Optimization of Debranched Waxy Rice Starch Preparation Using a Complete Central Composite Design

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ABSTRACT

An optimum condition for complete debranching of waxy rice starch (WRS) at pH 5 and 55°C was studied, using a complete central composite design (CCCD) of 3 factors, i.e., a starch concentration, a pullulanase concentration (Promozyme® 400L, Novozymes, Denmark) and an incubation time at 2 levels. The response of the study was a degree of hydrolysis. Statistical analyses indicated that only Promozyme® concentration and the incubation time influenced the degree of hydrolysis whereas the starch concentration did not exhibit a significant effect. A significant interaction between factors was not observed. The optimum condition for complete debranching of WRS was found to occur at 45 Pullulanase Unit Novo/g of starch for the Promozyme® concentration and 19 h for the incubation time. The optimum condition obtained from the CCCD study was applied to prepare debranched waxy rice starch (DBS) in a larger scale (800 g of WRS). The degree of hydrolysis of the DBS solution obtained from the larger scale was not different from that obtained from the small scale, indicating that the optimum condition from CCCD study could be readily applied for preparing DBS in the larger scale. After the DBS solution had been incubated at 4°C for 24 h, the DBS precipitant was separated by centrifugation and then dried. The yield of DBS precipitant obtained was about 78%. The loss of some DBS was suggested to occur during the precipitation of DBS due to some short-chain DBS which remained soluble in the solution. The debranched amylopectin in DBS precipitant consisted of 76% short and medium chains with degree of polymerization (DP) less than 34 and of 24% long chains with DP equal to or greater than 34.

Key words: Optimization, Waxy rice starch, Pullulanase, Complete central composite design, Debranched starch

INTRODUCTION

Starch consists of two types of polysaccharides, amylose and amylopectin. Amylose is an essentially linear polymer, consisting of glucose units linked together with alpha 1,4 glucosidic linkages. Amylopectin is a branched polymer, consisting of thousands of short linear-chain polyglucans, linked to each other by alpha 1,6 glucosidic bonds (Manners, 1989; Conde-Petit, 2001). The ratios between amylose and amylopectin in starch vary according to botanical sources. Some mutant genotypes of maize (*Zea mays*) and barley (*Hordeum vulgare*) contain as much as 70% amylose whereas other genotypes, called waxy, such as waxy rice, contain almost exclusively amylopectin (Bulé on et al., 1998).

The glycosidic linkages in starch molecules can be hydrolyzed by acids or enzymes. Enzymes that specifically catalyze the hydrolysis of α-D-1,6-glucosidic bonds or branched linkages of starch are known as debranching enzymes; the examples are isoamylase and pullulanase. When waxy starch is hydrolyzed by such enzymes, a lot of short linear-chain polyglucans, called debranched waxy starch, are produced (Allen and Dawson, 1975). Debranched waxy starches, particularly debranched waxy maize starch, have been found to be useful for various pharmaceutical applications. It showed a good performance as an excipient for programmed-release systems, both *in vitro* (Te Wierik et al., 1993b) and *in vivo* (Van der Veen et al., 1994). Debranched waxy maize starch is able to form inclusion complex with some drugs, such as prednisolone, and showed increased drug release from suppositories, compared with corresponding suppositories containing drug only (Te Wierik et al., 1994). Debranched waxy maize starch was also investigated for its ability to increase the dissolution of the drug. Drug, such as diazepam, released from capsules was slightly increased upon the application of debranched waxy maize starch. However, the limited increase in drug dissolution is caused by the limited solubility of debranched waxy maize starch. It is also found that the application of soluble fraction of debranched waxy maize starch, which has low degree of polymerization (DP), showed faster drug release (Te Wierik et al., 1993a). Alternatively, the use of debranched waxy rice starch, which has larger proportions of low DP than debranched waxy maize starch (Jane et al., 1999), would therefore display more advantages for this pharmaceutical application than debranched waxy maize starch.

At present, the information on debranching of waxy rice starch is limited and has not been investigated systematically. In this study, debranched waxy rice starch (DBS) was prepared by using pullulanase (Promozyme®, Novozyme, Denmark). The ability of Promozyme® to debranch starch depends on various environmental factors. Some factors such as pH and incubation temperature were well studied (Jensen and Norman, 1984). However, other factors, *i.e.*, starch concentration, Promozyme® concentration and incubation time vary according to the type of starch used and might need a specific study for a selected starch. In this study, the optimum levels of those factors for debranching waxy rice starch completely was investigated by a statistical design.

