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Full Length Research Paper

Preparation and properties of resistant starch from corn starch with enzymes

Qunyu Gao*, Suling Li, Huali Jian and Shizhong Liang

Carbohydrate Laboratory, College of Light Industry and Food Sciences, South China University of Technology, Guangzhou 510640, People's Republic of China.

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Corn starch was subjected to enzymatic biotechnology with thermostable α-amylase and pullulanase and followed by retrogradation, to prepare resistant starch. The properties of selected resistant starch (RS) samples were also investigated. The result showed that, appropriate amount (0.5 U/g) of thermostable α-amylase was good for resistant starch formation. The optimal condition for pullulanase hydrolysis was carried out with 0.8 PUN/g (dry starch) pullulanase in pH 5.5 starch gel at 60°C for 12 h. The highest yield of resistant starch could be obtained (19.02%) under optimal condition. The effect of pullulanase on resistant starch formation was the most significant. Compared with native starch, amylose content was increased by 17.0 to 28.1% and it did not increased with enhancement of resistant starch content. The native starch showed A-type X-ray diffraction pattern, whereas RS products exhibited B-type pattern with strong intensity at the peak of 17.2°C 2θ and two board peak at 20.1 and 23.9°C 2θ. The transformation enthalpy of the second peak increased gradually with increasing RS content and the transformation peak temperature and T_c-T_0 was also similar to this trend.

Key words: Resistant starch, thermostable α-amylase, pullulanase, properties.

INTRODUCTION

A long-held opinion that starch was completely digested and absorbed in the small intestine has been challenged (Englyst et al., 1983). Resistant starch (RS) has been defined as the sum of starch and starch degradation products that cannot digest in the small intestine (EURESTA GROUP, 1992). RS directly passes into the colon where it can be fermented by natural microflora to short-chain fatty acids such as butyric acid (Baghurst et al., 1996). RS is a non-caloric ingredient and does not contribute to increase in blood glucose. In this, it has physiological effects in the human body that are similar to that of dietary fiber, which has been shown to reduce risks for some diseases, including colon cancer, coronary heart disease and glycemia (Cairns et al.,1995; Ranhotra et al.,1996). In addition, RS does not hold much water and, thus, may be a preferred fiber source for use in low-moisture products such as cookies and crackers. Unlike traditional fiber sources, RS is free of gritty mouth feel and also does not alter flavor and textural properties of foods. These characteristics make RS a food ingredient of increasing interest.

Resistant starch is classified into four categories: physically inaccessible starch (RS1); RS granules (RS2); retrograded starch (RS3); chemically modified fragments (RS4) (Englyst et al., 1992; Tovar, 1992). In most cases, processing raw food materials would destroy RS1 and RS2, but it can produce RS3. RS3 is widely welcomed, because it can retain its functional characteristics when RS is used as a nutritional ingredient in cooked foods (Rosin et al., 2002).

The processing techniques combined with retrogradation of starch would produce resistant starch. After gelatinization and retrogradation, RS3 is formed due to increased interaction between starch molecular chains. It has been reported that, the linear chains can help to increase RS content after starch debranching (Berry,

*Corresponding author. E-mail: qygao@scut.edu.cn. Tel: +86-20-87113845.

Abbreviations: RS, Resistant starch; **RS1,** physically inaccessible starch; **RS2,** resistant starch granules; **RS3,** retrograded starch; **RS4,** chemically modified fragments; **DMSO,** dimethyl-sulphoxide; DSC, differential scanning calorimetry.

1986). Enzymatic and physicochemical investigation has shown that, amylose crystallization during starch retrogradation is responsible for RS3 formation (Berry, 1986; Bjorck et al., 1987; Sievert et al., 1989; Leloup et al., 1992). Studies on preparation of RS were widely done in many countries and there is a rapid development and great achievements about it. RS has also been commercially produced and marketed (Kitty Kevin, 1995). RS3 content quite associated with autoclaving and debranching the process.

