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## Effects of autoclaving and pullulanase debranching on the resistant starch yield of normal maize starch

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**Abstract:** In this study, resistant starch (RS), type 3, was prepared by the autoclaving and debranching of normal maize starch isolated from a selected ZP genotype. The objectives of this study were to optimize both starch autoclaving and debranching with pullulanase (Pro mozymeBrewQ) for the production of RS. Autoclaving at 120 °C (30 min) increased the RS content of all samples, whereas freezing at –20 °C did not have an obvious effect on the RS contents. The highest RS yield in the autoclaved starch samples was 7.0 % after three autoclaving–cooling cycles. After pullulanase debranching at 50 °C and retrogradation at 4 °C, the RS yields ranged from 10.2 to 25.5 % in all samples (depending on the hydrolysis time). Debranched starch samples with a maximum RS yield of 25.5 % were obtained after a debranching time of 24 h. This study showed that starch from the selected ZP maize genotype is suitable for pullulanase treatment and RS preparation but that additional studies with a greater number of different treatments (incubation time/temperature) are necessary to manipulate and promote crystallization and enhance RS formation.

**Keywords:** maize starch; autoclaving; debranching; pullulanase; resistant starch.

### INTRODUCTION

There is considerable interest in the nutritional implications of resistant starch (RS) in foods. The term “resistant starch” was used to designate mainly enzyme-resistant retrograded amylose but was expanded to all forms of starch that escape digestion and absorption in the small intestines.<sup>1</sup> The positive effect of RS in nutrition is based on the results of the fermentation process during which short chain fatty acids, primarily acetates, propionates and butyrates, are produced. They directly affect the large intestine by decreasing the pH value, which prevents the growth of pathogenic micro-organisms, and increasing the potential for mineral absorption. Fatty acids stimulate colonic blood flow and increase nutrient

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flow.<sup>2–5</sup> Besides physiological benefits in human, RS has been reported to have potential as a unique ingredient that can yield high-quality foods. For example, application tests of RS showed improved crispness and expansion in certain products and better mouth feel, colour and flavour as compared with products produced with traditional, insoluble fibres.<sup>6</sup>

Resistant starch can result from a highly retrograded amylose fraction, the quantity formed being directly proportional to the amylose content of the starch.<sup>7</sup> This type of RS has been classified as RS type 3 (RS3). The degree of RS formation in foods depends not only on the type of included starch and the adopted processing conditions but is also influenced by the duration and conditions of storage.<sup>8</sup>

RS3 is produced by gelatinization, which is a disruption of the granular structure by heating starch with an excess of water, and retrogradation, a slow recrystallization of starch components (amylose and amylopectin) upon cooling or dehydration. The generation of RS3 after this hydrothermal treatment is due to increased interaction between starch components. It has been shown that, after debranching of starch, the linear chains can contribute to a high RS content. In addition, partial acid hydrolysis and debranching of amylopectin are very effective in generating RS from various starches.<sup>9–11</sup> The advantages of debranching over mild acid hydrolysis include a shorter processing time, better processing control and higher RS yields.

Among different resistant starches, retrograded resistant starch (RS3) has great commercial importance, since its crystalline polymorphs exhibit an endothermic transition from 120 to 165 °C<sup>12,13</sup> that typically survives most, but not all, food-processing conditions. RS3 was initially hypothesized as a crystalline state of amylose double helices.<sup>14</sup> Later, the non-crystalline part of retrograded starch was also found to be enzyme resistant.<sup>15,16</sup>

