SCF) and the European Food Safety Authority (EFSA), is 600 μg, while the corresponding value suggested by the US Institute of Medicine is 1100 μg,[45] there is no risk of a consumer taking in excessive iodine by *Spirulina* consumption.

Spirulina contains substantial amounts of several B12 analogues (corrinoid forms, pseudovitamin B12) which do not fulfill specific functional roles of vitamin B12 for humans.[46] However, vitamin B12 bioavailability experiments with animals fed with *Spirulina* showed that *Spirulina* intake does not interfere with mammalian B12 metabolism, thus showing that the B12 analogues do not present inhibitory actions.[47]

Spirulina total nucleic acid contents of 4.2-6 % of microalga dry matter have been reported[12] (the RNA content is about 3–4 times higher than that of DNA),[6] which are higher than those of animal meat and various plant foods,[48] similar to those of unicellular algae but lower than those of bacteria and yeast.[6] Dietary nucleic acids highly influence serum uric acid levels because of purine metabolism. A maximum safe limit of RNA in the diet of 2 g day^{-1} has been suggested[48] since high levels of uric acid may result in pathological conditions.[49] Accordingly, a safety margin of 30 g maximum daily intake of *Spirulina* was recommended.[49]

3.5. Side effects

The Dietary Supplements Information Expert Committee (DSI-EC) reviewed recent information from human clinical trials, animal studies, and regulatory and pharmacopeial sources and analyzed adverse event reports regarding *Spirulina* to assess potential health concerns. The DSI-EC concluded that the available evidence does not indicate a serious risk to health or other public health concerns and assigned a Class A safety rating for *A. maxima* and *A. platensis*, thereby

permitting the admission of quality monographs for these dietary supplement ingredients in the United States Pharmacopoeia and National Formulary.[50] However, information is limited concerning interactions with pharmaceutical compounds or other dietary supplements. A few side effects have been reported from the ingestion of *Spirulina*, including headache, stomach ache, muscle pain, flushing of the face, sweating and concentration difficulties.[51] A few cases of severe side-effects have also been reported, including hepatotoxicity[52] and rhabdomyolysis.[51] Since *Spirulina* is an immunomodulatory supplement, it might affect disease severity in patients with autoimmune diseases. These patients have to avoid the consumption of *Spirulina*.[53,54] A case of anaphylaxis caused by the *Spirulina* pigment phycocyanin was also reported. Upon an oral challenge with increasing *Spirulina* doses, corresponding to four *Spirulina* tablets over a 3-h period, a 14-old year adolescent experienced diarrhea and erythema.[55] People with phenylketonuria should avoid the consumption of *Spirulina*.[51] It has been suggested that *Spirulina* when present in the diet exerted a neuroprotective effect in a mouse model of amyotrophic lateral sclerosis (ALS), by retarding or stopping motor neuron degeneration.[56] Nevertheless, as emphasized by the ALSUntangled Group,[57] at this time there is no evidence that *Spirulina* is effective for ALS and there appears to be real and theoretical toxicities that patients with ALS may encounter with it. Until better efficacy and safety studies are published, the ALSUntangled Group does not support the use of *Spirulina* in patients with ALS.[57]

It must be emphasized that the consumption of *Spirulina* of unknown origin or originating from countries that do not guarantee the quality and safety of the product should be avoided. The major areas of concern for the safe consumption of *Spirulina* are microbiological load, heavy metal content, pesticides, extraneous matter and cyanobacterial toxins.[13,50]

4. CONCLUDING REMARKS

Spirulina, a microscopic and filamentous cyanobacterium with widespread usage throughout the world as a dietary supplement and a potential solution for combating malnutrition, seems to offer significant health advantages to the consumer. The positive effects of *Spirulina* in relation to its immunomodulation and antiviral properties are based on clinical evidence but larger trials are required. The microalga chromophore phycocyanobilin, as a potent inhibitor of NADPH oxidase, may have versatile potential in the prevention and therapy of various diseases mediated by overactivity of this enzyme. *Spirulina* is accepted as a safe food supplement and the total number of side effects reported in the literature is relatively small. The iodine and nucleic acid content of *Spirulina* do not pose health risks assuming a consumption of *Spirulina* of up to 30 g per day.

ИЗВОД

ЗДРАВСТВЕНИ АСПЕКТИ МИКРОАЛГЕ *Spirulina* (*Arthrospira*) КАО ХРАНЉИВОГ СУПЛЕМЕНТА

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Спирулина (*Spirulina*), позната и као Артроспира (*Arthrospira*), је микроскопска филаментозна цијанобактерија која је дуго у употреби у исхрани. Комерцијално се производи у великим отвореним базенима, у контролисаним условима. Циљ овог прегледног рада је да изнесе нове податке о клиничком потенцијалу и примени спирулине, као и о резултатима који се односе на безбедност и споредне ефекте. Потенцијалне здравствене благодети спирулине потичу од њеног хемијског састава, укључујући протеине (највећи садржај протеина од све природне хране, 55–70 %), угљене хидрате, есенцијане аминокиселине, минерале (посебно гвожђе), есенцијалне масне киселине, витамине и пигменте. Три најважније компоненте спирулине које имају значајну улогу у побољшавању телесних функција код људи су протеин фикоцијанин, сулфати полисахарида и γ-линоленска киселина. Нови експериментални докази упућују на закључак да спирулина има имуномодулаторне и антивирусне ефекте. Према подацима америчког комитета ("*Dietary Supplements Information Expert Committee of United States Pharmacopeial Convention*"), досадашње клиничке студије не указују на здравствени ризик услед употребе спирулине, мада је пријављено неколико случајева са озбиљним пратећим појавама.

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SURVEY

Plant molecular farming: opportunities and challenges

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Abstract: Modern human life is impossible without products derived from classical, contemporary biotechnology. However, large-scale production of biotechnology wares opens a discussion about the economic impact, waste management, biosafety, and bioethical issues. Plant molecular farming offers a relatively inexpensive option for the yielding of many valuable products and demonstrates a number of advantages over classical technologies, but also raises the questions of further development perspectives, hazard identification and risk assessment. This review is focused on these two questions: opportunities offered and challenges faced by modern plant molecular farming systems.

Keywords: molecular farming; biohazard; plant biotechnologies.

CONTENTS

1. INTRODUCTION

During the last decade, plant molecular farming has become a widely used pipeline for the production of large variety of economically important components, among them bio-pharmaceuticals, enzymes, polymers, *etc*. Recently, several plant-derived biopharmaceutical proteins reached the late stages of commercial development. These products include antibodies, vaccines, human blood products, hormones and growth regulators.[1] For such products, plants offer practical and safety advantages as well as lower production costs compared with traditional systems based on microbial, animal cells or transgenic animals. With

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ever-increasing number of products in development, plant molecular farming is becoming even more competitive. In this review on the subject, recent technological developments in molecular farming are considered. An attempt has been made to give a broad overview on the exploited production systems and on several economic and biosafety issues.

2. BRIEF HISTORY OF MOLECULAR FARMING

The idea of producing valuable molecules using plants is as old as the first plant transformation. Since then a concept of how and which product can be efficiently produced in plants has rapidly evolved and developed. The first successes of genetic engineering could be defined as the first examples of molecular cloning – among them are human somatostatin, human somatotropin, and human insulin. The general idea that plant cells could be transformed was developed in early 1974.[2] Ten years later, the first successful agrobacterium-mediated plant transformation (Fig. 1) was reported by de Block *et al.*[3] At the very beginning, this technology was dedicated to the improvement the agricultural characteristics of crop plants, such as yield,[4] lipid content modification[5] and optimization of the amino acid composition,[6] or to provide agents for plant protection, such as biological insecticides,[7] engineered herbicides resistance[8] and pathogen resistance.[9] Since then, progress has been made in several different directions, including plant transformation systems and plant production lines. Since the initial commercial and scientific interest was focused on the production of proteins or secondary metabolites for internal uses in the transgenic plants, the main efforts

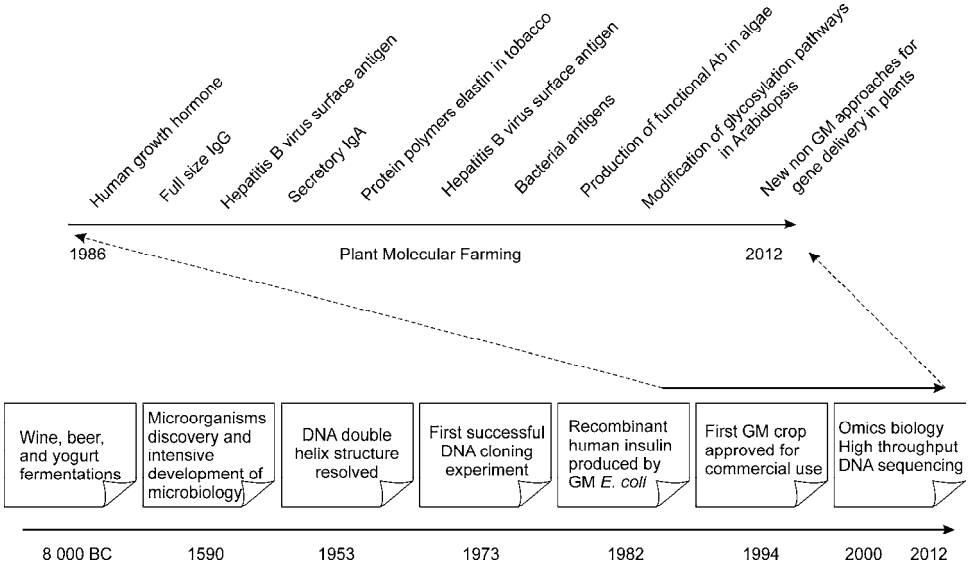

Fig. 1. A concise timetable of the development of molecular biotechnology and molecular plant farming.

were focused on improving transformation efficiency and broadening the host plant range.[10] In the latter respect, plant transformation systems were developed very rapidly. As a result, a number of agrobacterium-mediated transformation, direct gene delivery techniques[11] and pollen path[12] based systems have been deployed in different research or commercial plant genetic modification programs. Lately, the strategies for stable transformation of plants were more intensively substituted with the transient expression approach, which requires less time and allows some difficulties related to plant regeneration and somaclonal variations to be avoided. Most recently used transient expression systems are based on plant viruses "delivered" into the plant cell by *Agrobacterium* infiltration. The last decade of plant farming development can be truly called "a decade of plantibodies[13,14] and biofuel".[15] The intensive research in the field of biofuels and the production of human proteins in plants was initiated in both academic and research institutions and private companies.

3. ADVANTAGES OF THE PLANT MOLECULAR FARMING APPROACH

Expression of potentially valuable pharmaceuticals in plant-based systems has a number of advantages over the traditional biotechnological pipelines. Molecular farming provides payoffs with high technological, economical, social, and ecological impacts. As shown in Fig. 2, plant molecular farming has a remarkable potential for saving time and labor requirements and improving productivity and scalability. One of the important technological positives of this approach is related to the large variety of production systems available for this purpose. For instance, fully contained or open field systems can be easily implemented with some minor adjustments. The fully contained production pipelines do indeed require implementation of bioreactors with properties close to those of the microbiological fermenters. Furthermore, some of the contained production systems demand sufficient light in order to maintain the main plant functions, such as photosynthesis. Light has to be evenly distributed in the fermentation vessel, which in many cases is difficult to achieve or greatly increases the production costs. Therefore, this type of production is usually restricted to locations where the sunlight may be used as an energy source and the design of bioreactors is strongly influenced by the technical solutions implemented for algae cultivation. Alternatively, a genetically modified hairy root plant cultures may be used in dark vessels. However, this system is limited mainly to the production of secondary metabolites. Hairy roots induction is usually achieved by infecting plant tissues with the natural plant pathogen, *Agrobacterium rhizogenes* that causes so-called hairy root disease. The neoplastic roots produced by *A. rhizogenes* infection are characterized by high growth rate and genetic stability. These genetically transformed root cultures can produce high levels of secondary metabolites or amounts comparable to that of intact plants. Hairy root cultures promise

production of valuable secondary metabolites in many plants. The main constraint for the commercial exploitation of hairy root cultures is their scale-up, as a specially designed bioreactor that permits the growth of interconnected tissues unevenly distributed throughout the vessel would have to be developed. Since the hairy root cultures require a fully contained and controlled environment, the maintenance cost become equivalent to or higher than those required for conventional fermentation methods. Another *Agrobacterium* strain, *A. tumefaciens*, is recognized as applicable for plant molecular farming. *A. tumefaciens* is a natural plant pathogen as is *A. rhizogenes* but instead of forming hairy roots, it induces neoplastic growth of formations called "galls". Molecular farming employs genetically modified strains of *A. tumefaciens*. The genetic modification of this bacterial strain causes asymptomatic infection, which is coupled with the introduction of an additional gene set, coding the expression of a particular economically important product. Depending on the implemented experimental procedure, two types of results can be achieved: 1) stable plant transformation and 2) transient expression of a particular gene. Both methods differ in many aspects. The first method requires usage of a natural tumor-inducing plasmid, split in

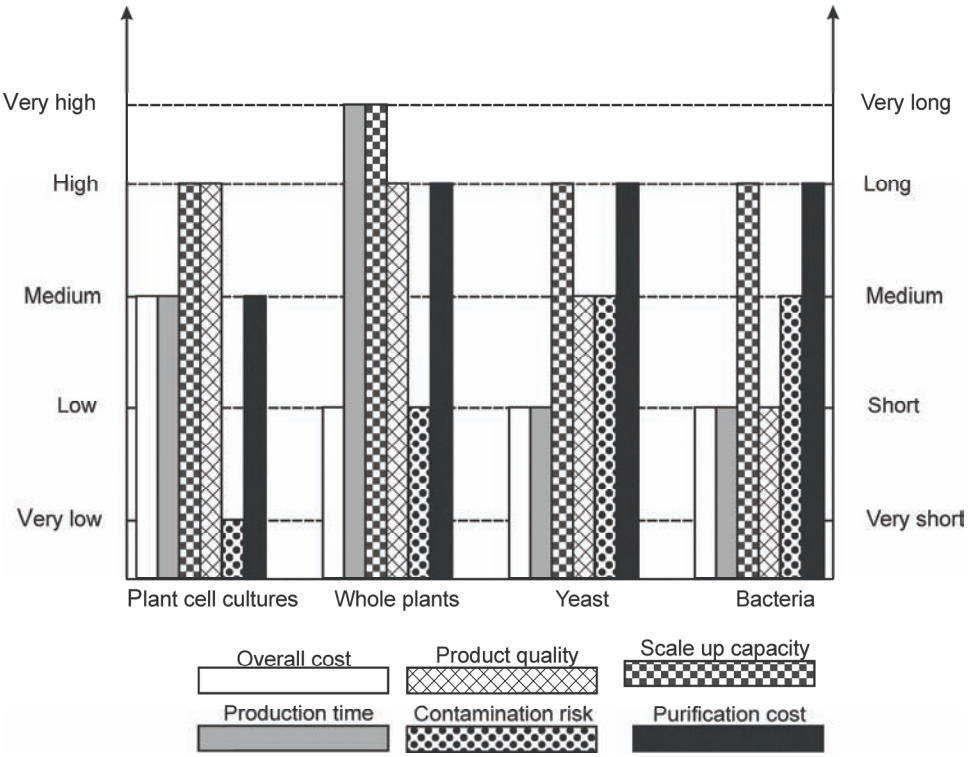

Fig. 2. A comparative analysis of molecular plant farming *vs*. traditional biotechnological production by Xu *et al*.[16] (with modifications).

binary vector systems, modified in such a way that only the desired part of the plasmid is incorporated into the plant genome. A stable transformation results in inheritable expression of the whole gene set, which usually confers expression of two genes – the gene of interest and a marker gene. The latter enables the positive or negative selection of the transgenic cells. The stable plant transformation also offers an additional opportunity for the translocation of the protein product into different cell compartments[17] and therefore, modification of the natural plant synthetic pathways becomes possible. In this respect, the system allows the production of not only recombinant proteins, but also the enhancement of the production of different secondary metabolites. Regarding the experimental procedure, stable plant transformation is more time and labor consuming but offers more sustainable expression in the next generations. Alternatively, transient expression offers a short-term high-level expression of a single gene encoding for the protein of interest. In this case, the expression cassette is usually cloned into the full length or partial plant virus genome, for instance tobacco mosaic virus, potato virus X (reviewed by Wagner *et al.*[18]), tobacco rattle virus (TRV),[19] *etc*., containing the gene of interest under control of a viral or other suitable promoter. The function of the viral genome in this scenario is to ensure the replication and subsequent transcription of its exogenously supplemented section. Depending on the employed viral component, a systemic or local gene expression may be observed. In most cases, the capacity of transient expression systems is limited to the production of recombinant proteins for a short-term period.