Among the statistical designs that have been used to search for optimum conditions in a multivariable system, a complete central composite design (CCCD) has been one of the most frequently-used methods. This statistical design provides more advantages than the conventional one. It requires less number of experiments and thereby, resulting in saving of

time, cost and manpower. Moreover, it helps in understanding the interactions among factors (Lewis et al., 1999; Montgomery, 2001). The CCCD was used to seek an optimum condition for preparing DBS in the present study. The optimum condition obtained from CCCD was tested for the preparation of DBS in the larger scale as well.

MATERIALS AND METHODS

Materials

Waxy rice starch (WRS) (10.01% moisture content, a gift from Cho-Heng Company, Thailand) contains starch 95.78 ± 2.94% (dry basis), crude protein 0.09 ± 0.02%, fat 0.12 ± 0.01%, crude fiber 0.32 ± 0.12% and ash 0.83 ± 0.06%. The fat content was reduced to 0.01 ± 0.005% prior to debranching by soaking WRS in 95% ethanol (1:3, w/w) for 24 h with continuous stirring. The apparent amylose content was 3.5%, following Knutson's method (Knutson, 1986). Pullulanase (Promozyme® 400 L, Novozyme, Denmark) was used without further purification for the preparation of DBS. The pullulanase activity of Promozyme® 400L was analyzed according to the analytical method provided by the company (Novo Industri A/S, 1983) and was found to be 299 Pullulanase Unit Novo (PUN)/ml. β-Amylase, Type 1-B: from sweet potato was purchased from Sigma Chemical Company, USA). Standard dextrans of different molecular weights (180, 3,260, 8,100, 18,300, 35,600, 55,500, 100,300, 164,200, 236,300, 332,800 and 500,500) for high-performance size-exclusion chromatography (HPSEC) were purchased from Fluka Chemie AG, Switzerland. All other reagents used in the experiments were of analytical grade.

Methods

1. The hydrolysis pattern of Promozyme® for waxy rice starch

WRS slurry (WRS (2.5 g) in 0.05 M acetate buffer solution pH 5 (47.5 g)) was gelatinized in a boiling water bath with continuous shaking for 30 min. The starch paste was allowed to cool down to 55°C before Promozyme® (50 PUN/g of starch) was added. The mixture was incubated in a water bath shaker (Polyscience Model 28I-M, USA) at 55°C and 120 rpm for 5 min, 10 min, 30 min, 1h, 2h, 4h, 8h, 18h and 24 h. After the determined incubation time, the enzymatic reaction was stopped by boiling the hydrolysate product for 20 min. The hydrolysis pattern of Promozyme® for WRS was investigated by high-performance size-exclusion chromatography (HPSEC).

2. A complete central composite design for debranching of waxy rice starch

A complete central composite design (CCCD) of 3 factors and 2 levels was used to investigate the optimum condition for debranching of WRS. The independent variables were a starch concentration (%, w/w), a Promozyme® concentration (PUN/g of starch, dry basis) and an incubation time (h). The low levels (coded value = -1) and the high levels (coded value = +1) were assigned at 5 and 10% (w/w) for WRS concentration (X1), 15 and 50 PUN/g of starch for Promozyme® concentration (X_2) and 8 and 24 h for the incubation time (X_3). The dependent variable (response) of this experiment was a degree of hydrolysis (D.H.).

The CCCD contained a total of 23 experiments (Table 1). The first 8 experiments (numbers 1-8) organized in a complete factorial design; the experiments numbers 9-14

conformed in a star design; and the experiments numbers 15-23 involved the replications of the central points. The real values for independent variables in each experiment were calculated from the coded values, using the following equation:

$$\text{Real value} = (C \times S) + M$$

where: C was the coded value of a given variable; S is half of the difference between high and low levels (real values) for that variable; and M is the average of high and low levels (real values at coded values = 0). Experiments were conducted in a randomized fashion. WRS slurry (50 g) was prepared by dispersing WRS in an acetate buffer solution (0.1 M, pH 5), containing sodium azide (0.1%, w/v) as a preservative, to a given concentration as shown in Table 1. The WRS slurry was gelatinized in a boiling water bath with continuous shaking for 30 min. The WRS paste was cooled down to 55°C before Promozyme® was added. The starch/enzyme mixture was incubated in a water-bath shaker (Polyscience Model 28I-M, USA) at 55°C, 120 rpm for a given period of time. The enzymatic reaction was then terminated by boiling the hydrolysate product in a boiling water bath for 20 min. The DBS obtained from all CCCD experiments were analyzed for the D.H.. The second– order (quadratic) model as shown below was applied to evaluate the relationship between the independent variables and dependent variables.