Although starches from diverse plants may be utilized, maize is the world's most abundant source that provides the majority of substrates used in the preparation of starch hydrolysates. Starch granules are quite resistant to penetration by both water and hydrolytic enzymes, due to the formation of hydrogen bonds within the same molecule and with other neighbouring molecules. However, these inter- and intra-hydrogen bonds can become weak as the temperature of the suspension is raised. As normal maize starch contains both linear amylose and branched amylopectin with short and long chains of different sizes, our approach was to degrade it through physical and enzymatic treatments to attenuate starch digestion properties. In the present study, debranching using pullulanase was applied to produce a sample with linear, low molecular weight and recrystallizable polymer chains. Debranching enzymes, such as pullulanase, rapidly hydrolyze only the  $\alpha$ -1,6-glucosidic bonds, releasing a mixture of long and shorter unit chains from the parent amylopectin molecule. In theory, a fast and efficient enzyme hyd-

hydrolysis requires pre-swelling the starch in water and full starch gelatinization. The objectives of this work were to check the possibility of RS production from normal maize starch from the selected ZP genotype and to optimize both starch autoclaving and debranching with pullulanase (PromozymeBrewQ) for RS production.

## EXPERIMENTAL

### *Materials*

Commercial maize starch was obtained from a local producer ("Jabuka", Pančevo, Serbia). Normal maize starch was isolated from the genotype ZP 434. Commercial debranching enzyme, pullulanase (PromozymeBrewQ, 400 PUN/ml) from *Bacillus acidipullulyticus* was obtained from Novozymes (Bagsvaerd, Denmark). One Pullulanase Unit Novo (PUN) is defined as the amount of enzyme that hydrolyzes pullulan, liberating reducing carbohydrates with a reducing power equivalent to one  $\mu\text{mol}$  glucose per minute under standard conditions. The resistant starch assay kit was purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland).

### *Starch isolation*

Starch from maize grain (ZP 434) was isolated by applying a 100-g laboratory maize wet-milling procedure.<sup>17</sup> The moisture, ash, crude protein and crude fat contents of the starch were determined using the oven method,<sup>18</sup> the AOAC method,<sup>19</sup> the microKjeldahl method<sup>19</sup> and the Soxhlet method,<sup>19</sup> respectively. The amylose content was determined by a rapid colorimetric method.<sup>20</sup>

### *Resistant starch production by autoclaving and cooling*

The samples were prepared by suspending 10% and 20% (w/v) of maize starch in 1000 ml of water. The suspensions were heated in a boiling water bath for 15 min with stirring and then transferred to an autoclave (Sutjeska, Belgrade, Serbia). The autoclaving conditions were: pressure, 1.1 bar, temperature, 120 °C, autoclaving time, 30 min and vessel volume, 60 dm<sup>3</sup>. After autoclaving, the samples were stored at 4 °C for 24 h. The samples were subjected to three autoclaving-cooling cycles. After the third cycle, the sample was divided in two; one part was stored at 4 °C and the other at -20 °C for 24 h. After each cycle, the RS was determined.

### *Conditions for enzymatic debranching of maize starch*

Prior to starch debranching, the optimal concentration of pullulanase was determined.

A maize starch suspension (20%, w/v) was gelatinized on a boiling water bath for 15 min under stirring. This gel was autoclaved at 120 °C for 30 min and then the gel was re-dissolved in distilled water to obtain a 10% (w/v) gel solution. The gel was cooled to 50 °C and pullulanase at different concentrations (0.25, 0.5, 1.0, 2.0 and 4.0%, calculated on dry starch weight) was added. The mixture was incubated with constant stirring for 24 h. Samples were taken out at different times and the reducing sugars (DE value) were determined by the Luff-Schoorl method (ISO 5377:1981).<sup>21</sup> At least three replicates for each hydrolysis time were conducted and good reproducibility was achieved since the differences between the triplicate measurements were less than 10%.

### *Resistant starch production by starch debranching*

Normal maize starch isolated from the genotype ZP 434 was debranched using an enzyme in concentration of 2% (calculated on dry starch weight), which was determined as optimal concentration. The maize starch gel was prepared as described in the previous section

and the reaction time was varied (1, 3, 5, 7, 9, 11 and 24 h); after these times, the samples were heated at 95 °C for 20 min, cooled down to room temperature and stored for 24 h at 4 °C or –20 °C. The samples were dried at 40 °C and stored in closed glass containers.

