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Isolation of thymus gland fractions and the determination of their biological activity

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Abstract: A calf thymus extract was prepared and fractionated into lipid and non-lipid fractions. The non-lipid fraction was isolated from the calf thymus extract using the Folch method. The components isolated from the non-lipid fraction were characterized by IR, NMR, biuret and HPLC method. The results of the analyses indicated the presence of peptides. The lipid fraction contained phospholipids, glycolipids and neutral lipids. The biological activity of both the isolated lipid and peptide fractions was determined by the *in vivo* hemolytic plaques method in Wistar rats with an involuted thymus. The peptide and phospholipid fractions of the thymus extract showed a significant increase of hemolytic plaques. The glycolipid and neutral lipid fraction failed to express a significant immunological response.

Keywords: thumus gland, peptides, immunomodulators, hemolytic plaques method.

INTRODUCTION

The thymus gland plays in important role in overall immunomodulation. Several authors reported in the early 1960s that the thymus appears to be a prerequisite for the normal development of immune response. Since the 1970s, the thymus gland began to be regarded as an endocrine gland. It is thought to be responsible for the development and regulation of T-cell immunity, acting through the endocrine mechanism.[1]

The thymus seems to exert its regulatory functions *via* the secretion of various noncellular, hormone-like products, called thymic peptides.[2] The peptides are reported to produce a number of effects on T-cells. Several studies have reported that thymic peptides can support the development of immature, precursor cells into fully competent T-cells. When the immune system is challenged,[3] thymic peptides seem to regulate the expression of various cytokine and monokine receptors on T-cells and induce secretion of IL-2, interferon alpha, and interferon gamma. So

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far, fractions of several thymic peptides have been isolated and identified pegged as thymosin fraction 5, different types of thymosins,[4] thymopoietin and its synthetic analog thymopentin–Tp-5; thymomodulin, thymostimulin–Tp-1 and thymic humoral factor–THF. Blood levels of these hormones are typically low in the elderly and aging, individuals prone to infection, patients afflicted with AIDS, cancer or osteoporosis, and individuals exposed to multiple stress factors. These hormones and factos have been studied in *in vivo* and *in vitro* experimental models, and more recently in valid clinical trials.[4] Clinical trials using thymic extracts have demonstrated their capacity to restore immunocompetence in cases when immunological deficiency is detected.

There are reports showing that the use of thymic peptide hormones in patients with chemotherapy-induced immunodeficiencies resulted in an increase of the circulating T-cells, recovery of T-cell subsets, and restoration of delayed hypersensitivity reactions.[5–7] Several clinical studies have demonstrated that the thymus peptide fractions contribute to improved status of certain cancers, including Kaposi's sarcoma, skin and breast cancer, as well as AIDS.[8–10] Other clinical studies indicate that some thymic peptides showed therapeutic promise for patients with allergies–asthma,[11] rheumatoid arthritis, some dermatological conditions, and bacterial, viral or fungal infections.[12,13]

No literature data could be found on studies of whole thymus extract investigated for its biological activity.

The goal of this study was to determine the biologically active fractions of calf thymus extract. This report describes the isolation and fractionation of the lipid and peptide components of calf thymus extract. The isolated components were characterized by the evaluation of structural and activity parameters, such as lipid and peptide contents, molecular weight of peptides, and biological activity of the isolated fractions by the *in vivo* hemolytic plaques method.[14–16]

EXPERIMENTAL

Chemicals and reagents

Chemicals used for extractions, column chromatography and TLC analyses were of analytical reagent grade, obtained from Merck. The calf thymus gland of animals aged 3, 6 and 12 months was obtained from an abattoir. The thymus was collected and thin layer frozen in order to achieve quick freezing. The frozen material was kept at –20 °C until used. All chemicals used for the instrumental methods were of high purity grade, obtained from Merck. A standard peptide mixture for HPLC determination was obtained from Sigma. The media for the biological analyses were obtained from Torlak, Belgrade.

Isolation of the calf thymus extract

The frozen calf thymus was ground in a meat grinder and then suspended in a medium polar organic solvent *n*-butanol, which stirring. The first step of the isolation was done by extraction of the active components into the organic phase. The second step of the isolation was performed in a flask, when the azeotropic vacuum distillation of the two system component (water and organic phase), occurred. Ballast material (animal tissue) was separated. The calf thymic extract was concentrated by evaporation and dried under vacuum.