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{22}X_3^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_1X_3+e$$

where: Y = D.H.; X_1 = WRS concentration (%, w/w); X_2 = Promozyme® concentration (PUN/g of starch); X_3 = incubation time (h); β = regression coefficient; e = random error.

The model coefficients were estimated with a multiple regression analysis, using computer software, 'Statgraphics Plus 3.0'. Three-dimensional response surface plots and contour plots were created, based on the model equation, using the same computer program in order to find the optimum condition for debranching of WRS.

Table 1. Experiments according to the complete central composite design for debranching of waxy rice starch.

No.	Coded values (real values)					
	X_1		X_2		X_3	
1	-1	(5.0)	-1	(15.0)	-1	(8.0)
2	+1	(10.0)	-1	(15.0)	-1	(8.0)
3	-1	(5.0)	+1	(50.0)	-1	(8.0)
4	+1	(10.0)	+1	(50.0)	-1	(8.0)
5	-1	(5.0)	-1	(15.0)	+1	(24.0)
6	+1	(10.0)	-1	(15.0)	+1	(24.0)
7	-1	(5.0)	+1	(50.0)	+1	(24.0)
8	+1	(10.0)	+1	(50.0)	+1	(24.0)
9	-1.68	(3.3)	0	(32.5)	0	(16.0)
10	+1.68	(11.7)	0	(32.5)	0	(16.0)
11	0	(7.5)	-1.68	(3.1)	0	(16.0)
12	0	(7.5)	+1.68	(61.9)	0	(16.0)
13	0	(7.5)	0	(32.5)	-1.68	(2.6)
14	0	(7.5)	0	(32.5)	+1.68	(29.5)
15	0	(7.5)	0	(32.5)	0	(16.0)
16	0	(7.5)	0	(32.5)	0	(16.0)
17	0	(7.5)	0	(32.5)	0	(16.0)
18	0	(7.5)	0	(32.5)	0	(16.0)
19	0	(7.5)	0	(32.5)	0	(16.0)
20	0	(7.5)	0	(32.5)	0	(16.0)
21	0	(7.5)	0	(32.5)	0	(16.0)
22	0	(7.5)	0	(32.5)	0	(16.0)
23	0	(7.5)	0	(32.5)	0	(16.0)

Where: X_1 = WRS concentration (%, w/w)
X_2 = Promozyme® concentration (PUN/g of starch)
X_3 = Incubation time (h)
All experiments were performed at pH 5, 55°C.

3. Evaluation of debranched waxy rice starch

(a) Degree of hydrolysis and number-average degrees of polymerization

The hydrolysis extent of WRS by Promozyme® was evaluated in terms of a D.H., which was calculated from the following equation:

$$\text{D.H.}(\%) = \left(\frac{\text{RS of WRS after hydrolysis} - \text{RS of Promozyme® blank}}{\text{TS of WRS after hydrolysis} - \text{TS of Promozyme® blank}} - \frac{\text{RS of WRS be fore hydrolysis}}{\text{TS of WRS before hydrolysis}} \right) \text{X } 100$$

The reducing sugar concentration (RS) and the total sugar concentration (TS) were analyzed according to the methods of Somogyi (1952) and Dubois et al., (1956), respectively. The number-average degrees of polymerization ($\overline{DP}_n$) of DBS were calculated by the following equation:

$$\overline{DP}_n = \frac{TS}{RS}$$

(b) % β-amylolysis limit

The % β-amylolysis limit of DBS was evaluated in order to investigate if WRS was completely debranched. The analysis procedure was slightly modified from that of Hood and Mercier (1978). WRS or DBS solution (1.5 ml; 0.2 %, w/v) was mixed with an acetate buffer solution pH 4.8 (0.3 ml; 0.2 M). β-amylase solution (0.2 ml; 20 units/ml) and deionized water (1.0 ml) were added and mixed, and the solution was incubated at 37°C for 48 h. The RS and TS of the β-amylolysis product were measured. The % β-amylolysis limit was calculated, using following equation:

$$\%\ \beta\text{-amylolysis limit} = \left(\frac{\text{RS of sample after hydrolyzed by } \beta\text{-amylase} - \text{RS of } \beta\text{-amylase blank}}{\text{TS of sample after hydrolyzed by } \beta\text{-amylase} - \text{TS of } \beta\text{-amylase blank}} \right) \text{ X 100 X 1.9}$$

The β-amylolysis product of the sample which was suggested to be completely debranched was submitted to HPSEC and high-performance anion-exchange chromatography (HPAEC) to study the molecular size distribution, using the same analysis conditions as for WRS and DBS.