#### *Resistant starch determination*

The RS content in the starch modifications was determined by an enzymatic method (AOAC 2002.02).<sup>22</sup> The samples were incubated with pancreatic  $\alpha$ -amylase and amyloglucosidase (AMG) for 16 h at 37 °C, during which time the non-resistant starch was solubilised and hydrolyzed to glucose by the combined action of the two enzymes. The reaction was terminated by the addition of an equal volume of ethanol and the RS was recovered as a pellet on centrifugation. This was then washed twice with ethanol (50 % v/v), and centrifuged. The RS in the pellet was dissolved in 2 M KOH by vigorously stirring on an ice-water bath. This solution was neutralized with a acetate buffer and the starch was quantitatively hydrolyzed to glucose with AMG. The glucose was quantified with glucose oxidase/peroxidase reagent (GOPOD), which gave a measure of the RS content of the sample.

#### *Statistical analysis*

The results are expressed by means of values  $\pm$  standard error of three separate determinations. The data reported for the effects of autoclaving on the formation of RS was assessed by analyses of variance (ANOVA) and the Duncan multiple test was used to identify any significant differences at the  $P < 0.05$  level between the means. The analyses were conducted using the statistical software package Statistica 8.1. (StatSoft Inc., USA).

## RESULTS AND DISCUSSION

#### *Proximate composition*

The basic properties of the starch samples used in this study are given in Table I. The commercial maize starch “Jabuka” and “ZP 434” did not significantly differ in their amylose content but they differed slightly in their ash and contents of crude protein and crude fat.

TABLE I. The properties of native starch samples (“Jabuka” and “ZP 434”)

Starch sample	Amylose, %	Moisture, %	Ash, %	Crude protein, %	Crude fat, %
“Jabuka”	26.5 $\pm$ 0.4	10.4 $\pm$ 0.2	0.10 $\pm$ 0.02	0.30 $\pm$ 0.05	0.10 $\pm$ 0.02
“ZP 434”	26.0 $\pm$ 0.3	8.4 $\pm$ 0.1	0.01 $\pm$ 0.00	0.26 $\pm$ 0.04	0.05 $\pm$ 0.00

Normal maize starch was chosen for this study because of its abundance and a low price compared with high amylose maize starches. Previously, it was found that there were not significant differences in the physicochemical characteristics between starches of different dent ZP maize genotypes.<sup>23</sup> The genotype ZP 434 ranks among the top hybrids produced in Serbia. Due to the limited quantity of starch isolated from “ZP 434”, commercial maize starch “Jabuka” was used in order to identify the optimum debranching conditions for RS formation. In the further steps, only starch from the selected maize genotype was used.

### Starch autoclaving

The formation of resistant starch type 3 (RS3) depends on many factors, *e.g.*, pH, temperature, incubation time, storage time, number of heating and cooling cycles, starch type, *etc.* The amylose content and the amount of water are directly correlated to the yield of resistant starch.<sup>2</sup> The gelatinization of starch granules during heat processing strongly influences their susceptibility to enzymatic hydrolysis. Even for starches with normal amylose levels, it is recognized that cooking at >100 °C can increase the RS3 yield. Repeated heat/moisture treatments are associated with a decrease in the hydrolysis limit of pancreatic  $\alpha$ -amylase and the increased RS3 formation.<sup>2</sup>

The effects of autoclaving and storage temperatures on the formation of resistant starch from normal maize starch ZP 434 are presented in Tables II and III, respectively. In this study, the starch concentration (10 % or 20 %) in the suspension did not significantly affect the RS yields but the number of autoclaving–cooling cycles did. After three autoclaving–cooling cycles, the RS yields increased by 2.1 % for both starch concentrations.