Corn starch is one of most abundant starch in China and other parts of the world in low prices. The objective of this study is to look for optimal conditions for RS formation from corn starch with experimental design. The properties of resistant starch including amylose content, crystalline structure and thermal properties were determined by the method of potentiometric titration, X-ray diffraction (XRD) techniques and differential scanning calorimetry (DSC), respectively. This study will provide an important guidance for producing RS in food manufacturing and a basis for further food application.

MATERIALS AND METHODS

Materials

Corn starch was obtained from Qinhuang Dao starch factory (Hebei Province, China). Thermostable α-amylase and pullulanase were kindly donated by Novozymes Company in Denmark. A total starch assay kit was purchased from Megazyme International Ireland Ltd., Co.Wicklow, Ireland, including thermostable α-amylase, amyloglucosidase, glucose determination reagent (GOPOD, made from glucose oxidase, peroxidase and 4-aminoantipyrine) and glucose standard solution.

Effect of α-amylase on RS formation

The experimental design was chosen to investigate the optimal conditions for RS formation from the autoclaved and debranched starch. At first, the effect of α-amylase on RS formation was investigated. Starch was mixed with water and starch concentration was adjusted to 6% (w/w), pH adjusted to 6.0- 6.4 and heated at 140°C for 20 min. During heating, different amount (0-5 U/g dry starch) of thermostable α-amylase was added. The gel was cooled to 45°C and pH was adjusted to 5.0, then pullulanase (1.6 U/g dry starch) was added to the gel and incubated at 45°C for 24 h. After hydrolysis and deactivation of enzyme, the starch paste was stored at 4°C for 24 h. The retrograded starch was dried at 45°C to approximately 10% moisture content and was ground. The appropriate amount of thermostable α-amylase for resistant starch formation was chosen for the following experiment.

Optimization of pullulanase debranching

An orthogonal $L_{16}(4)^4$ test design was used for optimization of the pullulanase debranched conditions. As seen in Table 1, the pullulanase debranched experiment was carried out with 4 factors and 4 levels. They were as follows: debranching temperature (35, 45, 55, 60°C), pH (4.5, 5.5, 6.5, 7.5), the amount of pullulanase (0.2, 0.4, 0.8, 1.6 U/g dry starch) and debranching time (12, 18, 24, 30 h). The range of each factor and level was based on the results of preliminary experiments. The optimization condition for RS formation was obtained from the orthogonal test design (Table 2).

RS determination

The method for determining RS contents was according to the analysis procedure provided by the Total Starch Assay Kit (AOAC Method 996.11). The sample (100 mg) was added to a glass test tube and 0.2 ml of aqueous ethanol (80% v/v) was added to wet the sample and aid dispersion. The tube was stirred on a vortex mixer. The sample was incubated in a boiling water bath for 6 min with 3 ml of thermostable α-amylase (stirred the tube vigorously after 2, 4 and 6 min). The tube was immediately added with 4 ml of 0.2 M sodium acetate buffer (pH 4.5), 0.1 ml of amyloglucosidase and was incubated in a bath at 50°C for 30 min. After incubation, an aliquot of this solution was centrifuged at 3000 rpm for 15 min. The residue obtained was washed with 50% ethanol twice and was centrifuged at 3000 rpm for 15 min. The sediment was solubillized in 2 ml of dimethyl-sulphoxide (DMSO) in boiling water bath for 5 min. The solution was quantitatively transferred to a 100 ml volumetric flask and was centrifuged at 3000 rpm for 15 min. 3 ml of GOPOD was added to aliquots (0.1 ml) of the supernatant and the mixture was incubated at 50°C for 20 min. Absorbance was measured using a spectrophotometer (Model 722, Shanghai Analytical Instrument Company, Shanghai, China) at 510 nm. The analyses were performed in triplicate.

Amylose content determination

The amylose content determination of native and resistant starch samples were according to the of iodimetric *method of* Schoch (1964).

X-ray diffraction (XRD)

Samples were analyzed using a D/Max-2200 X-ray diffractometer (Rigaku Denki Co., Tokyo, Japan). The samples were scanned with a CuKa target at 40 kV and 30 mA. X-ray diffraction patterns were recorded using a scintillation detector at a diffraction angle ranging from 6 to 60° (2θ) and scanning at the rate of 12°/min. The division of crystalline and sub-crystalline area was determined after Zhang et al. (2001). The degree of advanced and elementary crystallinity was quantified as the proportion of crystalline and sub-crystalline area to total area at angles between 6 and 60° Theta using the method of Nara and Komiya (1983).