(c) High-performance size-exclusion chromatography

HPSEC was used to observe the change in the molecular size of WRS after it was hydrolyzed by Promozyme®. WRS or DBS was dissolved in hot deionized water (0.5 %, w/v) by heating in a boiling water bath and filtered through an 8-μm membrane filter (Schleicher & Schuell, Germany) before injecting into a high-performance size-exclusion chromatograph, equipped with a refractive index detector (Shimadzu, Japan). The columns used were serially connected to one ultrahydrogel linear and two ultrahydrogels 120 serially connected in the given order. The column temperature was controlled at 40°C. Deionized water was used as an eluent at a flow rate of 0.8 ml/min. Dextrans of different molecular weights were used as references to calibrate the molecular weights.

(d) High-performance anion-exchange chromatography

A high-performance anion-exchange chromatograph with pulse amperometric detector (HPAEC-PAD; Dionex Bio-LC, USA) was used to study the chain length distribution of DBS. The condition used was modified from that of Blennow et al. (2000). DBS was dis-

solved in deionized water (1 mg/ml) by heating in a boiling water bath and filtered through a membrane filter (0.22 μm; Millipore, USA) before injection. The column used was Carbopac PA 100 (0.25 x 30 cm). The mobile phase was isocratic 150 mM sodium hydroxide from 0 to 130 min with a gradient profile of sodium acetate: 0-5 min (convex gradient) from 0 to 35 mM; 5-130 min (convex gradient) from 35 to 350 mM. The flow rate of the eluent was 0.25 ml/min.

RESULTS AND DISCUSSION

The hydrolysis pattern of Promozyme® for waxy rice starch

The HPSEC profiles of WRS and DBS, obtained by incubating gelatinized WRS (5%, w/w) with Promozyme® (50 PUN/ g of starch) at pH 5 and 55°C for various hydrolysis times, are shown in Figure 1. The peaks of WRS lay between the retention time of about 13 and 25 min, indicating that starch molecules were large and greatly varied in size. After Promozyme® had been added, WRS molecules were hydrolyzed into smaller molecules as evident by the decrease in WRS peak and the existence of peaks at longer retention time, 22.0 and 28.5 min. The peak at the retention time of 22.0 min (corresponding to DP~ 1220) was attributed to the fraction of starch hydrolysate product of large molecular size. The size of this peak decreased as the incubation time increased, indicating that the molecules in this fraction contained other branched chains, so they were subsequently hydrolyzed by Promozyme® into smaller molecules. The peak at the retention time of about 28.5 min (DP~ 11) became larger when the incubation time increased. The molecules in this fraction were not further hydrolyzed by Promozyme® and might be considered to be the end product of the hydrolysis. After 18 h, the peaks corresponding to WRS almost disappeared and the HPSEC profiles did not show a significant change, suggesting that the equilibrium had been reached. The HPSEC profile of DBS at 24 h showed two peaks at the retention time of 23.4 (DP~ 300) and 28.5 min (DP~ 11) which included a shoulder at 26.6 min (DP~ 22). The area of the latter peak (DP~ 11) accounted for 96.2% of the total area of the hydrolysate product, while that of the former was only 3.8%. This result indicated that DBS obtained at 24 h contained primarily short-chain polyglucans. The $\overline{DP}_n$ of the DBS obtained at 24 h was 18.9±0.8. The D.H. value of DBS obtained at 24 h was 5.02%. These values corresponded considerably well with the amount of the branched linkages in amylopectin molecules which were reported to consist of approximately 4-5% of the total glucosidic linkages (Manners, 1989; Conde-Petit, 2001). The % β-amylolysis limits of DBS was 97.6±1.9 %, indicating that WRS might be completely debranched by Promozyme®.