Another very promising alternative for genetic manipulation is the chloroplast transformation. It is accepted that the first plastids arose from endosymbiosis between a photosynthetic bacterium and a non-photosynthetic host[20] and therefore the chloroplast matrix environment is more bacteria-like, which provides better protein storage conditions for some proteins. The chloroplast genome is semi-autonomous: a large part of the chloroplast proteins are encoded by its own genome but, simultaneously, a small part of the genetic information is translocated to the nucleus of the plant cell – for example the large subunit of Rubisco is encoded by the chloroplast genome but the Rubisco small subunit encoding gene is located into to the plant nuclear genome.[21] Upon translation in the cytosol, the small subunit is subsequently transported into the chloroplasts.

Since the chloroplast transformation is mediated by homologous recombination of the transgene with the chloroplast genome, identification of spacer regions for integration of transgenes and the regulatory sequences is essential for experiment design. As the integration into the chloroplast genome is site-specific, the concerns of a position effect, frequently observed in nuclear transgenic lines,[22] are eliminated. As a result of the lack of transgene silencing, high levels of accumulation of transcripts have been reported.[23]

On the other hand, in most angiosperm plant species, plastid genes are maternally inherited[24] and therefore, transgenes in these plastids are not disseminated by pollen. An important advantage of plastid transformation is the ability to accumulate large amounts of foreign protein. Expression levels of up to 46 % of the total soluble protein were reported by De Cosa *et al.* and protein crystal formation was demonstrated.[25] Until now, chloroplast transformation has been implemented for the production of many valuable therapeutic proteins, such as Human interferon gamma (HIF-γ), Human somatostatin (hST), vaccines against antrax,[26] tetanus,[27] cholera,[28] *etc*. Chloroplast transformation has also been involved in production of a number of biomaterials such as monellin,[29] elastin-derived polymers,[30] *etc*.

In most cases, plant molecular farming systems require a certain degree of biosafety measures. After appropriate risk assessment, transgenic plants may be cultivated under regular field conditions according to the risk management programs developed for certain crops. In fact, only the cost of plant material processing is additional to the standard farming costs.

4. BIOSAFETY ISSUES RELATED TO MOLECULAR FARMING IN PLANTS

Considering that in most cases of molecular farming in plants or plant-derived cell/tissue cultures one is dealing with different degrees of genetically modified organisms, biosafety is gaining significant importance. The first two questions that require adequate answers are: 1) what is the hazard, respectively, how to identify the hazard and 2) what is the biological risk of implementation of such technology. Once the hazard is identified, exposure to the genetically modified event has to be determined. The risk is calculated using the formula:

$$\text{Risk} = \text{Hazard} \times \text{Exposure}$$

Hazard identification requires assessment of the potential gene transfer events, which are divided into two main classes: vertical and horizontal gene transfer. Vertical gene transfer is the movement of genetic material between at least partially sexually compatible plants. In this case, crops for molecular farming should be chosen with the minimization of gene flow in mind. This is the most prevalent form of transgene pollution and occurs predominantly *via* the dispersal of transgenic pollen, resulting in the formation of hybrid seeds with a transgenic male parent.[31] Hybrid seeds could also be generated with the transgenic plant as the female parent if the transgenic crops were fertilized by wild type pollen. In this case, transgene pollution would occur *via* seed dispersal, during growth, harvesting or transport.

Horizontal gene transfer represents genetic material exchange between sexually incompatible species that may belong to very different taxonomic groups. It is common in prokaryotes, resulting in the transfer of plasmid-borne resistance between different bacteria species. There are only few examples of natural gene transfer

between bacteria and higher eukaryotes. Since agrobacterium represents a special case where gene transfer occurs naturally from bacteria to plants, there is a perceived risk for horizontal gene transfer. In most cases, gene transfer from transgenic plants to soil bacteria or microorganisms in the digestive systems of herbivores is a subject of concern. The eventually transferred traits could have unpredictable effects on the relationships between different organisms, *i.e.*, render harmless bacteria pathogenic or make pathogenic species more difficult to control, *etc*.

Numerous potential solutions to the problem of transgene pollution caused by either horizontal or vertical gene transfer have already been developed.

When discussing biosafety issues related to genetically modified plants, it is necessary to recognize contamination with GM material during transport as an important hazard factor, especially in the case of post-harvest processing of the plant material (seeds, foliage, fruits, *etc*.). The risk of contamination becomes significant when genetically modified and conventionally produced plant materials or products are processed in the same installations or transported in the same vehicles. As complete cleaning is not possible in most cases, 0.8 % GM contamination in goods has been accepted as a threshold (this may differ in different countries).

5. CONCLUDING REMARKS

Research over the past 20 years has significantly increased knowledge in the field of molecular biology and especially the understanding of gene expression in higher plants, and technological development enabled plant derived cells or tissue to be cultured using different platforms. This has enabled a move from pure laboratory studies in model species to the exploration of a variety of different plants for the production of recombinant proteins and a number of secondary metabolites. Broad ranges of technical, pharmaceutical and industrial proteins have been produced in plants, some on a commercial basis. The main efforts were focused on overcoming the technical limitations of molecular farming, particularly by increasing low yields in some expression systems. However, there are several further challenges concerning the issues of environmental impact, biosafety and risk assessment, which reflect the release and agricultural-scale cultivation of transgenic plants, as well as the safety of the plant-derived products themselves.

И З В О Д

МОЛЕКУЛАРНИ АСПЕКТИ ГАЈЕЊА БИЉАКА – МОГУЋНОСТИ И ИЗАЗОВИ

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Модеран живот људи је немогуће замислити без производа проистеклих из класичне или модерне биотехнологије. Биотехнолошка производња великих размера отвара питања економског значаја, руковања отпадом, биолошке безбедности и биоетике. Га-

јење биљака на молекуларној основи нуди релативно јефтину могућност производње великог броја значајних производа и има одређене предности у односу на класичну производњу, али повлачи са собом и процену перспективе даљег развоја, идентификацију опасности и ризика овакве технологије. У овом ревијском раду описане су могућности и изазови примене система модерног молекуларног гајења биљака.

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577.152.3:547.458.87
Survey

SURVEY

Contribution of cell wall-modifying enzymes to the texture of fleshy fruits. The example of apple

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Abstract: The cell walls of fleshy fruits consist of polysaccharide assemblies (pectin, hemicelluloses and cellulose), the structure and interactions of which vary depending on the genetics of the fruit, and its stage and conditions of development. The establishment and the structural reorganization of the assemblies result from enzyme/protein consortia acting *in muro*. The texture of fleshy fruits is one of the major criteria for consumer choice. It impacts also post-harvest routes and transformation processes. Disassembly of fruit cell wall polysaccharides largely induces textural changes during ripening but the precise role of each polysaccharide and each enzyme remains unclear. The changes of cell wall polysaccharides during fruit ripening have mainly emphasized a modulation of the fine chemical structure of pectins by hydrolases, lyases, and esterases. This restructuring also involves a reorganization of hemicelluloses by hydrolases/transglycosidases and a modulation of their interactions with the cellulose by non-catalytic proteins, such as expansin. Apple is the third most produced fruit in the world and has been the subject of studies about fruit quality. This paper presents some of the results to date about the enzymes/ /proteins involved in fruit ripening with particular emphasis on apple.

Keywords: hemicelluloses; ripening; softening; polysaccharide-hydrolases; xyloglucan-transglycosidase.

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1. INTRODUCTION

Fleshy fruits are defined as fruits consisting mainly of a soft succulent tissue made of water-rich parenchyma, named pulp or flesh. They include two main types of fruit: drupes and berries. Drupes are fleshy fruits with a stone (cherry, plum, peach, *etc*.) that have only one seed and the endocarp is thick and very hard. On the other hand, berries are fleshy fruits with a thin endocarp that contains generally several seeds scattered in the pulp.

Apple (*Malus domestica*) is a particular case of fleshy fruit where a core containing several seeds is surrounded by the fleshy edible tissue. Since the latter does not originate from the pistil tissue but from the inferior ovary, it is referred to as an accessory fruit, previously called false fruit. Among fleshy fruits, apple is the third world production, after tomato and grape.[1] In Europe, Italy, Poland and France are the three main producers and cumulate 5.8 Mt. Apple fruit texture is a key quality trait orienting consumer choice as well as agro-industrial processes, impacting post-harvest routes and resistance to diseases.[2,3] Firm, crispy, mealy, juicy, crunchy are among the words describing fruit texture. These add to the criteria of visual appearance, sweetness and sourness and aromatic perceptions to determine consumer preference. Texture is defined as a group of properties sensed by the feeling of touch (hand, mouth) and related to the deformation, disintegration, and flow of the food under force.[4] Fleshy fruits contain 85 to 95 % water. Consequently, texture results from the structure and organisation of about 1.0 to 4.0 % of the macromolecular dry matter in cell walls.

Cell walls play a key role in cell protection and in the regulation of intercellular exchanges. Their thickness, molecular organization and structure shape the cells, assure cell–cell adhesion and provide the mechanical support to the cell for withstanding turgor pressure. On the tissue scale, the cell density and organization, as well as the number, size and distribution of intercellular spaces have consequences on the mechanical properties of fruit. On the organ scale, the proportion and distribution of different tissues (parenchyma, conducting tissue, etc.) affect the perception of fruit texture. Particular combinations of these features on different scales define the mechanical and physicochemical properties at the origin of the various texture descriptors. The understanding and control of the origin and evolution of these different combinations in ripening fruit represent a major challenge for the fruit sector in order to provide fruits with the desired textures.[5]

Texture evolves early on, starting at fruit development,[6] but it particularly changes during fruit ripening as the result of major cellular metabolic modifications and cell wall disassembly. These phenomena lead to softening, the mechanism of which was the subject of numerous studies in tomato, taken as a fruit model.[7,8] However, despite common molecular features with other fruits, tomato largely differs in structural and mechanical aspects from others, such as apple. In

particular, its cell wall composition and the enzymatic machinery involved in wall disassembly differ. Nevertheless, the discovery of regions of genomic synteny across distant species, such as, for example, strawberry (*Fragaria*) and apple (*Malus*) in the Rosaceae family[9] enable analogies between the fruits to be addressed.

Although softening of a fleshy fruit involves changes in water content and compartmentalization, this review will highlight the cell wall contribution to the texture in apple. After an overview of its cell wall and its constitutive polymers, the main changes occurring in the wall of the ripening fruit and the key enzymes and proteins involved in this process will be summarized.

2. STRUCTURE OF PLANT CELL WALL

Cell wall is a nanocomposite surrounding each plant cell exterior to the plasmalema. It corresponds to an assembly of biopolymers, mainly polysaccharides but also proteins and phenolic compounds. Fleshy fruits such as apple mainly contain parenchyma cells where the cell wall is of a primary type. Primary cell walls are thin, flexible and highly hydrophilic (65 % water).[10] In apple cell wall, as in other fruit parenchyma, three main polysaccharide families interact to form the primary cell wall: cellulose, hemicelluloses and pectin. Cellulose is a 1,4-β-D-glucan, which is associated by numerous hydrogen bonds to form long and rigid microfibrils. It is essentially insoluble in dilute acid and alkali, is highly resistant to physical, chemical and enzymatic degradations, and ensures wall rigidity.

The hemicelluloses are composed of three groups of polysaccharides: xyloglucan (XyG), (galactogluco)mannan (GgM) and glucuronoxylan (GuX). They are predominantly formed of neutral sugars and are soluble in dilute alkali. In apple, the most abundant hemicellulose is xyloglucan,[11] which can account for 20–25 % of the primary cell wall.[12] Its backbone is a 1,4-β-D glucan on which 75 % of the glucose residues (Glc) are substituted by mono-, di- or tri-saccharide side chains. The first sugar of these side chains is always a α-D-xylopyranose (Xyl). In apple, this first residue can carry β-D-galactopyranose or α-L-fucopyranose-(1,2)-β-D-galactopyranose disaccharide. Acetyl-esterification can occur on XyG. A nomenclature was proposed by Fry *et al.*[13] for the different structures encountered in xyloglucans, whereby one letter codes for the differently-substituted β-D-Glc residues of xyloglucan-derived oligosaccharides (Fig. 1).