Differential scanning calorimetry (DSC)

Differential scanning calorimetry experiments were run in a nitrogen atmosphere. Gelatinization temperatures were measured and recorded on a DuPont 1090 Differential Scanning Calorimetry (DSC) (DuPont Company, USA). Water (14 µl) was added with a microsyringe to starch (6 mg dry basis) in the DSC pans, which were then sealed, reweighed and kept at room temperature for 24 h to ensure equilibration of the starch samples and water. The samples were scanned from 30 to 180°C at 10°/min. An empty pan was used as a reference.

Statistical analysis

The test data were statistically analyzed using one-way analysis of variance (ANOVA) on the Statistical Package for the Social Sciences (SPSS) version 13.0 software for windows (USA). Least

Table 1. Factors and levels for orthogonal test.

Factor	Level			
	1	2	3	4
Temperature (℃)	35	45	55	60
pH	4.5	5.5	6.5	7.5
Amount of pullulanase (U/g)	0.2	0.4	0.8	1.6
Time(h)	12	18	24	30

Table 2. Analysis of $L_{16}(4)^4$ test results.

S/N	Temperature (℃)	pH	Amount of pullulanase (U/g)	Time (h)	RS (%)
1	35	4.5	0.2	12	7.78
2	35	5.5	0.4	18	9.25
3	35	6.5	0.8	24	4.54
4	35	7.5	1.6	30	14.02
5	45	4.5	0.4	24	17.84
6	45	5.5	0.2	30	7.56
7	45	6.5	1.6	12	15.06
8	45	7.5	0.8	18	5.02
9	55	4.5	0.8	30	14.23
10	55	5.5	1.6	24	15.72
11	55	6.5	0.2	18	4.78
12	55	7.5	0.4	12	5.56
13	60	4.5	1.6	18	17.54
14	60	5.5	0.8	12	19.02
15	60	6.5	0.4	30	16.99
16	60	7.5	0.2	24	5.27
K1	35.59	57.39	25.39	47.42	
K2	45.48	51.55	49.64	36.59	
K3	40.29	41.37	37.19	43.37	
K4	58.82	29.87	62.34	52.80	
R[a]	23.23	27.52	36.95	16.21	

[a] Refers to the result of extreme analysis.

significant difference (LSD) test was used to determine differences between means. P values < 0.05 were considered to be significant. Triplicate determinations were performed for each test and the results reported are mean values.

RESULTS AND DISCUSSION

Influence of thermostable α-amylase on RS formation

Native starch is a semi-crystalline entity; composed of loosely packed amorphous and densely packed crystalline regions. During starch gelatinization, most of the hydrogen bonds in crystalline regions of starch granule are broken, thus leading to the disentangling of double helices. Then the temperature and pH of starch paste were adjusted to appropriate value and the pullulanase was added to starch gel.

Compared with the control sample (0 U/g dry starch), it has been found that higher RS content can be achieved when thermostable α-amylase was added to starch paste during gelatinization (Figure 1). The impact of α-amylase on gelatinization starch and resistant starch formation is significant. The resistant starch of α-amylase hydrolysis was increased by 1.2 to 6.5%. The action of α-amylase on starch molecules was to cleave the α-1,4 glycosidic bonds of starch chains randomly, thus lowing the viscosity of starch paste rapidly. There is a good correlation between the viscosity of starch paste and the amount of α-amylase added. When little α-amylase was added to starch paste, its viscosity became higher and it