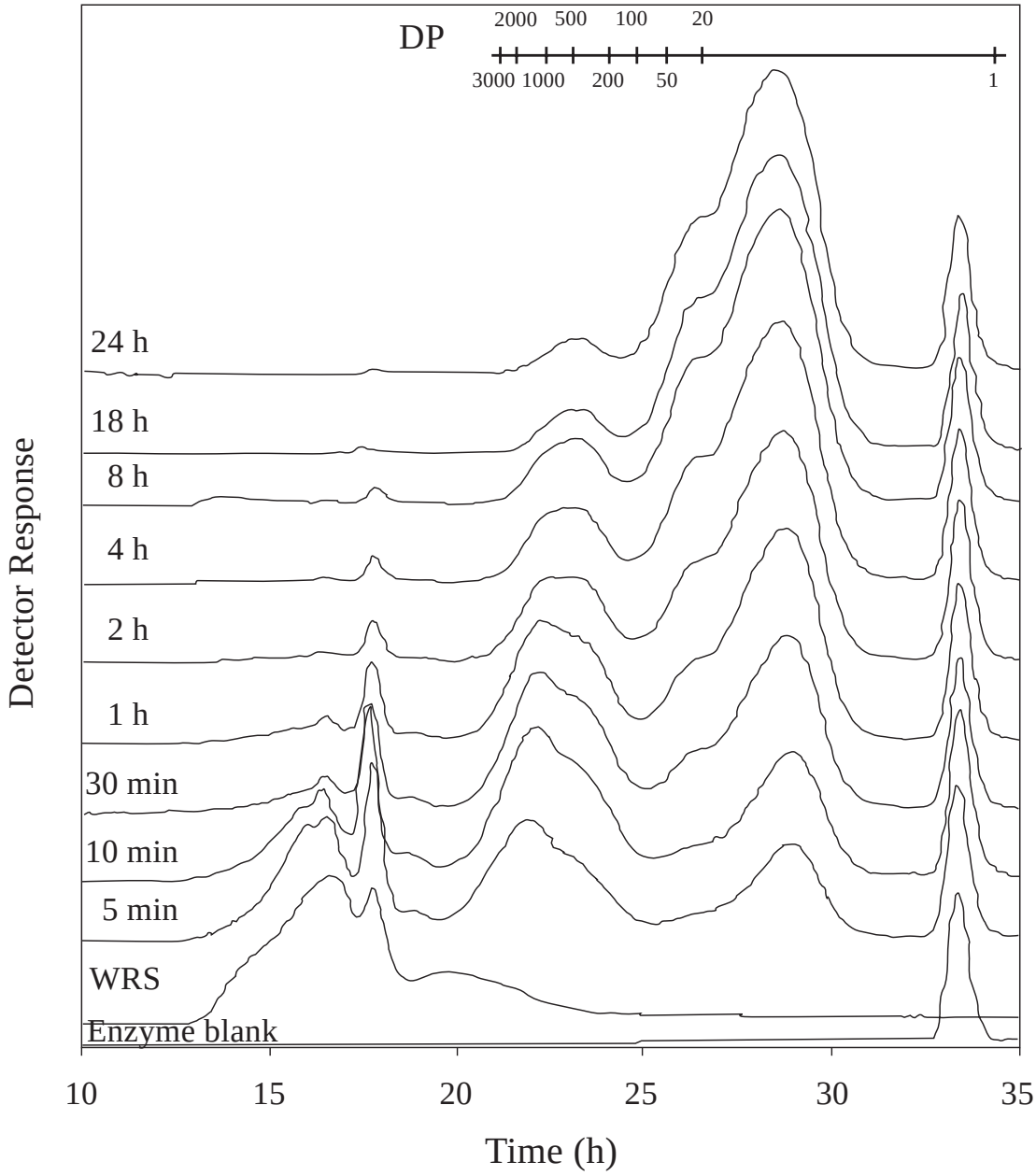

Figure 1. High-performance size-exclusion chromatograms of waxy rice starch and debranched waxy rice starch obtained by debranching gelatinized waxy rice starch (5%, w/w) with Promozyme® 400 L (50 PUN/g of starch) in an acetate buffer solution pH 5 (0.05 M) at 55°C at different incubation times; the scaled bar at the top of the figure provides the approximate DP of DBS, calculation based on standard dextrans of various molecular weights.