TABLE II. Effect of autoclaving at 120 °C on resistant starch formation from normal maize starch

Starch content in suspension, %	Autoclaving cycle	Resistant starch, %
10 I		4.8 <sup>c</sup> ± 0.2
	II 6.8	<sup>a</sup> ± 0.1
	III 6.9	<sup>a</sup> ± 0.1
20 I		4.9 <sup>c</sup> ± 0.1
	II 5.9	<sup>b</sup> ± 0.3
	III 7.0	<sup>a</sup> ± 0.2

<sup>a,b,c</sup> Means within a column followed by different letters are significantly different ( $P < 0.05$ )

TABLE III. Effect of storage temperatures (4 and –20 °C) on resistant starch formation from autoclaved and debranched starches

Sample	Resistant starch, %	
	<i>t</i> / °C	
	4 –20	
Autoclaved sample	7.0 ± 0.2	6.9 ± 0.1
Debranched sample (1 h)	10.2 ± 0.3	9.8 ± 0.1
Debranched sample (24 h)	25.5 ± 0.5	24.9 ± 0.2

Escarpa *et al.*<sup>24</sup> standardized the hydrothermal process in starch gelatinization, and they reported the RS content ranged from 7.6 for waxy starch (0 % amylose) to 36.4 % for purified amylose. These authors found that the RS yield increased with increasing amylose content in the starches under study. The differences in the results found in this study and those determined by other researchers might be due to the source of the starch and the conditions applied for the RS

preparation. Sievert and Pomeranz<sup>12</sup> determined RS values between 2.5 and 21.3 % for diverse starch sources, and with the Berry method,<sup>25</sup> the RS values ranged between 2.8 and 31 %.<sup>13</sup> Autoclaved wheat starch possessed 9 % RS compared with less than 1 % in uncooked wheat starch.<sup>26</sup> The autoclaved wheat starch contained 6.2 % RS (of dry matter); this increased to 7.8 % after 3 further boiling/cooling cycles.<sup>27</sup>

Generally, the content of resistant starch increases during storage, especially during low-temperature storage. Cold storage seems to support an increase in the RS content. In this study, the storage conditions (4 or  $-20$  °C) did not significantly affect the formation of RS of either autoclaved or debranched samples. These findings are in accordance with the results obtained by Hasjim and Jane,<sup>28</sup> who found that freezing at  $-20$  °C had little or no effect on the RS content of acid-modified starch samples.

#### *Effect of enzyme concentration on starch debranching*

An increased degree of debranching would enable the chains to align and aggregate and hence form perfectly crystalline structures, thereby leading to the formation of more RS.<sup>29</sup> Berry<sup>25</sup> reported that debranching of potato amylopectin with pullulanase before subjecting it to heating and cooling cycles substantially increased the RS3 content; this was attributed to an increase in the content of linear starch chains resulting from debranching.

As shown in Fig. 1, commercial maize starch “Jabuka” debranched with 4 % pullulanase exhibited the highest reducing value, followed by starch samples debranched with 2, 1, 0.5 and 0.25 % pullulanase. In the cases of 2 and 4 % en-