Xyloglucans are classified as ‘XXXG-type’ or ‘XXGG-type’ based on the number of backbone glucosyl residues that are branched.[14] Apple xyloglucan is of the XXXG-type with three Glc residues carrying a side chain and the fourth unsubstituted. In primary cell walls, xyloglucans extensively coat the cellulose microfibrils according to a reversible process involving hydrogen bonding (Fig. 2).[15]

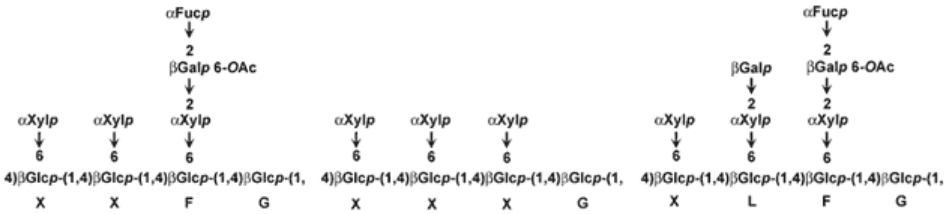

Fig. 1. Structure and nomenclature of apple xyloglucans.[13]

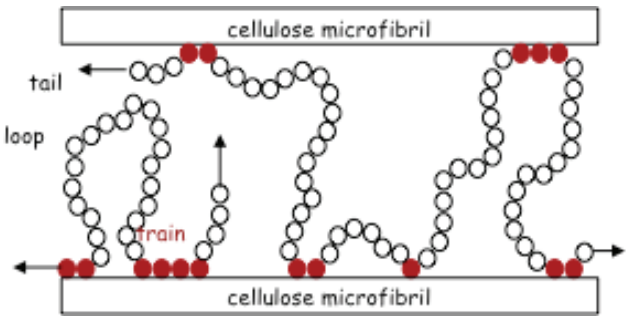

Fig. 2. Schematic representation of the interaction of xyloglucan and cellulose. Full circle, xyloglucan oligosaccharides that interact with cellulose microfibril (trains); empty circle, xyloglucan oligosaccharides that do not interact with cellulose microfibril (loops and tails), modified from Vincken *et al.*[15]

The second group of apple hemicelluloses is mannans. They are thought to account for only 3–5 % of the primary cell wall, as glucomannans or as galactoglucomannans (GgM). Glc and mannose (Man) form the backbone with an alternating or block-wise distribution, and the Man residues may carry Gal side chains.[12] Some structural data collected on GgM isolated from cultured tobacco or blackberry cells, or from kiwi fruit show a more or less alternating structure 1-4 linked β-D-Glc and β-D-Man. The Man residue can be substituted on *O*-6 with α-D-Gal or α-D-Gal (1-2) β-D-Gal and can be partially acetyl-esterified.[17–20] However, the fine chemical structure, properties and role *in planta* of GgM are still under discussion. They are able to form hydrogen bonds to cellulose[21,22] and are thus expected to share functions with XyG, although they are less abundant and generally shorter polysaccharides than XyG.[23]

The third group of hemicelluloses consists in glucuronoxylan. In tomato, GuX is co-extracted with GgM even under strong alkaline conditions, and enzymatic treatments of the extract provided evidence that GuX and GgM can be associated in a complex.[24] The function of GuX in the tomato wall remains to be established. The presence of GuX in apple has not been reported to date.

Pectin forms the third family of cell wall polysaccharides in the primary cell wall. Its high content in partly methyl-esterified galacturonic acid (GalA) and the

presence of rhamnose (Rha) are the distinctive features of this heterogeneous group of polysaccharides.[25] Pectin is a complex polysaccharide that can be envisioned as a multi-block co-biopolymer (Fig. 3). The simplest of these blocks is homogalacturonan (HG), an unbranched polymer of (1→4)-*α*-D-GalpA. Other minor galacturonans can be distinguished as, for example, rhamnogalacturonan II (RG-II) and xylogalacturonan (XGA), the latter being particularly present in apple pectin.[26] A second major block, rhamnogalacturonan I (RG-I), is composed of a repeating disaccharide unit [→2)-*α*-L-Rhap-(1→4)-*α*-D-GalpA-(1→]. RG-I is decorated primarily with arabinan and galactan side chains.

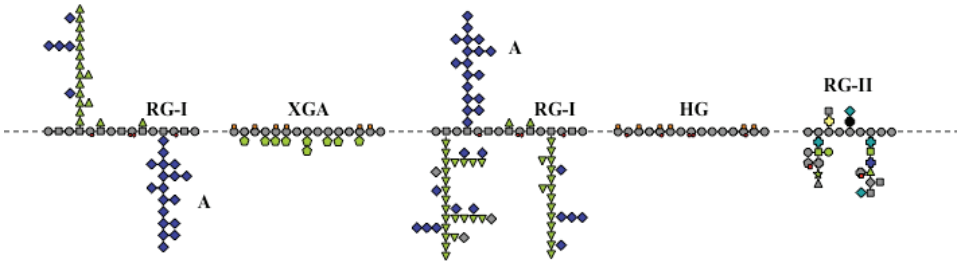

Fig. 3. Schematic representation of the main structural elements of pectin. HG: Homogalacturonan; RG-I: Rhamnogalacturonan I; XGA: Xylogalacturonan; RG-II: Rhamnogalacturonan II; A: Arabinan. Modified from Schols and Voragen.[25]

Homogalacturonan plays important roles in cell–cell adhesion and in controlling wall porosity. Depending on their degree of methylesterification and the distribution of the methyl-substituents, HG is able to dimerise *via* the presence of divalent cations, such as calcium. These interactions contribute to cell adhesion in the middle lamella region as well as to the control of wall porosity.[27] Rhamnogalacturonan I structural domains are also thought to contribute to the cell wall structure and cohesion through the ability of their side chains to interact with water.[28] On the other hand, it was shown that pectin side chains are able to link cellulose microfibrills. This was demonstrated *in vitro* by creating artificial composites by adsorption of extracted arabinan on the primary cell wall cellulose.[29] This demonstrated the ability of arabinan chains to stick to cellulose and a similar phenomenon is likely to occur *in planta*.

3. RIPENING-INDUCED CHANGES IN THE CELL WALL

Extensive depolymerisation and solubilization of pectins together with demethylation were correlated with the decrease in fruit firmness observed during ripening of many fruits.[30–32] Fruit ripening is also often accompanied with galactose loss.[31] On the other hand, no change in molecular weight was evidenced for hemicellulose groups such as GgM and GuX,[24] but could be observed for XyG.[7] In apple, galactose loss occurred mainly during maturation before ripening.[33] However, it was shown that in apple, the loss in galactose concerns not only pec-

tin, but also hemicelluloses. Indeed, during ripening, the galactose content decreased by 29 % in pectin and by 71 to 87 % in the hemicelluloses, according to the apple variety.[31] Nevertheless, there was no change in the molecular weight profile of the hemicelluloses and particularly xyloglucan.[34] More recently, galactose loss during ripening was confirmed in 14 and 17 apple genotypes collected over two years,[35] showing that it is a general feature of apple ripening. Moreover, important hemicelluloses modifications were observed during fruit construction and maturation. These results suggested that the galactose and mannose that disappeared during ripening originated from the same polysaccharide family. Simultaneously, acetyl-esterification decreased in xyloglucan.

3.1. Enzyme involvement in fruit ripening

Due to the complexity of cell wall polysaccharides, a large number of enzymes are involved in their modification or degradation. They can be grouped into three main categories. The first group corresponds to proteins that alter hydrogen bonds and is represented by expansins. The second category corresponds to depolymerases that cleave the polysaccharide backbone. This contains not only glycan hydrolases but also lyases that cleave uronic acid-containing polysaccharides by the β-elimination mechanism to generate an unsaturated uronic acid moiety at the new non-reducing end. This group also includes the transglycosidases that are specific for successively cleaving the polysaccharide backbone and transferring an oligosaccharide on the newly formed reducing end in a unique catalytic event, to allow chain extension. As an example, xyloglucanendotransglycosidase (XET) cuts and then ligates the XyG backbone during cell expansion.[36] The third category comprises the “shaving” enzymes that modify side chains or eliminate substitutions on the backbones of the polysaccharides. These include esterases that remove methyl- or acetyl-esters, and various glycosidases, such as galactosidases and arabinofuranosidases, which shorten the neutral side chains on pectin and hemicelluloses. These glycosidases catalyze the hydrolysis of glycosydic linkages to remove terminal residues specifically from the reducing or non-reducing end of oligosaccharides, polysaccharides or side chains.

To aid the occurrence of depolymerisation of matrix glycan, relaxation of the xyloglucan–cellulose network often occurs. This is thought to be the role of expansin, thanks to its ability to disrupt the hydrogen bonds between cellulose and xyloglucans.[37,38] This dissociation allows slippage of cell wall polymers, before the association reforms to restore the integrity of the cell wall network.[39] The resulting swelling of the cell wall is likely to improve diffusion of new polysaccharide components into the growing wall, as well as enzymes that cleave cellulose and pectins.[40] In tomato, overexpression or suppression of the ripening specific expansin *Exp*1 markedly affects texture.[38] In apple, the expansin activity pattern remains similar throughout all the developmental stages.[41]

Pectin depolymerisation during fruit ripening results from the combined action of endopolygalacturonase (PG, EC 3.2.1.15) and pectin methylesterase (EC 3.1.1.11) that were shown to increase drastically in several species.[42,43] In contrast, pectin-degrading enzymes (*i.e.*, endopolygalacturonase, pectin methylesterase and pectate lyase) are very low in apple.[41,44] Although the pectin content in apple is in the same range as in other fruits (1.5 to 2.5 % on a fresh weight basis),[45] it does not appear to be a major target for the ripening-involved enzymes. Nevertheless, *PG1* expression levels have been associated with softening in a range of cultivars,[46] and suppression of *PG1* in transgenic apple plants results in a firmer fruit.[47] Thus, the role of pectinolytic activities during apple ripening and softening remains to be elucidated. Other polysaccharide-degrading enzymes are likely involved in cell wall disassembly in apple.

Several different enzymes were followed from the apple fruit-set to the over-ripe stage.[41] In this study, xyloglucan endo-transglycosydase (XET) was found to be more active in the ripening fruit. This activity was shown to be particularly important in controlling tomato fruit softening.[48] In apple, the XET activity was shown to be consistent with the kinetics of XET genes expression during apple development, but it was not correlated with fruit growth.[49] In contrast, endoglucanase (EC 3.2.1.4), which contributes to cellulose and xyloglucan hydrolysis, seemed to be more prominent during growth than during ripening.[41] The study of the expression of cDNA encoding cell wall-modifying enzymes throughout the development process showed that many different enzymes, such as methylesterase, pectate lyase (EC 4.2.2.2), arabinofuranosidase (EC 3.2.1.55), endoglucanase and XET, were transcribed until late softening.[8] All the transcripts, except methylesterase, could be unambiguously detected by semi-quantitative RT-PCR in fruit during ripening. However, transcripts of endoglucanase were more abundant at fruit-set. Two XETs were detected in over-ripe fruit, one of them showing a ripening-related pattern. However, it has to be emphasised that none of the cDNAs identified in this work was fruit specific. It is also interesting to note that some hydrolases showed ripening-related expression patterns whereas limited or no polysaccharide depolymerisation was observed during apple softening.[34] Some of the hydrolases potentially involved in the softening process have been shown in other fruits to have only a limited effect on polysaccharide molecular weight. This is particularly true for a β-xylosidase (EC 3.2.1.37) in *Fragaria ananassa*, differently expressed in two strawberry cultivars with contrasted fruit firmness.[50] The softest cultivar showed an early accumulation of xylosidase transcripts and a higher translation to the protein during ripening. However, xylosidases are often described as bifunctional with both xylosidase and arabinofuranosidase activities. Consequently, it is rather difficult to know the precise role of these enzymes *in vivo*. Glycosidases such as β-galactosidase (EC 3.2.1.23) and α-L-arabinofuranosidase (EC 3.2.1.55) were related to the storability of

apple.[51] A high level of α-L-arabinofuranosidase activity was demonstrated to result from 3 different genes and was related to apple mealiness development during fruit storage.[52] The target cell wall polysaccharide of this activity and the resulting impact on the cell wall structure and mechanical properties involved in this texture defect remains to be established. As arabinan side chains in pectin are likely to interact with cellulose and contribute to the wall structure,[29] even a slight shortening of these chains by an arabinofuranosidase could affect this interaction and weaken the network. In addition, galactosidases, xylosidases, arabinofuranosidases and fucosidases potentially modify the XyG side chains structure and thus could control XET activity[53,54] and finally fruit texture.

3.2. CAZymes in Malus domestica

Previous results showed that variation of polysaccharide structure is associated with variations on some chromosomal regions that also control some variations in sensory as well as instrumental texture measurements.[55] However, the relationship between genetic variations, cell wall structure and apple texture are not known. Putative carbohydrate-active enzymes (CAZymes), including 26 glycosyl hydrolases (GH) and one polysaccharide lyase (PL) were identified in apple (www.cazy.org).[56] Among them, 20 GH and the PL could be involved in cell wall reorganisation (Table I). However, only 2 of them were identified at the protein level, the others being evidenced at the transcript level.

TABLE I. CAZymes in *Malus domestica*; GH: glycosyl hydrolase; PL: polysaccharide lyase

Enzyme	CAZy Family	Entry	Existence
β-Glucosidase	GH1	AEN94900	Evidenced at protein level
Endo-mannanase	GH5	C7A7X7	Evidenced at transcript level
Endo-mannanase	GH5	C7A7X8	Evidenced at transcript level
Endo-mannanase	GH5	C7A7X9	Evidenced at transcript level
Endo-glucanase	GH9	Q6V596	Evidenced at transcript level
XET	GH16	COIRH4	Evidenced at transcript level
XET	GH16	COIRH6	Evidenced at transcript level
XET	GH16	COIRH7	Evidenced at transcript level
XET	GH16	COIRH8	Evidenced at transcript level
XET	GH16	COIRH9	Evidenced at transcript level
XET	GH16	COIRI1	Evidenced at transcript level
XET	GH16	COIRI2	Evidenced at transcript level
XET	GH16	COIRI3	Evidenced at transcript level
XET	GH16	COIRI4	Evidenced at transcript level
β-1,3-Glucanase	GH17	Q6QCC8	Evidenced at transcript level
β-1,3-Glucanase	GH17	Q8RVM2	Evidenced at transcript level
β-1,3 glucanase	GH17	Q8RVM3	Evidenced at transcript level
Endo-polygalacturonase	GH28	P48978	Evidenced at transcript level
β-Galactosidase	GH35	P48981	Evidenced at protein level
α-Arabinofuranosidase	GH51	Q7X9G7	Evidenced at transcript level
Pectate lyase	PL1	Q6U7H9	Evidenced at transcript level

4. CONCLUSIONS

Cell wall softening is an essential feature of fruit ripening resulting from wall polysaccharide modifications. Diverse simultaneous structural changes in pectin, cellulose and hemicelluloses are thought to be responsible for the alteration of cell wall structure. These changes are the result of many different enzymatic activities, organized in consortia capable of a concerted action. However, the function of many of them has not yet been evidenced.