Lipid component isolation and characterization

Column chromatography. Merck silica gel 60 (70–230 mesh) in a glass column (3 x 75 cm) was packed and slurried in chloroform. All lipid fractions of calf thymus extract eluted with an equal volume of the organic solvent.[17] Each solvent fraction was evaporated under a stream of N_2 and dried to constant weight.

Thin layer chromatography. Characterization of each of the separated lipid fractions obtained from the calf thymus extract was determined using the TLC method, on Merck silica gel 60 pre-coated glass plates.

Peptide component isolation and characterization

Extraction. The fraction containing the biologically active peptides was isolated from the calf thymus extract using the Folch method.[18] After evaporation and lyophilization of the material, the peptide content was determined by the biuret method.[19]

IR-Spectra. IR Perkin Elmer 1725 XL, range of 4000–600 cm^{-1}.

NMR-Spectra. Varian Gemini 200 MHz in 10 % D_2O solution at 200 MHz.

HPLC-Method. HPLC system Hewlett Packard 1100 quaternary pump and diode-array detector. Chromatographic conditions – column: Zorbax SB-C18, 15 cm x 4,6 mm (5 μm); detection: 215 nm; flow: 1 ml/min; mobile phase: gradient: 0.1% TFA and ACN;

Temperature: 25°C; standard peptide mixture (Sigma 0545106).

Biological activity determination

The Cunningham and Szeberg direct hemolytic plaques method[14–16] was performed in Parker medium 199, with sheep red blood cells and Freund's adjuvant. A complement consisting of rabbit sera, sheep RBC and rat spleen cells was used as a test system. Plaque detection was performed under a light microscope in Cunningham compartments. Wistar rats (14–16 months), with involuted thymus were used for the biological experiments.

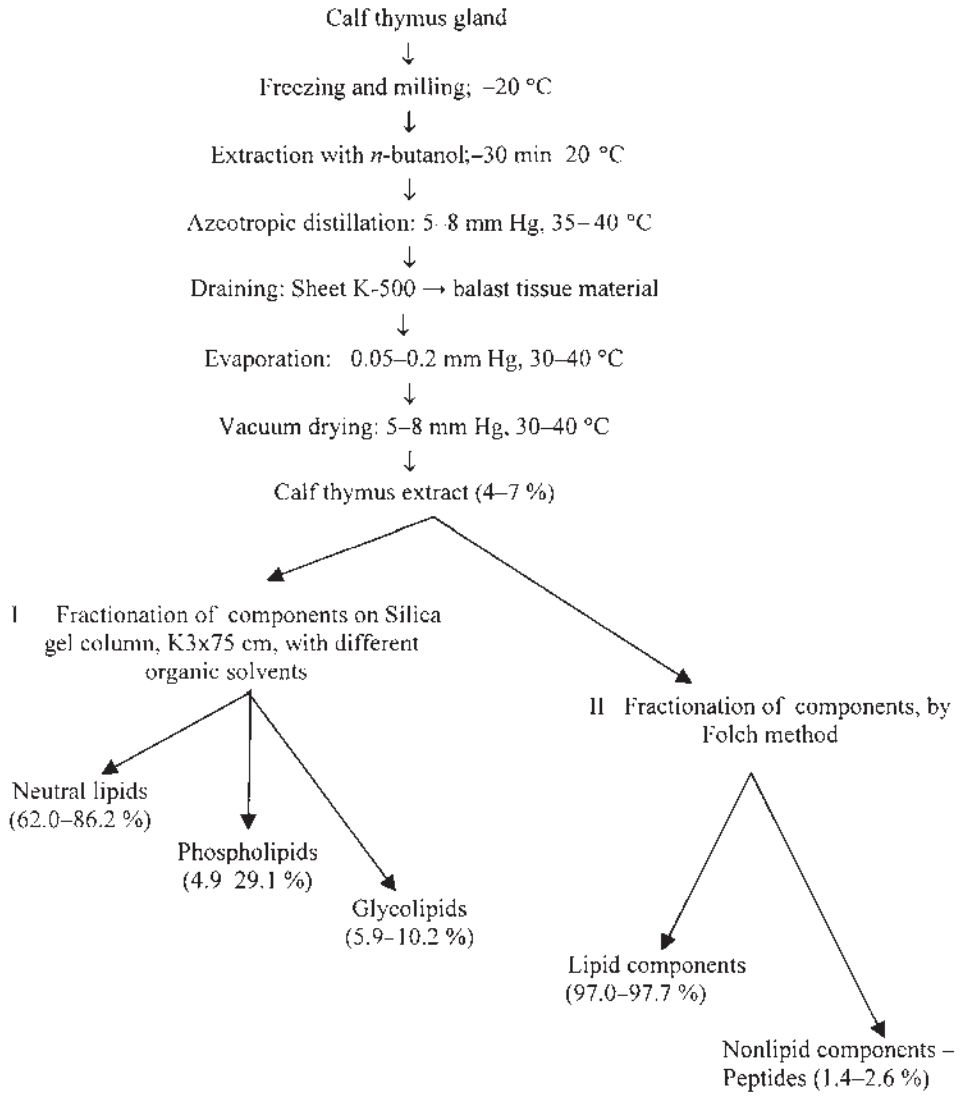
Rats were cared for in accordance with the principles enunciated in the Guide for Care and Use of Laboratory Animals, NIH publication No. 85-23.