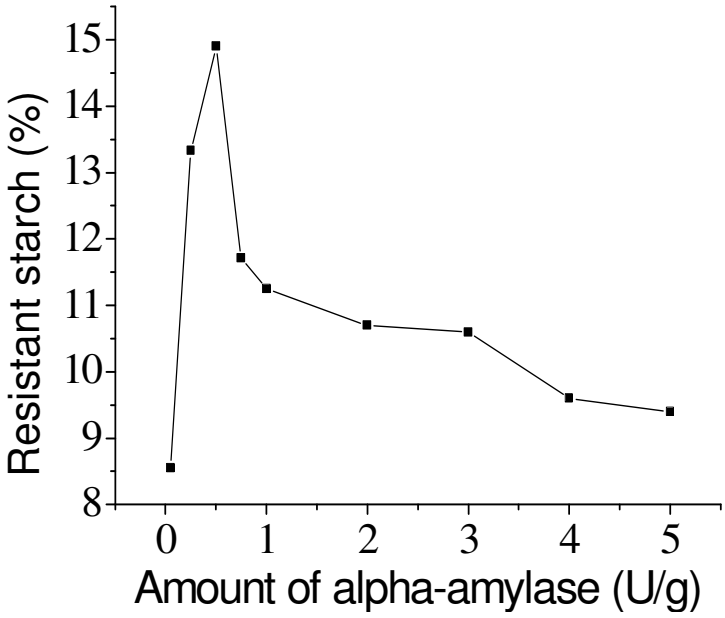

Figure 1. The effect of α-amylase on RS formation.

would be difficult for the linear chains to be close enough to form crystals; if too much α-amylase was added, the viscosity of starch paste would be lower and the paste was too dilute. The movement of linear chains was too fast. So the probability for the linear chains to be close to each other and stability became low. This is not good for RS formation. At the same time, the α-amylase will affect the length of the molecular chains and the formation of crystalline structure. It has been proved that RS could be formed easily when 0.5 U/g thermostable α-amylase was added and the highest RS content could be obtained (14.9%). Therefore, the addition of thermostable α-amylase before debranching with pullulanase is good for RS formation.

Optimization of pullulanase debranching parameters for resistant starch formation

The effect of pullulanase debranching parameters on resistant starch formation is presented in Table 2. Based on the results of orthogonal experiment, different condition has yielded different content of resistant starch in the range of 5.02 to 19.02%. The RS3 products with varied RS content could be attained by different conditions. The result indicated that, the amount of pullulanase was the most important factors for RS formation during debranching. The influence of amount of pullulanase and pH value on RS formation was more pronounced than those of reaction temperature and time among four factors. The significance of hydrolysis time on resistant starch formation is the least. Furthermore, the optimum pullulanase debranching conditions were found as follows: Temperature 60℃; pH 5.5; the amount of pullulanase 0.8PUN/g dry starch; hydrolysis time 12 h. Under the optimum conditions, the highest RS content was gained (19.02%) and it was far higher than that of native corn starch (0.67%).

Pullulanase catalyzes hydrolysis of the α-1,6 glycosidic bonds specifically. The hydrolysis of α-1,6 glycosidic bonds would produce more free linear chains in the hydrolysate. Upon pullulanase debranching and retrogradation, the linear chains similar to amylose, could participate in crystal formation by chain elongation and folding (Miles et al., 1985). These newly formed crystals were more perfect and firmer than the crystals of native starch granules and resist digestion in the small intestine. All these discussion was the foundation of RS preparation with enzymes.

Generally, the RS content of natural botanic starch increase with increasing amylose content, such as amylomaize starch (about 70 or 55% amylose), green bean starch (about 35% amylose). It is shown that, the amylose/amylopectin ratio in the native starch has some close relationship with RS content. In the same way, the crystals with different shape and property will reform by retrograded from the starch paste with the treatment of pullulanase. Thus, different pullulanase hydrolysis conditions exert different effect on amylose/amylopectin ratio, which will lead to the difference in RS content.