Further work is thus required to identify the enzymatic actors involved in cell wall remodelling and to link cell wall structure and apple texture. Now that the apple genome has been sequenced,[57] this will help the scientific community to identify the genes of interest for each mechanism.

ИЗВОД

УТИЦАЈ ЕНЗИМА КОЈИ МОДИФИКУЈУ ЋЕЛИЈСКИ ЗИД НА КОНЗИСТЕНЦИЈУ СВЕЖЕГ ВОЋА: ПРИМЕР ЈАБУКЕ

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Ћелијски зидови се састоје од полисахарида (пектин, хемицелулоза и целулоза) чије структуре и интеракције зависе од генетике воћа, као и стадијума и услова развоја. Успостављање и структурна реорганизација ових полисахаридних целина зависи од интеракција ензима/протеина који делују у зиду. Конзистенција свежег воћа је један од главних критеријума корисника приликом избора. Она зависи и од путева и процеса трансформације након брања. Разарање полисахарида ћелијског зида воћа веома утиче на његову конзистенцију током сазревања, али тачна улога сваког полисахарида и ензима се не зна. Промене полисахарида ћелијског зида током сазревања најизраженије су кроз промену хемијске структуре пектина дејством хидролаза, лијаза и естераза. Ово реструктурирање, такође, обухвата реорганизацију хемицелулозе дејством хидролаза/ /трансглукозидаза и модификацију интеракције ензима са целулозом посредством некаталитичких протеина као што је експанзин. Јабука је трећа воћка по величини производње у свету и њен квалитет се проучава. У овом раду су наведени подаци о ензимима/протеинима укљученим у сазревање воћа, уз посебан нагласак на јабуку.

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Biological activity of *Aronia melanocarpa* antioxidants pre-screening in an intervention study design

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Abstract: The beneficial effects of black chokeberry fruits and juices in health promotion and prevention of chronic diseases shown in both epidemiological and dietary intervention studies are often connected with their antioxidant activity. The aim of this study was to investigate the total phenolics and anthocyanins content, chemical antioxidant activity (DPPH-assay), antioxidant protection in erythrocytes and anti-platelet activity *in vitro* of three different chokeberry products: commercial and fresh pure chokeberry juice and a crude lyophilized water–ethanol extract of chokeberry fruits, as part of their pre-clinical evaluation. The obtained results indicated differences in chemical composition and antioxidant activity of the investigated products. Cellular effects, including both *in vitro* anti-platelet and antioxidant effects, were not directly correlated with the chemical antioxidant activity and the results obtained *in vitro* for anti-platelet effects were only partially consistent with the results obtained *in vivo*, in a pilot intervention trial. In conclusion, chemical analyses and *in vitro* experiments on foods and their bioactive substances are a valuable pre-screening tool for the evaluation of their biological activity. However, extrapolation of the obtained results to the *in vivo* settings is often limited and influenced by the bioavailability and metabolism of native dietary compounds or interactions with differrent molecules within the human body.

Keywords: chokeberry; erythrocytes; platelets; DPPH assay; flow cytometry.

INTRODUCTION

Black chokeberry (*Aronia melanocarpa*) belongs to the Rosaceae family. It is a native plant of North America and Canada, grown successfully in Europe since the beginning of the 20th century. The fruits and juices of black chokeberry (*A. melanocarpa*) are excellent sources of both nutritive and non-nutritive dietary

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compounds with numerous biological activities, including phenolics, vitamins and minerals. The phenolic compounds of chokeberries are procyanidins, anthocyanins, phenolic acids and tannins. Chokeberries are considered to be the best dietary sources of anthocyanins (25 % of total phenolics), one of the most powerful *in vitro* antioxidants. The fruits and juices of this plant have the highest antioxidant activity compared to other types of berries from the Rosaceae family.[1] A large number of dietary intervention studies showed the beneficial effects of the consumption of chokeberry juice and extracts on various risk factors for chronic diseases, including the parameters of oxidative stress,[2] total cholesterol, LDL, oxy-LDL, triglycerides, glucose, HbA1c, systolic and diastolic blood pressure,[3,4] platelet[5] and endothelial function.[6] The effects of black chokeberry consumption are often connected with their antioxidant activity. However, the very low bioavailability of anthocyanins observed after ingestion of anthocyanin-rich food questions the rationale for investigation of native compounds, instead of their metabolites, in *in vitro* models, in pre-screening for *in vivo* effects, including antioxidant activity. The aim of this study was to investigate the total contents of phenolics and anthocyanins, the chemical antioxidant activity (using the DPPH-assay), cellular antioxidant activity and anti-platelet activity *in vitro* and *ex vivo* of three different chokeberry products: commercial and fresh pure chokeberry juice and a crude lyophilized water–ethanol extract of chokeberry fruits.

EXPERIMENTAL

Chemicals

The following buffers and reagents were obtained from Sigma–Aldrich (Germany): phosphate-buffered saline (PBS), Folin–Ciocalteu's phenol reagent, gallic acid, potassium chloride, sodium acetate, 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), ferrous sulphate heptahydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Dulbecco's modified Eagle's medium (DMEM), hydrogen peroxide 30 % solution (H_2O_2), 3,4-dihydroxybenzoic acid (protocatechuic acid), calcein acetoxymethyl ester (calcein AM), foetal bovine serum (FBS), bovine serum albumin (BSA), adenosine diphosphate (ADP) and 2′,7′-dichlorofluorescein diacetate (DCF-DA). Monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated PAC1, phycoerythrine (PE)-conjugated CD62P, peridinin chlorophyll protein (PerCP)-conjugated CD61 and control immunoglobulin G-PE and immunoglobulin M-FITC were purchased from Becton Dickinson (USA).

Subjects

Whole blood was collected from subjects with metabolic syndrome for both *in vitro* experiments (n = 3) and *ex vivo* pilot study (n = 6; 3 males and 3 females). Metabolic syndrome was defined according to ATP III criteria.[7] All blood samples were taken by venipuncture according to the guidelines for blood sampling in platelet analysis. For the *ex vivo* pilot study participants were subjected to dietary intervention by acute intake of 200 mL of commercial pure chokeberry juice and blood samples were collected before and 2 h after the consumption. Whole blood was used for the isolation of plasma and platelets.

The study protocol was approved by the Ethical Committee of the Faculty of Pharmacy, University of Belgrade. The study was conducted in accordance with the revised Declaration of Helsinki. All participants provided written informed consent.

Samples

Three different chokeberry products were used in the study: commercial pure chokeberry juice (CCJ) (Aronia Antioxi, Nutrika, Serbia), fresh pure chokeberry juice (FCJ) obtained in the laboratory by squeezing of fresh fruits (with a yield of 0.53 mL g^{-1} of fresh fruits) and crude lyophilized water–ethanol (40/60 % vol.) extract (WECE) of chokeberry fruits. All investigated chokeberry products were prepared from the fruits of *Aronia melanocarpa* var. *rubina*, grown in western Serbia and harvested during October/November 2012. For the experiments, the juices were diluted to the working concentrations in water or defined buffers. The crude water–ethanol extract was further extracted (50 mg mL^{-1}) with water or methanol and soluble fractions (WCE and MCE, respectively) obtained by centrifugation were used in further experiments. For the cell-based assay, the methanol soluble fraction of investigated extract was evaporated to dryness in a rotary evaporator and the residue was reconstructed with water immediately before addition to the cells. The cell-based antioxidant activity and anti-platelet activity were also evaluated for protocatechuic acid, one of the major metabolites of cyanidin-3-glucoside.[8]

Determination of the total phenolics

Content of total phenolics in investigated samples was analysed using a modified Folin–Ciocalteu method.[9,10]. Juices and extracts were diluted in distilled water to the working solutions that gave absorbances within the standard calibration curve (0–600 μg mL^{-1} of gallic acid). The results are expressed as milligrams of gallic acid equivalents (GAE) per mL of juices or of the investigated extracts. Data are presented as mean ±*SD* for three replications.

Determination of the total anthocyanins

The total anthocyanin content (*TAC*) was quantified using the pH differential method described by Lee *et al*.[11] Briefly, the investigated juices (CCJ and FCJ) and extract fractions (WCE and MCE) were dissolved in a potassium chloride buffer of pH 1.0 and sodium acetate buffer pH 4.5. The absorbance of both buffer solutions was measured at 520 and 700 nm. The results were expressed as milligrams of cyanidin-3-glucoside equivalents (CGE) per mL of juices or g of investigated extract. All experiments were performed in triplicate.

Determination of the antioxidant activity

Radical scavenging activity of the investigated samples was analysed using the DPPH assay.[12] The data are presented as the concentrations of the samples that inhibited 50 % of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (0.05 mM) after 30 min of incubation, based on the decrease in absorbance measured at 517 nm.

The antioxidant activity of plasma obtained from whole blood before and 2 h after the dietary intervention with chokeberry juice was determined according to the method of Benzie and Strain,[13] as the ability of the plasma to reduce ferric ions. The data are presented as the Fe(III) concentration (mM) in the sample after reaction with the ferric tripyridyltriazine (Fe^{III}-TPTZ) complex, according to the calibration curve (0–2000 μM $FeSO_4$). All experiments were performed in triplicate.

Determination of the antioxidant protection of erythrocytes

The cellular antioxidant activity of the investigated samples was based on the antioxidant protection of erythrocytes exposed to reactive oxygen species (ROS). The cellular antioxidant protection assay was performed as previously described[14] with modifications regarding the exposure of erythrocytes to a lower level of extracellular ROS (1mM H_2O_2). Packed erythrocytes were isolated from the whole blood of the donors by three subsequent washings with

PBS. The obtained erythrocytes, re-suspended in PBS, were treated with serial dilutions of the investigated samples (1 h, 37 °C). After incubation, the cells were washed twice with PBS to remove extracellular antioxidants, and incubated with the intracellular dye 2′,7′-dichlorofluorescein diacetate (DCF-DA), washed again and treated with hydrogen peroxide (1 mM) for 30 min. The intracellular ROS levels were analysed by flow cytometry (FACSCalibur, BD, USA) based on the fluorescence of dichlorofluorescein (DCF), fluorescent product of DCF-DA in the reaction with intracellular H_2O_2. The results are expressed as the mean fluorescence intensity (*MFI*) of the total number of analysed erythrocytes (20000) and presented as mean ±*SD* of the data obtained in three subjects. All analyses were performed in duplicate.

Determination of platelet activation – in vitro

The platelet activation markers, P-selectin and GPIIb-IIIa, were measured by whole-blood flow cytometry according to a previously published protocol[15] with slight modifications for *in vitro* testing. In brief, after venipuncture, aliquots of dissolved (1:10 in Hepes-Tyrode Buffer, pH 7.4) anti-coagulated blood (3.2 % citrate) were incubated with serial (2×) dilutions of investigated samples (30 min, 37 °C) and subsequently incubated with CD61-PerCP (pan-platelet marker), CD62P-PE (anti-P-selectin) and PAC-1-FITC (antiGPIIb-IIIa) monoclonal antibodies with suboptimal concentration of platelet agonists (0.5 mM ADP) for 20 min in the dark, at room temperature. After the incubation with antibodies, the samples were fixed with paraformaldehyde solution (0.5 %) for 15 min and analysed. Sample analysis was performed using a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, USA). The results are presented as antigen positive platelets (%) in the platelet pool (20000 events).

Determination of platelet–endothelial cells adhesion – ex vivo

The effect of chokeberry juice consumption on platelet–endothelial adhesion was investigated *ex vivo* in a platelet–endothelial cell adhesion assay. EA.hy926, a continuously replicating cell line derived from primary human umbilical vein endothelial cells (HUVEC) was used as an endothelial cellular model.

EA.hy926 cells were cultured as a monolayer in DMEM supplemented with penicillin (192 U mL^{-1}), streptomycin (200 μg mL^{-1}) and 10 % heat-deactivated FBS. The cells were grown at 37 °C in 5 % CO_2 and humidified air atmosphere with twice-weekly subculture.

Platelet-coated surfaces were prepared as described previously[16] with modifications for *ex vivo* testing. Suspensions of platelets (0.1 mL containing 1×10^8 platelets in HEPES Tyrode's buffer), isolated from the whole blood before and after intervention, were added to plastic flat-bottomed micro-titre wells. The plates were incubated for 24 h at 4 °C. The day after, non-adherent platelets were removed by washing with PBS containing 1 % BSA. The same solution was used for the blocking of "free adherent" sites on the plastic (1 h at 37 °C). EA.hy926 were detached and re-suspended in PBS enriched with Ca^{2+} and Mg^{2+}. After staining with calcein-AM, 1×10^5 EA.hy926 cells were added to each platelet-coated well in the presence of thrombin (2 U mL^{-1}) and incubated for 1 h at 37 °C. The plates were then washed twice and the adherent cells were quantified in black 96-well plates with a fluorescence plate reader (Florosken Ascent FL, Thermo) with an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

Statistical analysis

All results are presented as mean ± standard deviation (*SD*). The data were analysed by the one sample *t*-test and $p < 0.05$ was considered statistically significant. The SPSS program, version 19 (SPSS Inc., Chicago, IL), was used for the analysis.

RESULTS AND DISCUSSION

Total phenolics content

The results of the total phenolics and anthocyanin content analysis, as well as the radical (DPPH) scavenging activity of the investigated samples are summarized in Table I, showing values expressed per mL of juices and g of dry weight (DW) of the investigated juices, as well as per ml of the investigated extract fractions and g of investigated extract (WECE) used for the purpose of comparison. The total phenolics contents of CCJ and FCJ were similar ($p = 0.79$), showing that processes included in the production of the commercial juice (pectinase treatment, filtration) did not influence the phenolics of the juice. The obtained results were in accordance with the results of Mayer-Miebach *et al.*[17] The contents of total phenolics in WCE and MCE, expressed as mg GAE per g of WECE dry weight (DW), were 4 and 2.5 times lower, respectively, than phenollics content in the juices, calculated on DW. It could be concluded that the phenolics present in 100 mL of juice could be provided with at least 35 g of the investigated extract, indicating that the extraction process should be further optimized and/or prioritizing juice as the optimal source of chokeberry phenolics in future clinical studies. Results showing the significantly higher phenolic content in MCE compared to WCE ($p < 0.001$) also indicate that compounds with the phenolic structure present in WECE were extracted more efficiently with less polar solvents (methanol *vs*. water).