RESULTS AND DISCUSSION

The entire process of isolation as described in the Experimental section exemplifies a simple procedure for the isolation of the calf thymus extract, while a detailed description of the procedure can be found in YU Patent Application P – 426 / 97. A general flow chart of the extraction and fractionation steps used is shown in Scheme 1. The procedure yielded 4–7 % of calf thymus extract, related to the thymus gland. The calf thymus extract was fractionated into lipid and non-lipid (peptide) components in order to enable their chemical and biological characterization. A high amount of total lipid components (97.0 – 97.7 %, w/w) and a very small quantity of peptides (0.29 – 0.55 %, w/w) were isolated from the calf thymus extract.

Isolation and separation of the lipid fractions from the calf thymus extract was achieved by column chromatography, whereby three major lipid fractions: neutral lipids, phospholipids, and glycolipids were obtained. The lipid fractions were analysed by TLC chromatography. The R_f values of each fraction were compared to reference standards.

Literature data clearly show that the source for the isolation of thymus peptides must be young animals or foetus.[9,12] The thymuses used as the starting material were isolated from calves aged 3, 6 and 12 months. The results of the fraction-



Scheme 1. Procedure for the isolation of the calf thymus components (see YU Patent Application P-426/97 for detailed description of the procedure).

ation showed a variance in the yields of each lipid and peptide component depending on age. The calf thymus extract has the neutral lipid fraction as the main component. The content of neutral lipids increases with age. The relative standard deviations for the neutral lipid fractions were very low (*RSD* = 1.26–2.6 %). The yield of the phospholipid fraction isolated from calf thymus extract decreases with age. The relative standard deviation for phospholipids and glycolipids isolated from 3–12 month old animal sources was variable and was especially high for the

12-month calf thymus. The yields of the fractions isolated from calf thymus extract (expressed in %), mean value ± *SD* and *RSD* values, are presented in Table I. The results obtained for the lipid fractions suggest that the neutral lipids content is a reliable parameter for the determination of the thymus gland quality.

TABLE I. Lipid and peptide fractions from the calf thymus extracts, according to age

Fraction	Yield of fractions/%					
	3 month		6 month		12 month	
	$X \pm \Delta X$	*RSD*/%	$X \pm \Delta X$	*RSD*/%	$X \pm \Delta X$	*RSD*/%
Neutral lipid	62.03 ± 0.93	2.60	79.53 ± 0.70	1.52	86.25 ± 0.54	1.26
Phospholipid	29.10 ± 1.05	6.24	7.97 ± 0.10	2.24	4.94 ± 0.63	25.50
Glycolipid	5.90 ± 0.62	18.15	10.19 ± 1.10	18.80	6.09 ± 0.97	32.10
Peptide	0.55 ± 0.03	11.64	0.36 ± 0.01	9.05	0.25 ± 0.01	6.80

Mean ± *SD* and *RSD*/%; (n = 3)

The main biologically active constituent of the non-lipid component was the peptide fracton. The peptide components were isolated from the calf thymus extract using the Folch method.[18] The quantity of isolated peptides was much higher in the samples of the young calf extracts. The relative standard deviation for the peptide fraction isolated from 3–12-month animal source was low (*RSD* = 6.8–11.6 %). These results indicate that the content of peptides can also be used as a reliable parameter for the determination of the quality of the raw animal material.