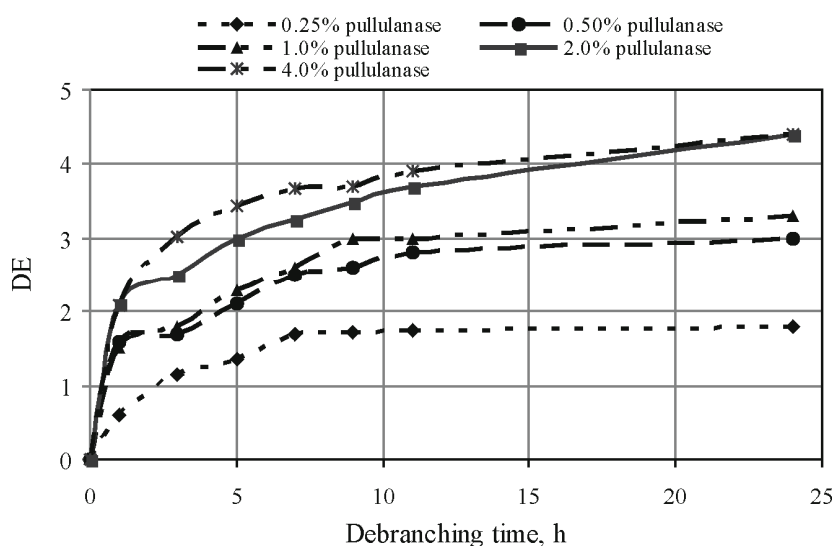


Fig. 1. Optimization of maize starch debranching; DE represents dextrose equivalent.

zyme, the reducing values resulting after 1 h of hydrolysis were not statistically different. The addition of the higher amount of enzyme did not result in complete debranching, thus a concentration of 2 % was used for starch debranching in this work. This result is in agreement with the findings of Hizukuri *et al.*<sup>30</sup> who reported that  $\alpha$ -amylase and pullulanase initially degraded potato amylose rapidly and then approached constant values on prolonged incubation with a large amount of enzyme.

The kinetics of starch debranching in a 2-L-reactor (Fig. 1) showed that after 9 h and longer, the glucose concentration was constant (for the lowest enzyme concentration) or slightly higher (for higher enzyme concentrations); however, starch debranching was performed for 11 and 24 h. After debranching for different times (seven samples), the starch solution was heated at 95 °C to deactivate the enzyme and RS was obtained.

#### Resistant starch content

Seven debranching times (1, 3, 5, 7, 9, 11 and 24 h) were applied to study the effect of debranching time on the content of RS (Fig. 2). A gradual increase in the RS content of the maize starch samples was observed with increasing debranching time, some of which were significant. Native maize starch from the genotype ZP 434 had a very low RS content (0.60 %) compared to the debranched samples. The RS content increased significantly from 0.60 to 25.0 % after 11 h of debranching. After a total debranching time of 24 h, the RS content slightly increased to 25.5 %. The RS content measured after 5 h was 18 %, showing that 70 % of the totally obtained resistant starch was produced during this time. When the content of debranched starch increased, higher amounts of short starch chains were produced. These linear fragments can contribute to starch retrogradation

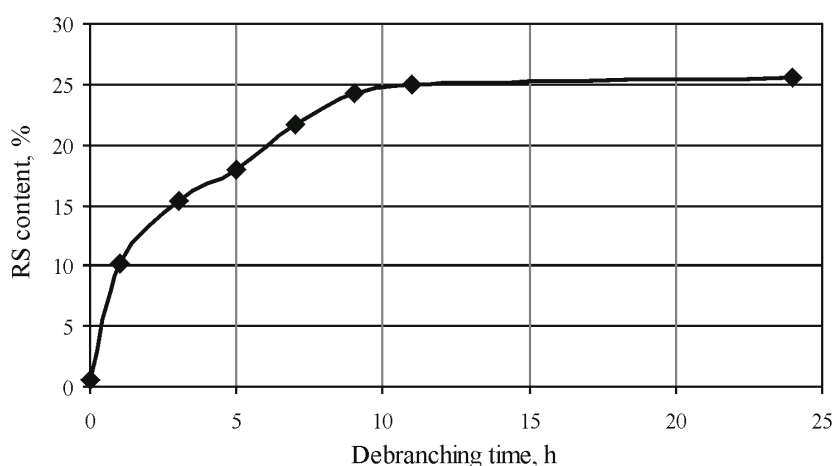


Fig. 2. Effect of debranching time on RS formation.

and decreased enzymatic susceptibility of starch, with the concomitant increasing in the RS content.

The RS contents of all debranched samples were significantly ( $P < 0.05$ ) higher compared to their respective autoclaved samples (control). Debranching was performed at 50 °C after which the samples were dried at 40 °C. The debranching and drying conditions were suitable for RS3 formation.