TABLE I. Total phenolics content, total anthocyanins content and radical scavenging activity of the analysed samples; CCJ – commercial chokeberry juice; FCJ – fresh chokeberry juice; WCE – water soluble fraction of the water–ethanol ($\varphi_{water} = 0.40$) extract of chokeberry fruits; MCE – methanol soluble fraction of the water–ethanol ($\varphi_{water} = 0.40$) extract of chokeberry fruits; GAE – gallic acid equivalents; DW – dry weight; CGE – cyanidin-3-glucoside equivalents

Samples	CCJ	FCJ	WCE	MCE
Total phenolics, mg GAE mL^{-1}	5.86±0.27	5.93±0.33	0.50±0.01	0.86±0.01
Total phenolics, mg GAE g^{-1} DW	41.2±1.9	43.3±2.4	10.15±0.22	17.12±0.18
Total anthocyanins, mg CGE mL^{-1}	0.15±0.02	2.18±0.09	0.0210±0.004	0.21±0.01
Total anthocyanins, mg CGE g^{-1} DW	1.07±0.14	15.91±0.65	0.42±0.02	4.12±0.18
IC_{50}[a] / µL mL^{-1}	0.44±0.03	0.52±0.03	7.41±0.46	4.12±0.38
IC_{50} / mg DW mL^{-1}	0.062±0.001	0.071±0.001	0.370±0.023	0.206±0.019

[a]Concentration of samples that inhibited 50 % of DPPH radical, based on absorbance measurements at 517 nm

Total anthocyanins content

Total anthocyanins (TA) content in FCJ was significantly higher than in CCJ ($p < 0.001$), supporting previously published data on the influence of storage and processing on the content of anthocyanins. Howard *et al.*[18] reported that both processing and storage of processed chokeberry products at ambient temperature

induced significant losses of anthocyanins. Anthocyanins in juices were more susceptible to the degradation processes compared to other products, due to the removal of skin and seeds. Degradation of anthocyanins is accompanied with an increase in the products of their polymerization, designed as polymeric pigments, but the precise mechanism is still unknown.

The TA contents of WCE and MCE were 0.42±0.02 and 4.12±0.18 mg CGE g^{-1} DW, respectively. The observed significant difference between the obtained values ($p < 0.001$), indicate that anthocyanins were more efficiently extracted with methanol and the content in MCE was more than 10 times higher than in WCE and almost 4 times higher than in CCJ calculated on DW, showing that 100 mL of CCJ is equivalent to approximately 3.7 g of extract.

Antioxidant activity (DPPH assay)

Antioxidant activity of juices and extracts was evaluated as the radical scavenging activity (RSA). After 30 min of incubation with 0.04 mM DPPH solution in methanol, 50% inhibition of absorbance measured at 517 nm (IC_{50} value) was obtained with 0.44±0.03 and 0.52±0.03 μL mL^{-1} of CCJ and FCJ, respectively, showing slight but significant difference between the obtained values (p = = 0.038) and surprisingly higher antioxidant activity of CCJ. Regarding investigated extract MCE was more effective than WCE as DPPH radical scavenger, with the IC_{50} value of 0.206±0.019 and 0.370±0.023 mg of WECE used for extraction (p = 0.007). RSA did not correlate with the anthocyanin content in investigated juices and extract fractions, indicating the influence of other bioactive substances present in the samples. In strawberries, DPPH radical scavenging activity was not significantly influenced by processing and storage and did not reflect the decrease in anthocyanin content.[19] However, RSA of extract fractions, based on quantities relevant for consumption, is negligible compared to the investigated juices and consequently could not be taken as optimal intervention sample.

Antioxidant activity of plasma, measured using the ferric reducing antioxidant power (FRAP) assay within *in vivo* pilot study in six subjects significantly increased after single intervention with commercial chokeberry juice (p = 0.001), with the obtained values of 1.51±0.26 mM Fe^{2+} compared to the baseline values of 1.29±0.23 mM Fe^{2+}. The effect of acute intake of flavonoid-rich juice consumption on FRAP value of plasma was observed previously, but the authors suggested that the effect may be due to changes in uric acid concentration.[20] Data on acute intake of chokeberry juice on antioxidant capacity of plasma is lacking although Pilaczynska-Szczesniak *et al*. have shown that long term chokeberry juice consumption reduced parameters of lipid oxidation and increased the activities of antioxidative enzymes in erythrocytes.[2]

Antioxidant protection of erythrocytes

Erythrocytes were used as a simple cellular model for the evaluation of the bioactive antioxidant effects of chokeberry against moderate oxidative stress induced by hydrogen peroxide, influenced by the uptake through the biological membrane and overall bioavailability. Isolated red blood cells were pre-incubated with serial dilutions of investigated samples and exposed to H_2O_2 (1 mM). Intracellular H_2O_2 levels were determined according to the measured DCF fluorescence using flow cytometry. As shown in a representative histogram presenting the decrease in DCF fluorescence in erythrocytes pre-treated with MCE (5 mg mL^{-1}), compared to the non-treated cells, after the subsequent H_2O_2 exposure (Fig. 1), the mean DCF fluorescence (horizontal axis) of the analysed cells, corresponding with intracellular ROS levels, is shifted to the left to lower values compared to the fluorescence of non-pre-treated cells, indicating scavenging of H_2O_2. Figure 2 shows the decrease in intracellular ROS presented as the inhibition of DCF fluorescence in the DCF-DA-stained cells pre-treated with different concentrations (1.25, 2.5 and 5 mg mL^{-1}) of water soluble fraction (WCE) or methanol soluble fraction (MCE) of water–ethanol chokeberry fruit extract and subsequently exposed to H_2O_2 (1 mM), compared to the fluorescence in control cells (without pre-treatment). Based on the results obtained, both WCE and MCE showed antioxidant activity against H_2O_2-induced oxidative stress in erythrocytes, and the effect was more pronounced for MCE, with the inhibition levels (%)

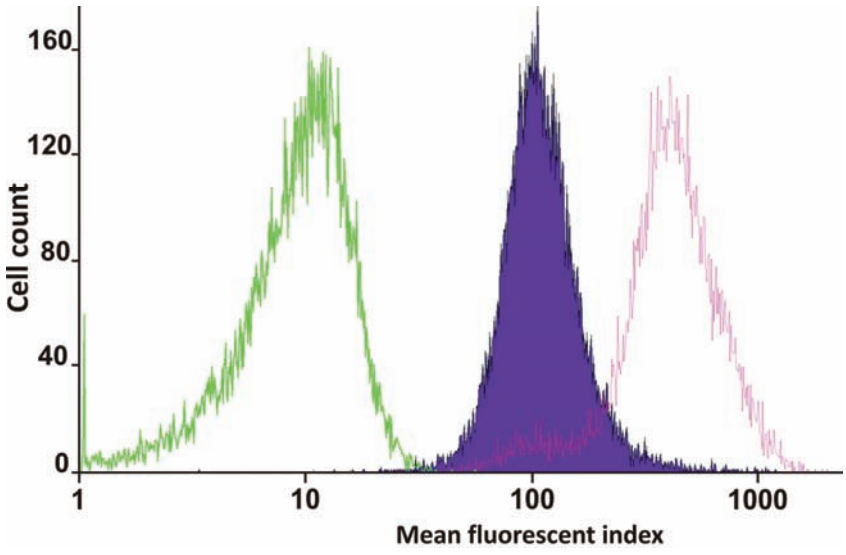

Fig. 1. Representative histogram showing DCF fluorescence decrease in erythrocytes pre-treated with the methanol soluble fraction (MCE) of chokeberry extract and subsequently exposed to H_2O_2 (full area), compared to the fluorescence of non-pre-treated cells, exposed to H_2O_2 (purple borderline) and cells without H_2O_2 exposure (green borderline).

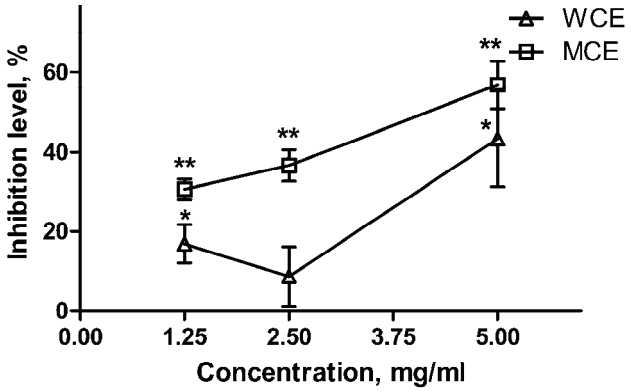

Fig. 2. Intracellular ROS decrease in erythrocytes pre-treated with different concentrations of the water soluble fraction (WCE) and methanol soluble fraction (MCE) of chokeberry extract and subsequently exposed to extracellular oxidative stress, compared to the control, non-pre-treated cells (significantly different from control cells: *p<0.05 and **p<0.01).

of measured fluorescence of 30.65±2.61 (p < 0.01), 36.67±3.98 (p < 0.01) and 56.83±5.96 (p < 0.01), for the investigated concentrations of 1.25, 2.5 and 5 mg mL^{-1}, respectively, compared to the values obtained for WCE of 16.89±4.78 (p < < 0.05), 8.69±7.45 (p = 0.18) and 43.42±12.08 (p < 0.05), for the same concentrations, respectively. The obtained results could be hypothetically explained with the higher amount of anthocyanins in the total content of phenolics of MCE. The main difference between the effects observed for MCE and WCE was the dose response, which was shown to be non-linear for WCE and linear for MCE, suggesting that the bioactive substances extracted with water or methanol could have antioxidant effects with different modes and activity. The inhibition level (%) was higher for MCE than for WCE at all the investigated concentrations, but was statistically significant only for 1.25 and 2.5 mg mL^{-1} (p < 0.05 and p < 0.01, respectively), showing that in higher concentrations, the cellular antioxidant activity could be influenced by the uptake of potential antioxidants into the living cell. The effects of investigated juices were evaluated in the concentration range of 0.25–75 μL mL^{-1}, with the maximum concentration hypothetically correlated with the concentration in plasma after the consumption of 200 mL of juice and the pre-assumption of complete availability of the bioactives present in the juices. Both juices showed antioxidant potential in concentrations corresponding to their IC_{50} values determined using DPPH assay, but the effect of FCJ was more pronounced (p = 0.049) with an inhibition level (%) of 63.7±8.4 at the IC_{50} concentration (0.44 μL mL^{-1}) compared to 47.3±5.7 obtained with CCJ at the IC_{50} concentration of 0.52 μL mL^{-1}. Interestingly, in the low concentration range (0.5–4 μL mL^{-1}), the observed antioxidant activity was inversely correlated with concentration, while at higher concentrations, a direct correlation could be observed.

Slatnar *et al*. investigated the cellular antioxidant effects of different berry juices using *Saccharomyces cerevisiae* as an *in vitro* cellular model, DCF-DA staining but without ROS exposure. They showed that the results obtained in the yeast cells were markedly different from the data obtained by the DPPH assay and concluded that the major factors found to influence *in vivo* antioxidant activity were not only the cellular availability of the polyphenols present in juices but also the ratio of specific polyphenol present in the juices and consumed by the cell, with the favourable effects of the high anthocyanin content and low content of hydroxycinnamic acids. They also found that when the uptake of polyphenols by the yeast cells was low, the antioxidant activity increased. High hydroxycinnamic acid uptake with low anthocyanin intake induced higher intracellular oxidation.[21] The cellular antioxidant activity of protocatechuic acid (PCA), a simple phenolic acid with *in vitro* antioxidant properties, was also investigated and it was found to be one of the major metabolites of cyanidin-glucosides.[8,22] The decrease in intracellular ROS, based on the inhibition of DCF fluorescence in DCF-DA stained cells pre-treated with different concentrations (10, 100 and 1000 μM) of PCA and subsequently exposed to H_2O_2 (1 mM), compared to the fluorescence in control cells (without pre-treatment) is shown in Fig. 3. Antioxidant protection of erythrocytes by PCA showed a statistically significant inverse dose response correlation (p = 0.0065), with the lowest dose (10 μM) that induced the highest reduction in intracellular ROS (52.09±7.29), suggesting that antioxidant activity of PCA is not mediated by its direct antioxidant action. The published data on the antioxidant properties of PCA within cellular systems and in animal models are controversial. Nakamura *et al*. showed dose and time dependant effects of PCA in animal models of skin tumours, with beneficial ef-

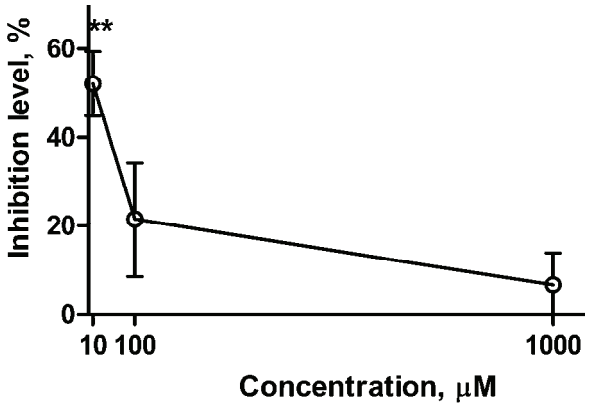

Fig. 3. Intracellular ROS decrease in erythrocytes pre-treated with different concentrations of protocatechuic acid (PCA) and subsequently exposed to extracellular oxidative stress, compared to the control, non-pre-treated, cells (significantly different from control cells: $^{**}p < 0.01$).

fects of lower doses and pro-oxidant effects of higher doses and longer exposure.[23] At doses higher than 10 mM, PCA was also found to induce oxidative stress in both transformed and malignant cells from oral tissue, but in lower non-toxic doses (2.5 mM), it sensitized cells to the pro-oxidant stimuli.[24] Opposing results showing protective effects of PCA isolated from natural source against H_2O_2 induced oxidative stress both *in vitro*, in PC12 cells, as well as *in vivo* in animal models were reported by Shi *et al.*[25] These discrepancies could be partly explained by the reported induction of cellular antioxidant enzymes by the lower concentrations of PCA.[26]

Inhibition of platelet activation – in vitro

The influence of the analysed samples on the expression of two activation markers, P-selectin and GPIIb-IIIa, on the platelet surface after *ex vivo* action of a suboptimal concentration of ADP was investigated. The effects were focused on the activities of WECE regarding its further use in clinical trials. As shown in Fig. 4, P-selectin expression in the whole blood platelets incubated with different concentrations (1.25, 2.5 and 5 mg mL^{-1}) of the water soluble fraction (WCE) and the methanol soluble fraction (MCE) of the water–ethanol chokeberry fruit extract and subsequently treated with ADP (0.5 μM) was not significantly different from the expression of this activation marker in activated non-pre-treated (control) cells. Inhibition levels (%) of P-selectin expression determined after incubation with 1.25, 2.5 and 5 mg mL^{-1} of WCE were –12.64±5.52, –4.84±13.09 and 1.51±5.52, respectively, while with MCE, the obtained values were –2.86±5.99, 1.37±3.72 and 7.50±5.73, respectively, and none of the observed changes were statistically significant. Linearity of the dose-response curves for the investigated concentrations and P-selectin expression were not observed either. Inhibition of

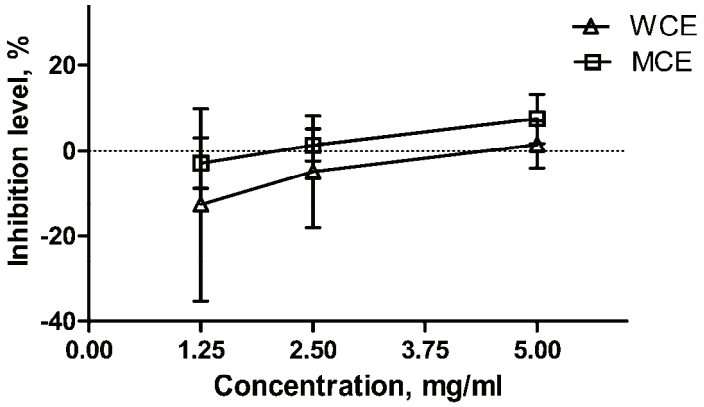

Fig. 4. Inhibition of P-selectin expression in ADP-stimulated platelets pre-treated with different concentrations of the water soluble fraction (WCE) and methanol soluble fraction (MCE) of chokeberry extract compared to control, non-pre-treated cells.

the GPIIb-IIIa activation marker was influenced by the pre-treatment under the same experimental conditions with inhibition levels (%) of –2.96±1.83, 4.25±3.95 and 18.83±8.36 for WCE, and –4.27±2.46, 4.28±1.49 and 21.18±8.79 for MCE, for concentrations of 1.25, 2.5 and 5 mg mL^{-1}, respectively. Although the statistical significance was compromised by the high inter-individual variations, dose-response linearity could be observed (Fig. 5). CCJ and FCJ did not influence the expression of either of measured antigens in analysed concentration range (0.25––75 μL mL^{-1}). An effect of PCA in investigated concentration range (10–1000 μM) was also not observed (data not shown).