Using the biuret method, the quantity of isolated peptides present in the non-lipid component of the calf thymus extract was found to be 17–25 % (w/w). The results of the analyses showed that a small quantity of peptides (0.29 – 0.55 %, w/w), was present in the calf thymus extract. The non-lipid fraction was analyzed by IR and NMR spectroscopy. The spectral analyses showed bands indicating the peptide structure: 3300–2300 cm^{-1} ν(OH) + ν(NH), strong, broad; 1600–1560 cm^{-1} ν(C=O), strong band; 1410 cm^{-1} ν_s(C=O), weak band.

The one-dimensional ^{1}H-NMR spectrum of the thymus peptide sample was recorded in D_2O. Spectral analysis showed the bands: amide and aromatic 6–10 ppm; H_α 4–6 ppm; H_β, $H\gamma$ 2–2.5 ppm; methyl groups ~ 2 ppm. The presence of peptides in the non-lipid compound was confirmed by the HPLC method. The results of the comparison of the retention times of the standard peptide mixture showed that the molecular weights of the peptides present in the calf thymus extract were below 1500 D (Fig. 1).

On the basis of the presented results, it was confirmed that thymus extracts contained peptide components. This is in good agreement with literature data on the thymus exerting its regulatory functions through the secretion of various non-cellular products, the so-called thymic peptides.[2]

The biological activity of the calf thymus extract fractions was determined by the *in vivo* screening method of direct hemolytic plaques.[14–16] Wistar rats (14–16