The process and mechanism of crystal reformation are very complicated and many factors are reported to affect the amylose retrogradation and therefore RS formation. Not only the amylose/amylopectin ratio, but also the molecular weight of amylose is a key factor affecting crystallization. During starch retrogradation, amylose

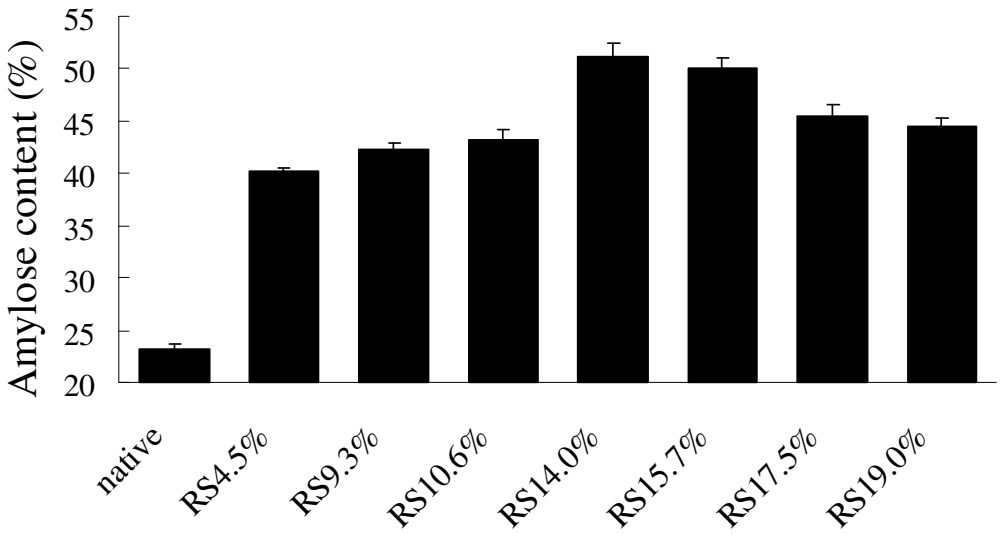

Figure 2. Amylose content of native and resistant starch.

crystallization occurs through chain elongation by double helical formation between amylose molecules near the terminal regions of the chains and chain folding; the elongated amylose chains fold and facilitate helix-helix aggregation/packing by formation of interhelical hydrogen bonds. The intimate packing of starch double helices results in crystal formation (Wu and Sarko, 1978). The crystal formation steps described earlier will happen constantly after the amylose chains in starch paste close to each other. However, the moving speed of each chain is different. The amylose molecules which have lower molecular weight move more quickly than those with greater molecular weight. Because the probability of collision is very small and it is difficult to stabilize, the chain with low molecular weight, amylose whose chain is too short for RS formation. So the content of RS depends on both the content and the molecular weight of the amylose. The hydrolysis conditions of pullulanase could be optimized with orthogonal experiment. The result indicates that, the amount of pullulanase was found to be the most important factor in all.

Amylose contents

Amylose content of native and selected resistant starch samples is shown in Figure 2. The amylose content of native starch was only small amount (23.2%). However, compared to native starch, the amylose content in RS3 was increased by 17.0 to 28.1%. The difference between amlyose content of native and resistant starch samples was significant. The result indicated that, the effect of pullulanase debranching on gelatinization starch was pronounced. It is known that, RS3 was associated with highly retrograded amylose chains. Pullulanase can hydrolyze α-D-glucosidic linkages of pullulan, a linear molecule, but also cleaves α-(1→6) branch linkages of amylopectin and glycogen (BeMiller and Whistler, 2009).

An increase in amylose content was observed from RS4.5 to RS14.0%, but a decrease was found from RS14.0 to RS19.0%. The RS contents did not always increase with the amylose content. More amylose chains are expected to facilitate recrystallization (Leong et al., 2007). It was suggested that, the crystal structure of the B-polymorph of amylose was based on double-stranded helices (Wu and Sarko, 1978). But during retrogradation, the moving speed of each chain with different molecular weight (MW) was different. The moving speed of high MW chains was less than that of low MW chains. Since the motion with too fast speed limits the chance of molecular collision, the amylose chain with too low MW will be unfavorable for starch retrogradation or crystal formation. Apart from the amylose content, the RS content is linked to polymerization degree of starch, chains length distribution and amylopectin. In general, the RS3 sample with high amylose content had high RS content, whereas the sample with low amylose content had low RS content accordingly. In other words, high amylose content is favorable for resistant starch formation.