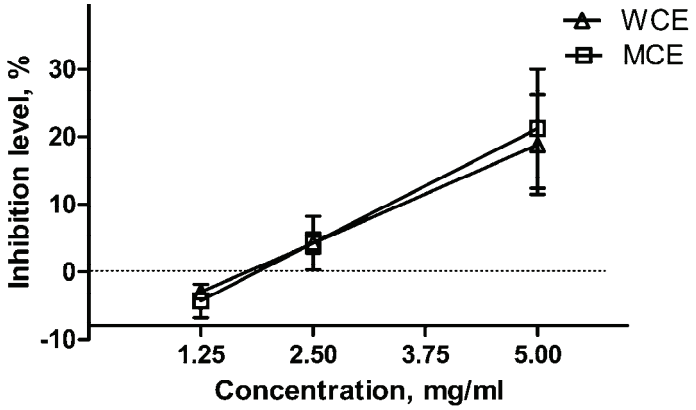

Fig. 5. Inhibition of GPIIb-IIIa expression in ADP-stimulated platelets pre-treated with different concentrations of the water soluble fraction (WCE) and methanol soluble fraction (MCE) of chokeberry extract compared to control, non-pre-treated cells.

Expressions of the platelet surface antigens, P-selectin and GPIIb–IIIa, are sensitive indicators of platelet activation.[15] P-selectin is a protein constitutively expressed in the *α*-granules of platelets and following platelet stimulation, it becomes expressed on the platelet surface. The active role of P-selectin in thrombosis, coagulation and its crucial role in the pathogenesis of atherosclerosis implies mostly its role in leukocyte recruitment (notably monocytes) as an inflammation process, mediated *via* its interaction with PSGL-1 (P-selectin glycoprotein ligand) constitutively expressed in almost all leucocytes.[27] GPIIb-IIIa is an activation-dependent receptor for fibrinogen and it mediates homotypic platelet aggregation with a crucial role in thrombosis.[28] Based on the results of previous studies, dietary bioactive compounds, including polyphenols, can modulate platelet activation in response to agonist, both *in vitro* and *ex vivo*.[29] Numerous studies reported beneficial effects of chokeberry products or extracts on platelet function *in vitro*, including reduction of nitrative and oxidative stress in platelets[30–32] and platelet aggregation,[33,34] but anti-platelet effects regarding expression of GPIIb-IIIa in response to suboptimal ADP were not investigated previ-

ously. Influence of chokeberry fruit extract on P-selectin expression after ADP action was previously investigated.[35] Regarding the anti-platelet activity of PCA, previous studies showed that PCA selectively inhibited shear stress-induced platelet activation and aggregation but did not inhibit platelet aggregation induced by ADP and other endogenous agonists (collagen, thrombin). The proposed mechanism of the observed effects of PCA is the blockage of the von Willebrand factor binding to the activated glycoprotein Ib.[36]

Inhibition of platelet–endothelial cells adhesion – ex vivo *pilot study*

The *ex vivo* effects of CCJ consumption were assessed by employment of the platelet–endothelial cells adhesion assay, according to a previously described procedure. Adhesion of platelets, isolated 2 h after the consumption of CCJ, to endothelial cells (EA.hy926) in culture in the presence of thrombin was inhibited in all subjects compared to the adhesion of the platelets isolated before the consumption. The mean value of inhibition (%) obtained in six subjects was 37.83±22.64, showing significant inhibition of platelet adhesion after chokeberry juice consumption (p = 0.009) with a marked inter-individual variation of the obtained values. The results obtained in this pilot study indicate a rationale for future investigation of the observed effects in a larger population and within a controlled study.

As platelet–endothelial adhesion is considered to be a GPIIb-IIIa dependant process,[37] the results obtained *in vivo* are consistent with the results obtained for GPIIb-IIIa inhibition *in vitro.* It is noteworthy that the results of the present pilot study investigating the effects of CCJ consumption on both P-selectin and GPIIb-IIIa expression after *ex vivo* suboptimal ADP action, evaluated by flow cytometry, were also observed (data not shown). The *in vitro* effects of chokeberry extract on platelet–endothelial cells adhesion was previously investigated by Luzak *et al*., who reported that incubation with a low concentration (5 μg mL^{-1}) of chokeberry extract increased the efficacy of human umbilical endothelial cells in culture to inhibit ADP activated platelet adhesion *in vitro*.[35] In the performed study, the effects of chokeberry consumption targeted on the function of platelets were investigated. The assay itself has numerous advantages in the screening of different agents for *in vitro* anti-platelet effects targeted at the inhibition of GPIIb-IIIa or endothelial cell-mediated inhibition of platelet aggregation or effect of the consumption food and food bioactives on *ex vivo* agonist-induced platelet activation and the consequential platelet adherence to endothelial cells in culture.

The only reliable option to confirm the beneficial effects of anthocyanin-rich food in health promotion and the prevention of chronic diseases and to prove the hypothesis that the effects mostly rely on anthocyanins is to conduct a human intervention study designed as a randomized placebo-controlled trial. Numerous constrains on intervention trials with anthocyanins within food matrix include the

design of the placebo, the rapid degradation of the bioactive substances, the relevancy of their antioxidant activity in pre-screening for relevant effects of cardiovascular diseases (CVD) and surrogate outcomes.

Biological relevance of direct antioxidant effects of polyphenols in the prevention of chronic diseases, including cardiovascular disease, has recently been re-evaluated. More often experts in the field accept the opinion that the direct antioxidant effect could not explain the numerous health effects observed in both intervention and epidemiological studies.[38] This opinion is based on their poor bioavailability, low concentrations in blood compared with other antioxidants and the decline in their antioxidant activity following ingestion. Based on the results obtained in this study, the *in vitro* antioxidant potential is not reliable for the prediction of numerous effects relevant in CVD prevention.

The degradation of anthocyanins within berry products (especially juices) could be an issue in long term trials and additional strategies for mitigating anthocyanins losses during the intervention period are required. The extract of chokeberries evaluated in this study did not provide satisfactory results compared to the juice and cellular *in vitro* effects were obtained in high non-relevant doses regarding formulation in dosage forms or juice supplementation, suggesting that further optimization in the extraction process is needed. Two cell-based assays, investigating the antioxidant potential and anti-platelet effects were also not correlated for the evaluated fractions of extracts, showing that antioxidant activity is not always a prerequisite for other cellular effects as previously suggested.[38] The *in vitro* anti-platelet effects, although evaluated at high concentrations of extract, are supported by the pilot *ex vivo* trial, but the final conclusions could be made only in an intervention trial. Subjected to intense metabolic transformation, the bioactives could also provide metabolites with opposing effects, compared to the native forms. The effects of long-term consumption of chokeberry extract on platelet aggregation induced by ADP *ex vivo* were recently reported in patients with metabolic syndrome,[5] but the effects on specific activation markers and both homotypic and heterotypic platelet aggregation, with optimal and suboptimal agonist action should be further investigated.

In conclusion, a multifaceted approach in preclinical investigation is the optimal strategy in the screening for potential candidates for health promotion and prevention of chronic diseases, but it still could not guarantee the effects *in vivo*.

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ИЗВОД

ИСПИТИВАЊЕ БИОЛОШКЕ АКТИВНОСТИ АНТИОКСИДАНАСА АРОНИЈЕ (*Aronia melanocarpa*) У ДИЗАЈНИРАЊУ ИНТЕРВЕНТНИХ СТУДИЈА

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Повољни ефекти плодова и сокова ароније у промоцији здравља и превенцији болести показани су у многим епидемиолошким и интервентним студијама и веома често се повезују са антиоксидативним деловањем њихових састојака. Циљ ове студије је био да се испита антиоксидативно деловање на еритроците и анти-тромбоцитно деловање три различита производа добијена од плода ароније: комерцијалног сока, свеже цеђеног сока и лиофилизованог водено–етанолног екстракта плода ароније, у *in vitro* експерименталним условима. Добијени резултати поређени су у односу на садржај укупних полифенола и антоцијана, као и на антиоксидативну активност одређену DPPH тестом. Резултати су указали на значајне разлике у хемијском саставу и антиоксидативној активности испитиваних производа, али директна веза са ефектима на ћелијама, укључујући и антиоксидативно деловање на еритроците и анти-тромбоцитно деловање *in vitro*, није показана. Резултати добијени *ex vivo* у оквиру пилот студије, са једним од испитиваних узорака, једним делом су потврдили резултате добијене *in vitro*. На основу резултата добијених испитивањем антиоксиданаса ароније показано је да хемијска анализа и *in vitro* експерименти на ћелијским моделима имају велики значај у процени њихове биолошке вредности. Екстраполација добијених резултата у ситуацију *in vitro* је, међутим, често отежана и под утицајем биорасположивости и метаболизма дијетарних биоактивних супстанци.

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Review

REVIEW

The modifications of bovine *β*-lactoglobulin – effects on its structural and functional properties

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Abstract: Due to its excellent techno-functional properties, high nutritional value and low cost, *β*-lactoglobulin (BLG), the main protein in whey, is a frequently used additive in wide range of food products. It is also considered as an acid-resistant drug carrier for the delivery of pharmaceutical and nutraceutical agents. However, BLG is the main allergen of milk. A variety of methods has been explored for the modification of BLG in attempts to improve its functional properties and to decrease its allergenicity. Due to its compact globular structure, BLG is relatively resistant to modifications, especially under mild conditions. BLG can be modified by physical, chemical and enzymatic treatments. Although chemical modifications offer efficient routes to the alteration of the structural and functional properties of proteins, they are associated with safety concerns. In the last decade, there is a tendency for application of novel non-thermal physical processing methods, as well as enzymes in order to obtain BLG derivatives with desirable properties. The objective of this review is to overview the chemical, physical and enzymatic processing techniques utilized to modify BLG and their effects on the structural and functional properties of BLG.

Keywords: *β*-lactoglobulin; modification; chemical; physical; enzymatic; functional.

CONTENTS

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1. INTRODUCTION

β-Lactoglobulin (BLG) is the major whey protein found in the milk of most mammalian species, representing 60 % of the total whey proteins. BLG (Fig. 1) is a globular protein consisting of 162 amino acid residues with a molecular weight (MW) of 18.3 kDa. BLG has two intramolecular disulfide bonds and one free thiol group, which plays an essential role in the antioxidant activities of BLG.[2] At different pH values, BLG preserves more or less the same secondary structure but it can adopt various tertiary structures.[3] BLG undergoes time- and temperature-dependent denaturation above 65 °C, which is accompanied by extensive conformational transitions that expose highly reactive thiol and amino groups. Bovine BLG belongs to the lipocalin protein family. It binds a wide range of small hydrophobic ligands, acting as a transporter. The binding of retinol and fatty acids to BLG have been widely implicated in its proposed physiological functions.[4] BLG and its peptide fragments have various bioactivities. It was reported that the various peptides derived from the proteolytic digestion of BLG have inhibitory activity against the angiotensin-converting enzyme.[5] Antimicrobial, immunomodulating, opioid and hypocholesterolemic activities have also been documented.[5]

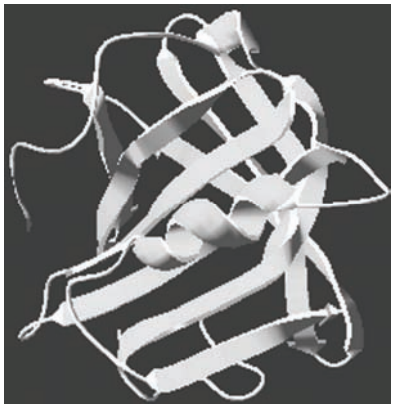

Fig. 1. Schematic representation of the structure of bovine *β*-lactoglobulin drawn using Swiss Pdb Viewer (http://www.expasy.org/spdbv/).[1]

BLG has desirable techno-functional properties determined by its physicochemical properties, such as viscosity, gelation, foaming, solubility and emulsification. This protein has a high nutritional value and may serve as a carrier for lipophilic nutrients and drugs. It is frequently used as an additive in a wide range of food products. However, BLG is the main allergen of milk and presents a significant health risk for patients allergic to milk. Due to its compact globular structure, BLG, as many other food allergens, is resistant to digestion.[6]

In the last decade, the requirements of food technologists, nutritional scientists and consumers for techno-functional, tropho-functional and hypoallergenic attributes of food products have been continuously rising. Different methods for

BLG modifications were studied in attempt to broaden its application in food and pharmaceutical industry by improving its techno-functional properties, as well as to increase its digestibility and reduce its allergenic potential. This review summarizes the effects of chemical, physical and enzymatic modifications of BLG on its structural and functional characteristics.

2. CHEMICAL MODIFICATIONS

Chemical modifications of proteins are not welcome in the food industry despite the application of non-toxic reagents, because of the complex procedures required to remove unreacted chemicals and the products of side reactions. However, chemical modifications offer efficient ways of imparting desirable properties to proteins.