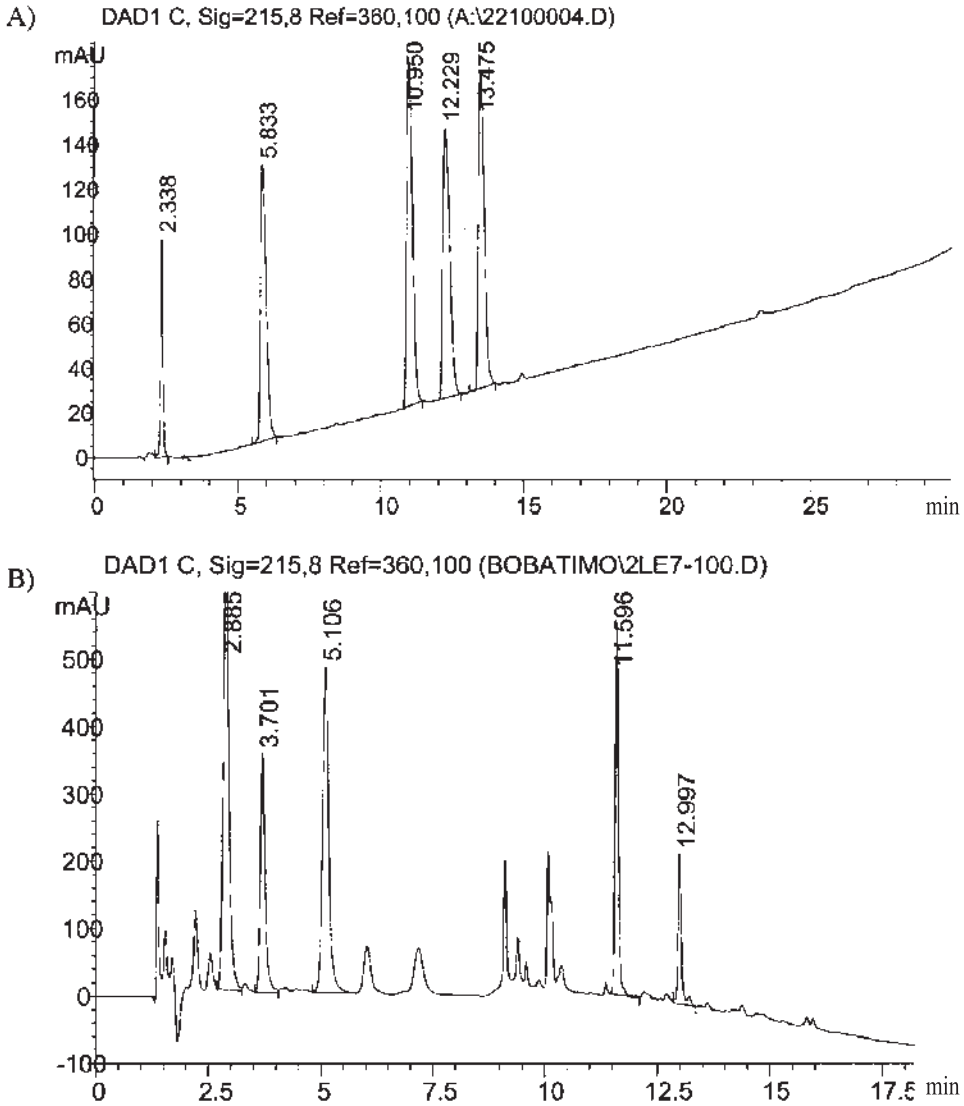


Fig. 1. Elution profiles of the standard mixture and representative sample. (A) Standard mixture Sigma: t_r = 2.338 min, Gly–Tyr; t_r = 5.833 min, Val–Tyr–Val; t_r = 10.950 min, Leu-Enkephalin; t_r = 12.229 min, Met–Enkephalin; t_r = 13.475 min, Angiotensin II. Standards were disolved in the initial mobile phase; (B) a sample obtained from the non-lipid component isolated from the calf thymus extract at 0.1 mg/ml concentration.

months old), with an involuted thymus were used in the experiments. The method for direct hemolytic plaques is based on the complement system (C), which is the principal humoral mediator in antigen and antibody reactions. Interactions occurring at the time of the complement activation result in biological activities associated directly with inflammatory processes and a variety of cell types, bacteria and

viruses. The complement may also intensify the activity of both humoral and cellular effector systems, such as histamine release from mast cells, controlled leukocyte movement, and phagocytosis.

The activation of the total component C1–C9 sequence is required to produce lysis of antibody sensibilized leukocytes. The standard hemolytic test uses sheep erythrocytes (E), rabbit and rat antibodies to sheep erythrocytes (A), and a fresh guinea pig complement.

The localized hemolysis in agar (plaque method) was used to detect antibody synthesis by individual cell, thus making the identification of cells (B-lymphocytes) producing IgM anti-erythrocyte antibodies possible.

The experimental model that was used to demonstrate the immunostimulating effect of calf thymus fractions involved rats (14–16 months) with a naturally involuted thymus gland and significantly lower antibody synthesis, as compared with young rats (2 month) used as positive controls. Older rats (14–16 months) receiving a placebo instead of the immunostimulating components were used as negative controls.

The test animals were given appropriate dose of subcutaneous thymus fractions every other day for 12 days, while the controls (young rats) were given physiological saline.

The animals were immunized with sheep erythrocytes and sacrificed 4 days post-immunization. A live spleen cell suspension was prepared. An appropriate number of lymph cells was mixed with sheep erythrocytes, which were in excess, but whose number was also known. The guinea pig complement was than added to the mixture, and the so-obtained mixture was poured into a Cunningham compartment (incubated at 37 °C for 1 h). In this period of time, anti-erythrocyte antibodies were produced by the cells to coat the sheep erythrocytes. The activation of the complement system resulted in sheep erythrocyte lysis and any antibody-synthesizing cell was surrounded by a circular area of lysis (plaque).

The capability of IgM antibodies synthesis was partially regained in the older animals with a naturally involuted thumus gland after the administration of the thymus peptide and phospholipid fraction, as shown by the hemolytic plaque producing cell count (Table II). The numbers approached those obtained in younger animals with a preserved thymus gland (positive controls).