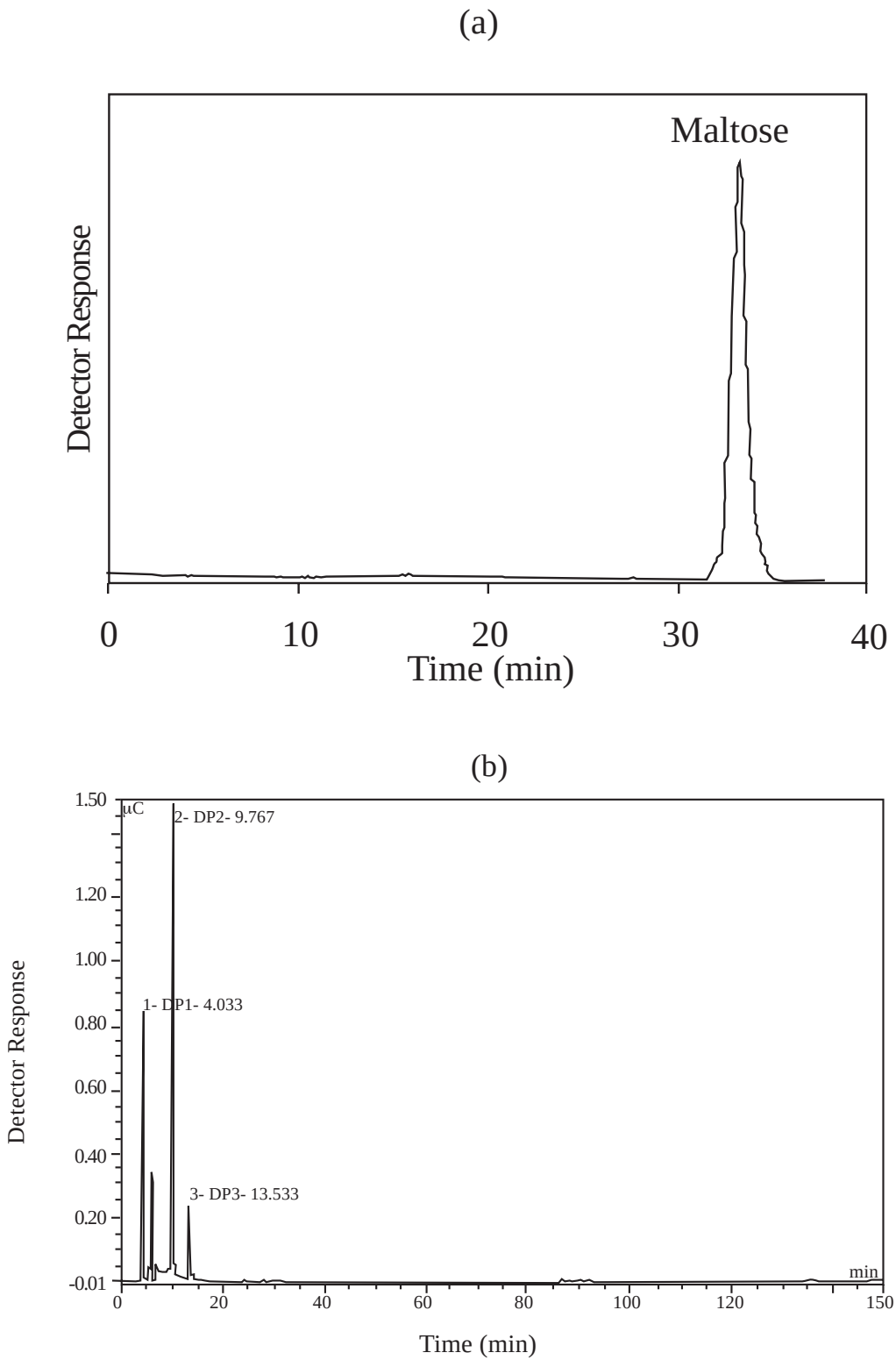

Figure 2. The molecular size distribution of β-amylolysis product of debranched waxy rice starch, prepared by debranching waxy rice starch (5%, w/w) with Promozyme® (50 PUN/g of starch) at pH 5, 55°C for 24 h, as investigated by (a) high-performance size-exclusion chromatography and (b) high-performance anion-exchange chromatography; where DP1=Glucose; DP2=Maltose; and DP3=Maltotriose.

The β-amylolysis product of DBS obtained at 24 h was analyzed, using HPSEC and HPAEC-PAD systems. The HPSEC chromatogram (Figure 2(a)) showed that there was no other peak but a single peak at the retention time of 33.1 min, which corresponded to the retention time of maltose. The HPAEC chromatogram (Figure 2(b)) showed three peaks corresponding to glucose, maltose and maltotriose. Both HPSEC and HPAEC results suggested that β-amylase was able to completely hydrolyze DBS that was obtained at 24 h. It also indicated that WRS (5%, w/w) was completely debranched by Promozyme® (50 PUN/g of starch) at pH 5, 55°C within 24 h. The presence of glucose and maltotriose in HPAEC chromatogram was attributed to the β-amylolysis of DBS chains of odd-number DP. Maltotriose was produced after the last β-maltose had been removed from the main chain. However, β-amylase was able to hydrolyze maltotriose to 1 glucose and 1 maltose but with difficulty (Hizukuri, 1996). As a result, both glucose and maltotriose were present as degradation products.