X-ray diffraction pattern and crystallinity

The X-ray diffraction patterns of native and the selected RS3 samples are presented in Figure 3. Native starch showed A-type crystalline pattern with strong diffraction peaks at 15.7, 16.8, 18.4 and 23.9° 2θ (Figure 3, curve A). But there are some differences in diffraction pattern found on RS. RS3 had strong diffraction at the peak of 17.2° 2θ and had with two board peak at 20.1and 23.9° 2θ (Figure 3, curve B, C, D, E). After enzyme debranching

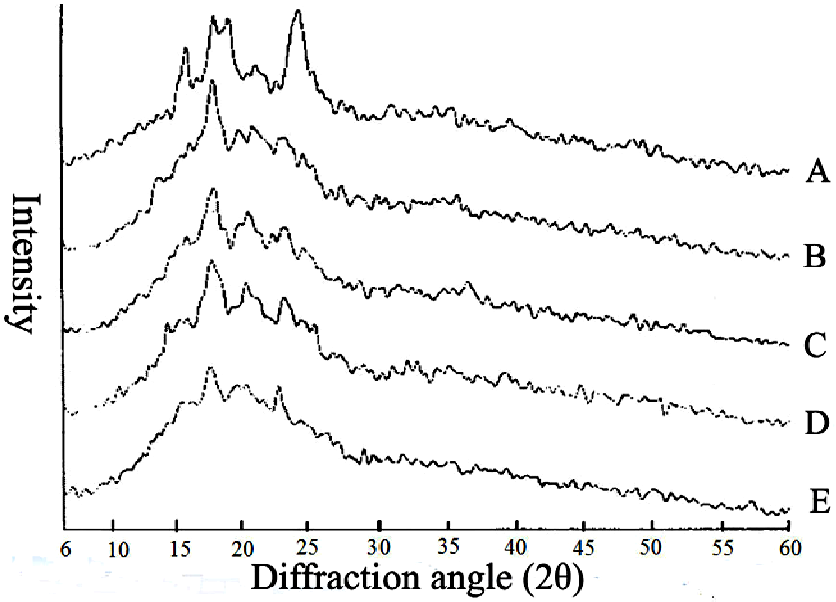

Figure 3. X-ray patterns of native and resistant starch samples. (A) Native starch; (B) RS 4.5%; (C) RS 19.0%; (D) RS 9.3%; (E) RS 10.6%.

Table 3. Degree of crystallinity of native and resistant starch.

Sample	Degree of crystallinity (%)		
	Advanced	Elementary	Total
Native starch	15.79	23.55	39.34
RS 4.5%	8.41	36.38	44.79
RS 9.3%	9.18	37.11	46.29
RS 10.6%	9.46	39.51	48.97
RS 19.0%	9.79	43.39	53.18

and retrogradation, RS3 showed B-type X-ray diffraction pattern. Gonza´lez-Soto et al. (2007) suggested that, the RS3 developed peaks, due to the process of starch chain re-ordering or retrogradation. Since the peaks observed were associated to the retrogradation behavior and therefore produces RS, the X-ray diffraction pattern in the RS sample showed the trend in that of the RS content. Pongjanta et al. (2009) and Leong et al. (2007) reported that, not only amylose chains were recrystallized, but also linear debranched amylopectin chains, which would help form RS crystalline structure in the period of heating and cooling.

The crystallinity of native starch was less than those of RS3 (Table 3). After the treatment of amylase, pullulanase and retrogradation, the crystallinity of RS3 was increased significantly. The extent of increase in crystallinity was 5.45 to 13.84%. The degree of crystallinity of RS 19.0% was the greatest. The greater its crystallinity, the higher the content of RS. With the increase in RS level, the crystallinity of RS3 was increased correspondingly. In addition, the content of advanced crystallite was keeping with this trend. The result showed that, the degree of general crystallinity and the relation between the content of different types of crystallites and the content of RS was consistent with this tendency. But the rules might not be applied to RS from different preparation methods. The division of advanced crystallite and elementary crystallite was only based on different granularity, irrespective of their compactness and firmness. The result showed that, new crystalline structure was formed, which will limit the enzyme from hydrolyzing the starch.