The results of the screening biological analyses indicated that the most potent component of calf thymus extract was the peptide fraction, an observation congruent with literature data on the action of thymic peptide. The results also showed that the phospholipid fraction exhibited significant biological activity, whereas the activity of the glycolipids was noticeably weaker. Using the described *in vivo* method of hemolytic plaques, no significant increase of hemolytic plaques with the neutral lipid fraction was detected. Thus, it can be concluded that the estimated biological activity of the calf thymus extract originates from the activity of the peptides, whereas the activity of the lipid components (mainly phospholipids) has an

adjuvant effect. The statistical significance of these results was confirmed by valid statistical methods, such as Student's t test and variance analysis.

TABLE II. Biological activity of the different thymus fractions

Thymus fraction	Dose	Number of hemolytic plaques	Plaques increase/%	Statistical significance
Calf thymus	100 mg/kg s.c.	1404	23.8	$t < 0.05$
extract		1031	20.3	$t < 0.05$
Neutral lipid	83 mg/kg s.c.	999	–	–
Glycolipid	2.87 mg/kg s.c.	1185	4.5	$t < 0.05$
Phospholipid	13.8 mg/kg s.c.	934	9.0	$t < 0.05$
Peptide	50 mg/kg s.c.	1543	36.6	$t < 0.05$
Placebo	100 mg/kg s.c.	1134	–	–
		857		
		1008		

The presented results indicate that the increased number of hemolytic plaques can be considered as non-specific evidence of potential immunostimulant activity. Further experiments, with more specific tests, are necessary to study the broader implications of these immunomodulating effects.

CONCLUSION

It can be presumed that the thymus gland is suitable material for the isolation of biologically active raw pharmaceutical material. The major compounds of the calf thymus extract were isolated and partially characterized. The lipid components of the calf thymus extract were fractionated by means of chromatographic methods into neutral lipids, phospholipids and glycolipids fractions. The chemical structure of each of the lipid components was defined. The non-lipid components were isolated from the calf thymus extract using the Folch method. The chemical structure of the peptides was confirmed by IR and NMR spectroscopy, the biuret method and the HPLC technique.

The biological activity of the fractions of the calf thymus extract was determined by the *in vivo* screening method of direct hemolytic plaques. According to the results of the biological analyses, while no significant increase of hemolytic plaques was detected with the neutral lipid fraction, the phospholipid and glycolipid fractions had low, but still significant, biological activity which varied with the age of animal raw material. Using the described *in vivo* method of hemolytic plaques, the most potent component of calf thymus extract was found to be the peptide fraction.

It is believed that the estimated biological activity of the calf thymus extract originated from the activity of the peptides, while the lipid components (mainly the phospholipids and glycolipids) had a probable adjuvant effect.

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ИЗВОД

ИЗОЛОВАЊЕ ФАРМАКОЛОШКИ АКТИВНИХ ФРАКЦИЈА ИЗ ТИМУСА И ОДРЕЂИВАЊЕ ЊИХОВЕ БИОЛОШКЕ АКТИВНОСТИ МЕТОДОМ ХЕМОЛИТИЧКИХ ПЛАКА

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У раду је описан поступак изоловања екстракта телећег тимуса и одређивање биолошке активности његових компоненти. Поступак изоловања се базира на екстракцији замрзнутог телећег тимуса са средње поларним органским растварачем. Добијени екстракт тимуса је фракционисан на липидне и нелипидне компоненте у циљу одређивања носиоца биолошке активности. Липидна компонента екстракта је применом хроматографске методе (силика гел 60, Merck) фракционисана на неутралне липиде, гликолипиде и фосфолипиде. Хемијска природа сваке изоловане липидне компоненте је потврђена применом танкослојне хроматографије. Фракција која садржи биолошки активне пептиде је изолована из екстракта телећег тимуса, приеменом методе по Folch-у. Пептидна структура изоловане компоненте је утврђена применом биуретске, IR, NMR и HPLC методе. Биолошка активност појединачно изолованих компоненти је потврђена применом *in vivo* методе са хемолитичким плакама. За одређивање биолошке активности употребљени су Wistar пацови старости од 14–16 месеци, са инволуираним тимусом. Утврђено је да пептидна и фосфолипидна фракција показују значајно повећање хемолитичких плака, док неутрални липиди и гликолипиди не показују адекватан имунолошки одговор.

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