The content of amylose (37 %) determined after debranching corresponds well with findings reported by Escarpa *et al.*<sup>24</sup> who used potato starch with a similar amylose/amylopectin ratio as in the present samples. As can be seen in Fig. 2, the RS content after 5 h of debranching was 18 % compared to 18.2 % reported by Escarpa *et al.*<sup>24</sup> These authors observed increased RS yields when the amylose content in the studied starches was higher. Recently, the results of Öztürk *et al.*<sup>31</sup> indicated that 48 h of debranching of high amylose maize starches was suitable for RS formation. The results of González-Soto *et al.*<sup>32</sup> demonstrated that debranching of banana starch with pullulanase (Promozyme D) for 5 h was sufficient to obtain a high RS level (18 %). In another study,<sup>25</sup> an RS content of up to 46.8 % was determined in potato amylopectin after debranching, drying and heat processing. The differences in the results found in this study and those determined by other researchers might be due to the source of the starch and the conditions used for the RS preparation.

Based on the obtained results, it can be concluded that a high level of debranching occurred during the first 11 h of debranching; thus, a 11-hour debranching with pullulanase was sufficient to obtain a high RS level.

#### CONCLUSIONS

Autoclaving at 120 °C (30 min) increased the RS content in all samples, whereas freezing at -20 °C had no obvious effect on the RS contents. The highest RS yield in the autoclaved starch samples was 7.0 % after three autoclaving-cooling cycles. After debranching of starch with pullulanase at 50 °C and subsequent retrogradation at 4 °C, RS yields ranging from 10.2 to 25.5 % were found in all samples (depending of the hydrolysis time). The RS determination showed that starch debranched for 24 h had the highest RS level and that approximately 70 % of the starch had been hydrolyzed after 5 h. An enzyme concentration of 2 % was sufficient for starch debranching, giving the highest amount of liberated glucose. Debranching of maize starch for 11 h was sufficient to obtain a high content of RS in the products.

This study showed that the selected starch from the ZP maize genotype was susceptible for pullulanase treatment and RS preparation but additional studies with a greater number of different treatments (incubation time/temperature) are necessary to manipulate and promote crystallization and hence enhance RS formation.



## ИЗВОД

## УТИЦАЈ ОБРАДЕ У АУТОКЛАВУ И ХИДРОЛИЗЕ ПУЛУЛАЗОМ НА ПРИНОС РЕЗИСТЕНТНОГ СКРОБА ИЗ НОРМАЛНОГ КУКУРУЗНОГ СКРОБА

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У овом раду, резистентан скроб (РС), тип 3, припремљен је обрадом у аутоклаву и хидролизом са пулулазозом из нормалног кукурузног скроба који је изолован из одабраног ЗП генотипа кукуруза. Предмет истраживања је био да се изврши оптимизација третмана обраде у аутоклаву и хидролизе пулулазозом (Promozy meBrewQ) у циљу добијања РС-а. Обрада у аутоклаву на 120 °C (30 min) је утицала на повећање садржаја РС-а у свим узорцима, док температура чувања од -20 °C није имала утицаја на његов садржај. Највећи принос РС-а у аутоклавираним узорцима скроба је био 7,0 % након три циклуса обрада у аутоклаву-хлађење. Након хидролизе пулулазозом на 50 °C и ретроградације на 4 °C принос РС-а у свим узорцима се кретао од 10,2 до 25,5 % (зависно од времена хидролизе). Хидролизоване узорци скроба са максималним приносом РС-а од 25,5 % одређени су након 24 часа инкубације. Ово истраживање показује да је скроб из одабраног ЗП генотипа кукуруза погодан за хидролизу пулулазозом и добијање РС-а, али су неопходна даља истраживања са већим бројем различитих третмана (време/температура инкубације) у циљу побољшања процеса кристализације и повећања приноса РС-а.

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