Thermal properties

Differential Scanning Calorimeter (DSC) was used to determine the gelatinization temperature and enthalpy of native and resistant starch (Table 4). It is suggested that, the endothermic transitions in the range of 42 and 72°C is probably due to melting of retrograded amylopectin, whereas other endothermic transitions appeared in approximately 120 to 170°C owing to melting of

Table 4. DSC characteristics of native and resistant starch samples.

Peak	Parameter	Native	RS 4.5%	RS 9.3%	RS 10.6%	RS 19.0%
Peak 1	T_0 (℃)	79.79	65.02	65.06	60.02	60.86
	Tp (℃)	91.79	81.03	81.03	77.40	88.79
	T_c (℃)	105.52	95.37	95.37	97.40	105.46
	T_c-T_0 (℃)	25.73	30.35	30.31	37.38	44.60
	ΔH (J/g)	4.47	1.96	2.02	0.85	2.37
Peak 2	T_0 (℃)	-	125.63	127.31	124.79	127.31
	Tp (℃)	-	143.32	156.93	161.02	164.35
	T_c (℃)	-	163.34	171.49	172.75	181.35
	T_c-T_0 (℃)	-	37.71	44.18	47.96	54.04
	ΔH (J/g)	-	3.83	6.20	6.70	8.90

[a,]The transition temperatures reported are the onset (T_0), peak (T_p) and conclusion (Tc) of the gelatinization endotherm; [b,] the enthalpy of gelatinization (ΔH) was estimated by integrating the area between the thermogram and a base line under the peak and was expressed in terms of joules per unit weight of dry starch (J/g); [c,] Indium was used for calibration.

retrograded amylose (Sievert and Pomeranz, 1990). Native starch had only one endothermic transition and the onset temperature for melting is about 79.79℃. However, the RS3 samples had two endothermic transitions. One small endothermic transition has been attributed to retrograded amylopectin. The onset temperature for melting retrograded amylopectin is in the range of 60.02 to 65.02℃ and the RS 10.6% was the lowest of selected samples.

The treated corn starch displayed another endothermic transition from 124.79 to 181.35℃. It is likely that this appearance of endothermic transitions is associated with melting of recrystallized amylose or retrograded amylose. The melting for retrograded amylose was at higher temperatures (124.79 to 127.31℃) and there was no significant difference in the amount of onset temperature between the samples. For the first small endothermic transition, the gap between T_c and T_0 increased with the enhancement of RS, but there is no distinct relationship between enthalpy and RS content. The difference in T_c-T_0 and enthalpy was varied widely from amylopectin structure of RS samples. For the second endothermic transition, an increase in Tp, T_c-T_0 and enthalpy of peak 2 was found with increasing RS contents. The sample containing 19.0% resistant starch provided the highest value (8.9 J/g).

According to Cooke and Gidley (1992), molecular (double-helical) and crystalline order structure are disrupted at the same time during gelatinization. This comparison of enthalpy values indicated that, the enthalpy of gelatinization mainly reflects the loss of molecular (double-helical) order. After the debranched treatment, amylose chain association increased with increasing RS content. The increased value of T_c and T_0 could be associated with structural differences among RS samples. The increased enthalpy of RS reflects more double-helical chains which are produced with the treatment of debranching and retrogradation. It is known that, enthalpy of reorganization structure tended to be lower than enthalpy of disordering. Consequently, the reorganization structure seemed to be ordered and compact and this reformed structure could explain as RS3 samples' resistance to the enzyme hydrolysis.

Conclusions

RS was prepared from corn starch by the treatment of autoclaving, enzyme hydrolysis and retrogradation. It is very important to control the amount of thermostable α-amylase and the hydrolysis conditions during the course of preparation. It has been demonstrated that the content of RS could be improved greatly with enzymes. The highest RS content could be obtained (19.02%) under optimal conditions. After debranching and retrogradation, the amylose content obtained was higher than that of native starch. The RS content is not increased with increasing amylose content and higher amylose content is favorable for RS formation. Compared to native starch, the diffraction peak at 17.2 °2θ was sharper with increased cystallinity. A new endothermic transition appeared with increased gelatinization temperature and enthalpy on resistant starch. This is associated with reorganization of the starch chain and newly formed structures by retrogradation.

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