Acylation and alkylation. Acylation by anhydrides of monocarboxylic acids replaces ammonium cations with neutral acylamino groups, resulting in electrostatically neutral groups. Acylation by anhydrides of di- or tricarboxylic acids leads to a conversion of cationic into anionic residues, which alters the net charge of a protein. A large degree of acylation can affect the secondary and tertiary structure of the protein.

It was demonstrated that acetylation and succinylation of BLG influenced not only its tertiary structure, but also its secondary structure. These modifications resulted in decreased hydrophobicity and increased content of beta sheet structures and random coils. Both acetylated and succinylated BLG were less stable against guanidine hydrochloride and urea denaturation.[7] The surface pressure of low-extent succinylated BLG was higher than that of native BLG, but higher levels of succinylation reduced the surface pressure.[8] Foam formation and stability of BLG were reduced following its succinylation.[8] Succinylated BLG was found to be a suitable natural excipient for the formation of delayed release tablets or tablets containing probiotic bacteria. Furthermore, succinylation of BLG improved its survival under gastric conditions.[9] In addition, succinylation of BLG decreased its solubility and its charge density at acidic pH values, resulting in decreased tablet erosion (protein loss) at pH 1.2. Solubility of succinylated BLG compared to unmodified protein increased at pH 7.5, making it suitable for the protection and intestinal release of gastro-sensitive compounds. Many studies have shown that acylated milk proteins with increased negative charge are potent antiviral compounds, having a therapeutic potential for local administration. Modification of BLG by 3-hydroxyphthalic anhydride (3HP) yielded compounds that exerted antiviral activity *in vitro* against herpes simplex virus type 2, influenza virus, human immunodeficiency virus 1 (HIV-1) and simian immunodeficiency virus (SIV).[10]

BLG modified with vanillin and benzaldehyde exhibited very low solubility near the isoelectric point and possessed improved emulsifying activity in the aci-

dic pH range, but lower emulsion stability in the alkaline pH range.[11] Alkylation of BLG enhanced its binding affinity for retinoids.[12] BLG alkylated by allyl isothiocyanate (AITC) gave BLG–AITC conjugates, which at pH 7.1 possessed loose and partially unfolded structures, whereas at pH 4.0, they exhibited features typical for a molten globule. BLG–AITC conjugates showed better emulsifying and foaming properties than those of unmodified BLG.[13] The immunogenicity of chitosan-conjugated BLG was reduced compared to native BLG. The linear epitope profiles of these conjugates were similar to those of BLG, but the antibody response to each epitope was dramatically reduced due to the masking of B cell epitopes.[14] The anti-BLG antibody response in mice was markedly decreased following immunization of the animals with BLG–carboxymethyl dextran (CMD) conjugates. Although linear epitope profiles of BLG–CMD conjugates were similar to those of BLG, the antibody response for each epitope was dramatically reduced as a consequence of the effective shielding of epitopes by CMD. BLG conjugated with poly(ethylene glycol) (PEG) had decreased antigenicity, while the allergenicity of the conjugate was weaker than that of the native protein.[15] Oxidative sulfitolysis combined with covalent binding of PEG to BLG resulted in a conjugate with improved emulsifying activity and emulsion stability at acidic as well as at neutral pH values.[16]

It is known that covalent linking of lipids to proteins results in increased hydrophobicity of proteins, which then display an improved capacity to form and stabilize emulsions and foams. Stearic acid-modified BLG, thus, possesses an increased hydrophobicity, while the solubility of the conjugate depends on its content of stearic acid. The emulsifying and foaming properties of these conjugates were improved at low and medium levels of stearic acid incorporation.[17] BLG with a low content of stearic acid demonstrated increased binding of IgE and IgG when compared to the native protein, which was due to the increased exposure of antigenic sites. However, the ability of BLG with medium content of stearic acid to bind antibodies was reduced. Finally, a high level of fatty acid attachment to BLG resulted in its denaturation and inability to bind to antibodies, due to the reduction or destruction of the available allergenic sites. A decrease in the digestibility of the modified BLG accompanied by an increased lipophilization was also observed.[18]

Esterification. Proteins modified by esterification gain a more positive charge as the number of ionizable carboxyl groups becomes reduced. Moderate esterification of BLG induces not only slight changes in its secondary structure, but also its tertiary structure acquires properties that are characteristic of the molten globule state. This leads to opening of the BLG molecule to the cleavage of the peptide bond. Esterified BLG is hydrolyzed rapidly by pepsin, which is due to 22 new sites of pepsin cleavage introduced by esterification.[19] Fourteen cleavage sites are pepsin-specific and their unveiling is due to the imposed ter-

tiary structure changes. Eight of the new cleavage sites are esterified carboxylates recognized by pepsin.[18] The methyl-, ethyl-, and butyl-esters of BLG showed enhanced surface activity, as well as hydrophobic probe binding activity, the most pronounced effect being that of methyl esters.[20]

The positive charges on the protein molecules enable them to interact with viral proteins or viral DNA, affecting viral replication, transcription or translation and, consequently, viral infectivity. Highly esterified BLG at pH 7 showed DNA-binding capacities comparable to those exhibited by native basic proteins, such as lysozymes and histones. Esterification of BLG enhanced its antiviral activity against the avian influenza A virus (H5N1), the influenza virus A subtype H1N1 and the HSV-1 virus.[21] Peptic hydrolyzates of esterified BLG also displayed antiviral activity.[22]

Glycation by Maillard reaction. Glycation by the Maillard reaction is a ubiquitous reaction of condensation of a reducing sugar with the amino groups of proteins. The products of Maillard reaction could improve the functional and/or biological properties of a protein, which could be utilized in the food industry. The glycosylation of BLG in Maillard reaction under mild conditions does not alter the protein structure, except a slight increase in its Stokes radius and an increase in the temperature of denaturation. Since the Stokes radius of denatured BLG is not significantly smaller than that of glycosylated BLG, it was proposed that its non-polar residues associate with the sugar moieties in the unfolded state, thereby preventing their solvent exposure and affecting the aggregation propensity and the type of aggregate formed.[23] Glycation of BLG in the Maillard reaction by several alimentary sugars induced oligomerization of BLG monomers.[23] Among the sugars used, pentoses (arabinose and ribose) induced the highest degree of modification, while hexoses (glucose, galactose and rhamnose) were less reactive and lactose generated the lowest degree of modification.[24] Proteins substituted with pentoses formed polymers stabilized by sugar-induced covalent bonds. When other sugars were used, a proportion of the aggregated proteins were stabilized only by hydrophobic interactions and disulfide bonds. In the presence of less reactive sugars, heating of BLG induced only minor structural modifications, in contrast to more reactive sugars such as pentoses. Dry-state glycation of BLG did not significantly alter the native-like BLG behavior, while treatment in solution led to important structural changes. Denatured BLG monomers associated covalently *via* their free thiol group and in a subsequent step, non-covalent polymerization of the unfolded homodimers and swollen monomers occurred through hydrophobic interactions.[25]

Moderate glycation of BLG in the early stages of the Maillard reaction had only a small effect on its recognition by IgE, whereas a high degree of glycation had a clear “masking” effect on the recognition of epitopes.[26] The antigenicity of BLG glycated by glucose and oligoisomaltose was reduced.[27] High levels of

BLG glycation in the Maillard reaction impaired BLG proteolysis by simulated gastrointestinal digestion and, consequently, increased the IgG- and IgE-reactivities of the hydrolysates, regardless of the employed carbohydrate. However, protein aggregation during the advanced stages of the Maillard reaction had a masking effect on the BLG epitopes, counteracting the negative effect of the lower digestibility of the glycated protein on its allergenicity.[28] After mice immunization with BLG modified by acidic oligosaccharides, the anti-BLG antibody response was markedly diminished in the vicinity of the carbohydrate-binding sites,[29] which was due to reduced T cell response because of reduced susceptibility of the conjugates to processing enzymes for antigen presentation. High levels of BLG glycation with galactose, tagatose, and dextran impaired its proteolysis and, consequently, increased the IgG- and IgE- reactivities of the hydrolysates, regardless of the used carbohydrate. The different increases in susceptibility of glycated BLG to pepsinolysis were related to the alteration of the conformation of the protein when glycation was performed with highly reactive sugars.[30]

Glycation of BLG with highly reactive pentose sugars, such as arabinose or ribose, improved BLG emulsifying properties, while its foaming properties were improved when glycation occurred with hexose sugars, such as glucose or galactose.[24] Glycation of BLG with different sugars induced an increased ability for scavenging radicals, with ribose and arabinose having the most pronounced effect.[31] Maillard reaction products, formed in the reaction of hydrolyzed BLG with glucose, exhibited increased radical scavenging activity, which reduced its iron chelating activity.[32] BLG modified by glucose-6-phosphate showed a greater thermal stability and a greater emulsifying activity than the native protein.[33]

SH-group modifications. BLG has two intramolecular disulfide bonds that may be responsible for its allergenicity. After reduction of one or both of its disulfide bonds by thioredoxin, BLG became strikingly sensitive to pepsin and lost its allergenicity.[34] Modification of the buried thiol group of BLG by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) destabilizes the rigid hydrophobic core and the dimer interface, thereby producing a monomeric state that is native-like at pH 2.0 but is molten globule-like at pH 7.5. Upon reducing the mixed disulfide of DTNB–BLG with dithiothreitol, intact BLG was regenerated.[35] In order to cleave the disulfide bonds, BLG was modified by sulfitolysis, but it was hydrolyzed by pepsin and trypsin more readily than the intact protein.[36]

Phosphorylation. Phosphorylation of BLG with $POCl_3$ in the presence of either triethylamine or hexylamine resulted in a slightly cross-linked BLG with increased negative charge. The phosphorylation resulted in disordering of the secondary structure of BLG and shifting BLG solubility minima toward lower values.[37] The addition of butylamine produced phosphobutylated BLG with excellent foaming and emulsifying properties, while the use of longer aliphatic pri-

mary amines (C8–C12) gave rise to reduced solubility, poor foaming and weak emulsifying properties.[38]

Non-covalent chemical modifications. Ligand binding to BLG may have a stabilizing effect on the protein. Susceptibility of BLG/retinol or BLG/retinoic acid complexes to trypsin was significantly lower than that of unconjugated BLG, due to the more compact structure.[39] When bound to gallic acid, BLG displays a lower degree of hydrolysis than native BLG, but gallic acid induces oxidation of the four methionine residues in BLG to methionine sulfoxides.[40] On the other hand, binding to BLG can protect some ligands from oxidation; this suggests that BLG could be employed as an effective carrier of oxidation-sensitive hydrophobic drugs and nutriceuticals. Binding of folic acid to BLG improves its photostability.[41] Binding of resveratrol to BLG, although partially disrupting its tertiary structure, provides a slight increase in the photostability of resveratrol and a significant increase in its hydrosolubility.[42] Epigallocatechin-3--gallate (EGCG) in thermally-induced BLG–EGCG conjugates are protected from oxidative degradation.[43] BLG/retinoid complexes exhibited an increased thermal stability and a higher resistance to UV light-induced oxidation.[44] It was reported that BLG degrades more rapidly in the presence of catechin-enriched polyphenols from green tea. However, laccase polymerized green tea catechins (GTC) adversely affected protein digestion of BLG and the protecting effect of polyphenols correlated well with the ability of proteins to form insoluble complexes with oxidized catechins.[45] In a study of non-covalent interactions between BLG and polyphenols from extracts of green and black teas, coffee and cocoa, it was demonstrated that stronger non-covalent interactions delayed pepsin and pancreatin digestion of BLG and induced the β-sheet to α-helix transition at neutral pH values. The positive correlation between the strength of protein–polyphenol interactions and the half time of protein decay under gastric conditions was found, as well as a masking of the total antioxidant capacity of protein–polyphenol complexes.[46] BLG saturated with palmitic acid possessed a higher gelling temperature and gelation time, implying that differences in the amount of bound fatty acids could be an important source of variability in the gelation behavior of BLG.[47] Phosphatidylcholine (PC) binding increased BLG thermostability, accelerated heat-induced gelation, and reinforced the mechanical strength of gels.[48] PC protected BLG from degradation under duodenal conditions and altered the pattern of the digestion products. PC binds to a secondary fatty acid binding site in BLG, thus blocking the action of proteases, rather than occupying the central calyx.[49] Interaction of flavor compounds with BLG have an influence on the release of flavor from food. BLG possesses at least two binding sites for aroma compounds. The longer the hydrophobic chain of the aroma compound, the greater is the affinity of BLG for the compound. This results in a decreased release rate and a lower final headspace aroma concentration.[50]

3. MODIFICATION BY PHYSICAL METHODS

During a physical treatment, an irreversible change occurs only above a given threshold of treatment intensity. The formation of covalent and non-covalent protein aggregates contributes to the irreversibility of the process. Below the threshold, a number of reversible modifications occur, but after the perturbation is over, the protein returns to a native-like form. Since comprehensive reviews that deal with the effects of thermal treatment on the structure and function of BLG already exist,[51] in this review, focus is directed on the modifications of BLG caused by non-thermal physical methods.