An optimum condition for preparing debranched waxy rice starch

A CCCD of 3 factors and 2 levels were applied for seeking the optimum condition for preparing DBS. After debranching WRS with Promozyme® under various CCCD conditions, the experimental data were analyzed with a multiple regression analysis (least square estimation) to study the relationship between response (D.H.) and independent variables (WRS concentration, Promozyme® concentration and incubation time). The analysis of variance of the regression is shown in Table 2. Both the linear terms and the quadratic terms (polynomial terms) were evidently significant at confidence levels greater than 99%. Lack of fit was not statistically significant. Therefore, the second order model was sufficient to explain the relationship between the independent variables and dependent variable within the experiment region. The regression coefficients of the regression model as well as their t-values are presented in Table 3. Promozyme® concentration (X2) and incubation time (X3) were the significant factors on the D.H. whereas starch concentration (X1) did not exhibit a significant effect. These results indicated that the concentration of starch did not influence the action of Promozyme® to debranch WRS. A significant interaction between independent variables was not observed. However, the elimination of the non-significant terms to simplify the model is rarely necessary in response surface modeling where the fit of the total model is more important than the significance of the individual coefficient (Lewis et al., 1999). As a consequence, all parameters were included in the model equation which can be expressed as follow:

$$\text{D.H.} = 0.99 - 0.003X1 + 0.11X_2 + 0.11X_3 - 0.00014X_1^2 - 0.00098X_2^2 - 0.0033X_3^2 - 0.00054X_1X_2 + 0.0033X_1X_3 - 0.00015\,X_2X_3$$

(adjusted $R^2 = 0.8899$)

Table 2. Analysis of variance of the regression: complete central composite design.

Source	Sum of Square	Df	Mean Square	F-ratio	F-sig (p=0.01)
Total	9.3001	22			
Model	8.6951	9	0.9661	20.75	4.19
Linear	6.5164	3	2.1721	46.67	5.74
Quadratic	2.1786	6	0.3631	7.80	4.62
Residual	0.6051	13	0.0465		
Lack of fit	0.2820	5	0.0564	1.40	6.63
Pure error	0.3230	8	0.0403		

Table 3. Significance of regression coefficients for degree of hydrolysis.

Variable	Regression coefficient	*t*-value	*p*-value
Constant	0.99	1.11	0.2859
X_1	-0.003	-0.019	0.9851
X_2	0.11	5.45	0.0001*
X_3	0.11	2.56	0.0235***
X_1^2	-0.00014	-0.016	0.9871
X_2^2	-0.00098	-5.54	0.0001*
X_3^2	-0.0033	-3.94	0.0017**
X_1X_2	-0.00054	-0.31	0.7605
X_1X_3	0.0033	0.87	0.4009
X_2X_3	-0.00015	-0.28	0.7805

Significance of regression coefficients at confidence levels greater than *99.9%, **99% and ***95% by *t*-test

X_1 = WRS concentration (%, w/w)

X_2 = PromozymeR concentration (PUN/g of starch)

X_3 = Incubation time (h)

The regression equation was used to calculate the predicted D.H. values for all CCCD experiments and the results are shown in Table 4. It can be seen that the predicted D.H. values were close to the experimental D.H. values. The % differences between the experimental D.H. values and predicted D.H. values were mostly lower than 5%, which indicated that the regression equation could predict the D.H. obtained from the experiments with a high accuracy.

Table 4. The experimental degree of hydrolysis and the predicted degree of hydrolysis of debranched waxy rice starch obtained under various central composite design conditions and their differences.