Gamma irradiation. γ-Irradiation of BLG in solution promotes the formation of linear oligomers, which consist of dimers of BLG (tetramers, hexamers, *etc*.), the oligomerization being partially caused by intermolecular cross-linking between tyrosyl radicals.[52] The changes in the secondary and tertiary structure of BLG induced by γ-irradiation are similar to the alterations observed in BLG that had been treated thermally under mild conditions.[53] Both changes result in the reduced solubility of the protein and increased agglomeration. Application of γ-radiation up to a dose level of 10 kGy did not affect the molecular-weight distribution of BLG, but reduced its solubility and increased its antigenicity.[54]

Pulsed electric field (PEF) and electrolysis. Application of high-intensity PEF to BLG resulted in the partial denaturation of the protein and its aggregation, including covalent cross-linking. PEF treatment increased the thermal stability of BLG by 4 to 5 °C, as well as its gelation rate.[55] After the treatment by electrolysis, BLG on the cathode showed markedly mitigated allergenic properties, attributed to the dislocation of the allergenic peptides from the protein surface.[56]

Ultrasound. High intensity ultrasound under uncontrolled temperature conditions induced changes in the secondary structure of BLG, which was accompanied by the formation of dimers, trimers and oligomers, and by an increased susceptibility to pepsin digestion. Although sonication under controlled temperature conditions induced changes in the secondary structure of BLG, it did not induce protein oligomerization or a change in its capacity to bind retinol.[57] The sonication-induced BLG forms had larger hydrophobic surfaces than native BLG and, thus, more easily underwent cross-linking with phenol oxidase. Sonication had only a minor effect on the ability of BLG to bind to IgE, both *in vitro* and *in vivo*.[57] The reactive thiol content and surface hydrophobicity of BLG increased continuously during sonication, suggesting an unfolding of the dimer structure, as an initiation step, with exposure of the thiol groups and the hydrophobic regions. Minor secondary and tertiary structural changes were also observed.[58] A noticeable synergism between ultrasound and heat on BLG denaturation was observed.[59] It was shown that even under conditions that were not very favorable for the Maillard reaction, such as neutral pH and reaction in solution, BLG could be glycated with the aid of high intensity ultrasound. The ultrasound-promoted

BLG glycation did not have a drastic impact on its secondary or tertiary structure. Forms of BLG obtained in the Maillard reaction forced by ultrasound exhibited radical scavenging ability and possessed a greater ferrous ion-chelating activity and better reducing power than the native protein.[60]

UV irradiation. UV irradiation of BLG induces changes in its molecular size distribution and a disruption of the ordered structure of BLG. This led to changes in the BLG antigenicity and to altered activity of BLG in the regulation of immunoglobulin production.[61] After 24 h of UV-irradiation, 18 % of the protein had been denatured with some protein aggregation. Changes in the secondary structure of BLG were similar to those that occurred in the early phase of heat-induced denaturation. The number of exposed sulfhydryl groups increased, but the number of total sulfhydryl groups decreased together with some photo-oxidation.[62]

High hydrostatic pressure (HHP). Stapelfeldt *et al.*[63] proposed a three step pressure denaturation model for BLG in neutral solution at ambient temperature: an initial pressure-melted state (up to 50 MPa) with the partial collapse of the inner calyx and solvent exposure of the free thiol group, followed by a reversible denaturation with exposure of the hydrophobic regions (half-denaturation at 123 MPa) and irreversible denaturation with the thiol–disulfide exchange becoming increasingly important at higher pressures. In the initial stage of HHP-induced aggregation of BLG, only dimers and trimers arose due to SH/S–S interaction.[64] The high-pressure denaturation of BLG led to increased reactivity of the thiol group that was buried inside the native globule. This was a result of the exposure of the thiol group to the protein surface. The release of monomers seems to represent one of the earliest events, while association of the transiently modified monomers stabilized the denatured form of the protein.[65] Up to 200 MPa, a large number of monomeric BLG molecules was formed, which carried exposed thiol groups and which had an increased surface hydrophobicity. At pressures larger than 200 MPa, the hydrophobicity of the protein surface continued to increase, while the exposure of thiol groups decreased, due to the formation of covalently linked oligomers. Pressure-induced BLG oligomerization and formation of disulfide bonds resulted from SH/S–S interchange reactions rather than from oxidation of the thiol groups. At a pressure between 100 and 300 MPa, there was a significant increase in relative hydration of BLG due to structural changes of the protein. The number of water molecules associated with BLG molecule was also increased. Only a small decrease in the hydration of BLG was observed at higher pressures, due to the irreversible denaturation and aggregation.[66] Mazri *et al.*[67] found the reaction order for HHP-induced BLG denaturation to be $n = 1.5$, which is the same as that found for thermal denaturation of BLG. This unusual value has been attributed not only to a complex reaction mechanism, which involves not only many consecutive and/or concurrent steps, including the dissociation of

BLG dimer into monomers, but also to unfolding of monomers followed by the aggregation. Kolakowski *et al.*[68] demonstrated that the pressure-induced unfolding of BLG was significantly lower at 4 °C than at 25 °C and was without aggregation. They suggested the existence of different BLG conformations under different *p*/*T* conditions: the native conformation at 0.1 MPa/20 °C, a pressure-denatured state at 200 MPa/20 °C and a cold-denatured state at 200 MPa/–15 °C.

Although BLG exhibits a considerably enhanced susceptibility to proteolysis under HHP, the addition at ambient pressure of proteases to pressure-treated BLG gave only a modest increase in proteolysis. The products of chymotrypsin hydrolysis obtained under pressure were different from those obtained at atmospheric pressure without immunochemical reactivity, which indicated that chymotrypsin effectively hydrolyzed the hydrophobic regions of BLG that were transiently exposed during the pressure treatment.[69] The rate of pepsin hydrolysis of BLG (negligible at 0.1 MPa) increased considerably when a pressure up to 300 MPa was applied. However, this was not accompanied by qualitative changes in the profiles of the peptides obtained after hydrolysis. HHP treatment at 600 and 800 MPa resulted in a rapid digestion of BLG by pepsin.[70] HHP treatment increased the binding of BLG to rabbit anti-BLG IgG antibody, but it did not affect the binding of BLG to IgE from patients allergic to BLG. There was no apparent relationship between these responses and the degree of protein aggregation.[71]

The viscosity of BLG solutions increased with pressure due to the unfolding of BLG monomers and their aggregation. BLG modified by high-pressure displayed a reduced emulsifying capacity and a reduced foamability when compared to native BLG. This was attributed to an increase in both BLG hydrophobicity and its potential for aggregation.[72] Furthermore, a pressure-treated BLG showed a greater capacity for protein–protein interactions in the adsorbed layers of interfaces. The binding of retinol to BLG was enhanced by an increase in pressure up to 150 MPa, but at higher pressures, it decreased and disappeared altogether at 300 MPa. Once dissociated, the BLG–retinol complex did not re-associate after decompression at neutral pH.[73]

4. ENZYMATIC MODIFICATIONS

The employment of enzymes offers many advantages, including the ability to perform modifications under physiological conditions with great specificity and stereoselectivity, but without undesirable side reactions. Enzymatically modified proteins are well accepted by consumers, as enzymes are natural products. However, due to their labile activity and difficulties in their purification, enzymes are still underexploited as protein modifiers. Exposure to different pH conditions, heat treatments, and adsorption to interfaces are all potential ways to in-

duce conformational changes, which could then increase accessibility of BLG to enzymatic catalysis.

Cross-linking enzymes. A protein that undergoes cross-linking by modifying enzymes should have a flexible structure. As a globular protein with a compact structure, BLG is poorly accessible to cross-linking enzymes. Therefore, for an efficient cross-linking, it is necessary to partly unfold the BLG molecules, either prior to or during the cross-linking. The addition of mediators can help, for example, the addition of small phenolic compounds during the cross-linking of BLG by phenol oxidases.

Transglutaminase (TG). Proteins can be cross-linked using transglutaminase, which catalyzes an acyl group transfer between the γ-carboxamide group of glutaminyl residues in proteins and the ε-amino group of lysyl residues, forming an isopeptide bond. Moderate TG treatment of BLG in the presence of dithiothreitol (DTT) led to a decrease in the thermal stability of BLG. In contrast, treatment of BLG in an excess of TG resulted in a remarkable increase in the thermostability of the protein, regardless of the presence or absence of DTT. The modulation of the thermal stability of BLG by TG treatment may be attributed to partial unfolding of the protein molecule and subsequent re-arrangement of its conformation.[74] BLG modified by TG in the presence of cysteine possesses increased foamability and index of surface hydrophobicity, although less than heat-treated BLG.[75] Subjecting BLG to slightly alkaline (pH 7.5) and alkaline (pH 9.0) conditions results in a limited cross-linking by TG, which is due to a minor population shift towards a molten-globule state. However, upon heat treatment followed by cooling, the cross-linking of BLG was associated with a molten-globule-like conformation that showed a small change in the secondary structure and a significantly disturbed tertiary structure.[76]

Tyrosinase. In tyrosinase-induced cross-linking of a protein, the oxidation proceeds as a two-step reaction. Monophenols are first oxidized to diphenols, after which, quinones are formed. Thalmann *et al*.[77] reported that BLG can be cross-linked by tyrosinase from *Agaricus bisporus* only in the presence of a low molecular weight phenolic compound, which probably acted as a bridging agent between the protein subunits. It appears that the cross-linking of BLG by *Trichoderma reesei* tyrosinase (TrTyr) may occur even without the presence of phenolic mediators at pH 9, but not at pH 7.5. The increased accessibility of BLG to the tyrosinase at pH 9 was associated with small changes in the protein secondary structure and a loosening of the hydrophobic core, rather than with a major unfolding of BLG, which occurs in the higher pH range (between 10 and 12).[78] At neutral pH, BLG was susceptible to TrTyr only after heat treatment, as the BLG adopted a molten-globule-like conformation that enabled accessibility to TrTyr. Heat-treated BLG molecules adsorbed on an air/water interface and then enzymatically treated favored intra-molecular over inter-molecular cross-linking within

the packed BLG layer, which makes the adsorbed molecules even more rigid and less free to reorganize.[75]

Laccase. The application of laccase in the cross-linking of proteins is affected by the accessibility of their phenolic moieties, as proteins generally contain a small number of phenolic groups. In the presence of ferulic acid, laccase was able to form irreducible intermolecular cross-links in BLG, as well as to induce oxidative modifications. The latter included: dityrosine formation, formation of fluorescent tryptophan oxidation products and formation of carbonyl derivatives of histidine, tryptophan and methionine, which resulted in the protein molecules exhibiting a higher surface tension.[79] The allergenicity of BLG cross-linked by laccase in the presence of phenolics originating from sour cherry decreased, whereas the digestibility of the remaining BLG monomers increased under conditions that simulated the human gastrointestinal tract. The tryptic hydrolysates of cross-linked BLG showed a 57 % increase in radical-scavenging activity when compared to the control BLG.[44] The cross-linking of BLG in the presence of phenolics from apple (APE) rendered cross-linked BLG. Laccase treatment of BLG caused a bi-phasal pepsin–pancreatin digestibility of the monomeric and cross-linked protein to decrease, thus decreasing its nutritional value.[80] Cross-linking of BLG by laccase in the presence of polyphenols from green tea extended the half-life of BLG that was subjected to *in vitro* digestion by pepsin.[81]

Proteolytic enzymes. Whey protein hydrolysates can be practically used in the production of infant formulas for atopy. It is known that only mixtures of amino acids are completely non-allergenic products and that other hypoallergenic products available on the market still contain peptides, which may trigger allergic mechanisms. Hydrolysis could partly reduce the immunogenicity of native BLG, as it destroys the three-dimensional structure of BLG by removing some of its epitopes.[82] Proteolysis by trypsin resulted in extensive degradation of BLG, which then had a remarkable reduction in IgE binding capacity.[83] In addition, peptides obtained by tryptic hydrolysis of BLG induced a specific oral tolerance in mice.[84] The application of high pressure enhanced this proteolysis, which resulted in a decrease of BLG antigenicity and also a decrease in its ability to bind IgE from serum.[85] Furthermore, the application of a mixture of different proteases possessing different specificities proved to be more effective in enhancing the hydrolysis of BLG and also in reducing its residual antigenicity.[86] A contaminating proteolytic activity in the enzyme preparations used for cross-linking removed a peptide from the *N*-terminus of BLG, making it more susceptible to pepsin digestion and reducing its allergenic potential.[87]

Fragments of the β-barrel domain of BLG, obtained by proteolysis, have a lower surface hydrophobicity and slightly higher surface and interfacial tension. As a consequence, these proteolytically formed oligopeptides possess higher emulsifying activity index, higher emulsion stability and better foaming proper-

ties.[88] A limited tryptic proteolysate of BLG formed much stronger gels with a lower gel point and higher rate of gelation, which were due to a more efficient association of the fragments that had unfolded domains and also to the existence of structured domains with lower thermal stability.[89] An improvement of the functionality of BLG hydrolysates, compared to the intact protein, was caused by the presence of peptides, as higher concentrations of molecular species were present in the hydrolysates. However, BLG treated by alcalase first forms transparent gels, before the non-covalently linked protein aggregates appear.[90] BLG hydrolysate possesses a higher anti-oxidative capacity and iron-binding capacity than native BLG, mostly due to Met and Tyr residues.[91]

5. CONCLUSIONS

Research devoted to modifications of BLG is attracting more and more scientists who work in the food industry. This is mainly because BLG is the most abundant protein in whey, which is a by-product of cheese production and, thus, a cheap source of this highly valuable nutrient. In addition, the excellent inherent functional properties of BLG enable a wide range of applications of this protein in the food and pharmaceutical industries. However, there is an increasing prevalence of food allergies worldwide, which includes an allergy to milk. Having a compact globular structure, BLG is relatively resistant to modifications, especially under mild conditions. On the other hand, its resistance to modifications helps it retain its desirable functional characteristics after a controlled modification.

Alterations in the digestibility of BLG induced by modifications can have several consequences. On the one hand, the increased digestibility may reduce its allergenic potential. The alteration in the peptide profile, which is obtained after BLG digestion in gastrointestinal tract, could also lead to a loss of one set of health-promoting biopeptides and gaining another. On the other hand, some of BLG modifications reduce its digestibility and are, therefore, suitable for the application in weight-loss food products. Many methods for modifications of BLG target its lysine residues. As lysine is an essential amino acid, extensive modifications of lysines lead to a substantial loss of the nutritional quality of milk products. Any change in BLG structure induced by modifications may increase allergenicity of this molecule by exposing hidden epitopes or by creating new epitopes. Therefore, an allergenicity assessment is necessary to test the safety of modified BLG molecules.

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ИЗВОД

МОДИФИКАЦИЈЕ БЕТА-ЛАКТОГЛОБУЛИНА – ЕФЕКТИ НА ЊЕГОВЕ СТРУКТУРНЕ И ФУНКЦИОНАЛНЕ ОСОБИНЕ

ДРАГАНА СТАНИЋ-ВУЧИНИЋ и ТАЊА ЋИРКОВИЋ ВЕЛИЧКОВИЋ

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Бета-лактоглобулин (БЛГ) је главни протеин сурутке који се често користи као адитив у великом броју прехрамбених производа захваљујући својим изванредним техничким и функционалним особинама, високој нутритивној вредности, као и ниској цени. Овај протеин се показао као погодан носач за лекове и хранљиве супстанце, јер је отпоран на киселу средину у гастро-интестиналном тракту. Међутим, БЛГ је и главни алерген млека. Са циљем унапређења функционалних особина и смањења алергености овог протеина до сада је развијен велики број метода за његову модификацију. Захваљујући својој компактној глобуларној структури БЛГ је релативно резистентан на модификације, нарочито оне под благим условима. БЛГ може бити модификован физичким, хемијским и ензимским методама. Мада су хемијске модификације ефикасан начин промене структурних и функционалних особина протеина, у вези са њима се често поставља питање безбедности. Током последње деценије постоји све већа тенденција ка примени нових третмана заснованих на физичким нетермалним методама, као и на примени ензима, како би се добио БЛГ са жељеним особинама. Циљ овог прегледног рада је приказ хемијских, физичких и ензимских техника које се користе за модификацију БЛГ, као и њихових ефеката на структуру и функцију БЛГ.

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