No.	X_1	X_2	X_3	Experimental D.H. Value	Predicted D.H. Value	Residual	% difference
1	5.0	15.0	8.0	3.24	3.13	0.11	3.60
2	10.0	15.0	8.0	3.43	3.19	0.24	7.40
3	5.0	50.0	8.0	4.52	4.53	-0.01	-0.17
4	10.0	50.0	8.0	4.48	4.50	-0.02	-0.42
5	5.0	15.0	24.0	3.60	3.45	0.15	4.39
6	10.0	15.0	24.0	3.92	3.78	0.14	3.71
7	5.0	50.0	24.0	4.66	4.76	-0.10	-2.18
8	10.0	50.0	24.0	5.02	5.00	0.02	0.40
9	3.3	32.5	16.0	4.40	4.43	-0.03	-0.61
10	11.7	32.5	16.0	4.52	4.68	-0.16	-3.44
11	7.5	3.1	16.0	2.29	2.61	-0.32	-12.16
12	7.5	61.9	16.0	4.94	4.81	0.13	2.70
13	7.5	32.5	2.6	3.48	3.61	-0.13	-3.55
14	7.5	32.5	29.5	4.24	4.30	-0.06	-1.38
15	7.5	32.5	16.0	4.38	4.56	-0.18	-3.87
16	7.5	32.5	16.0	4.78	4.56	0.22	4.91
17	7.5	32.5	16.0	4.26	4.56	-0.30	-6.50
18	7.5	32.5	16.0	4.82	4.56	0.26	5.79
19	7.5	32.5	16.0	4.43	4.56	-0.13	-2.77
20	7.5	32.5	16.0	4.42	4.56	-0.14	-2.99
21	7.5	32.5	16.0	4.76	4.56	0.20	4.47
22	7.5	32.5	16.0	4.57	4.56	0.01	0.30
23	7.5	32.5	16.0	4.62	4.56	0.06	1.40

All CCCD experiments were carried out at pH 5, 55°C.
X_1 = WRS concentration (%, w/w)
X_2 = Promozyme® concentration (PUN/ g of starch)
X_3 = Incubation time (h)
Experimental D.H. value = D.H. obtained from the experiments
Predicted D.H. value = D.H. calculated from the regression equation
Residual = Experimental D.H. value ñ predicted D.H. value
% Difference = Residual x 100 /predicted D.H. value

Additional experiments under conditions different from those used to construct the model equation were carried out in order to evaluate the model validity (Table 5). Due to the fact that a starch concentration was not a significant factor that contributed to D.H., it was kept constant at 7.5% (w/w) (Coded 0) in all additional experiments. The first four experiments in Table 5 were performed at conditions in which all values of Promozyme® concentration and incubation time ranged within the domain of the study, while other four experiments were carried out at conditions outside the domain ranges. It can be seen that the predicted values of D.H. were close to the values obtained from the real experiments. The experimental values were between the lower and upper limits of 95% confidence interval for the predicted D.H.. Therefore, the obtained model equation can be used to predict the condition for preparing DBS with a desired D.H. within the range of experimental domains. Moreover, it can be observed that the D.H. values predicted from the model equation at conditions outside the domain range were close to the experimental values. As a result, this model equation can be applied to predict the D.H. of DBS prepared with Promozyme® concentration and incubation time outside the domain (coded values between -1.68 to 1.68) with high accuracy.

Table 5. Validity test: the experimental degree of hydrolysis and the predicted degree of hydrolysis of debranched waxy rice starch obtained at conditions different from those used for model construction.

No.	Coded (real) values						Experimental D.H. values	Predicted D.H. values	95% Confidence level	% difference
	X_1		X_2		X_3					
1	0	(7.5)	-0.5	(23.75)	-0.5	(12.00)	4.00	3.99	3.50-4.49	0.25
2	0	(7.5)	-0.5	(23.75)	+0.5	(20.00)	4.12	4.20	3.71-4.70	-1.90
3	0	(7.5)	+0.5	(41.25)	-0.5	(12.00)	4.33	4.66	4.16-5.15	-7.08
4	0	(7.5)	+0.5	(41.25)	+0.5	(20.00)	4.46	4.85	4.35-5.35	-8.04
5	0	(7.5)	-1.68	(3.07)	-1.68	(2.55)	1.56	1.60	0.78-2.42	-2.50
6	0	(7.5)	-1.68	(3.07)	+1.68	(29.45)	2.47	2.41	1.59-3.23	2.49
7	0	(7.5)	+1.68	(61.93)	-1.68	(2.55)	3.75	3.92	3.10-4.74	-4.34
8	0	(7.5)	+1.68	(61.93)	+1.68	(29.55)	5.09	4.49	3.67-5.32	13.36

A three-dimensional response surface plot for D.H. generated by fitting the equation model is presented in Figure 3(a). It can be observed that the D.H. increased upon increasing the concentration of Promozyme® from 15 to about 45 PUN/g of starch. At any incubation time, a relationship between Promozyme® concentration of and D.H. was not linear; the more Promozyme® concentration applied, the less increase of D.H. was obtained. Any further increase of Promozyme® concentration beyond 45 PUN/g of starch did not show a significant increase in the D.H. Similarly, the D.H. increased when the incubation time was increased. The hydrolysis of WRS progressed at a slower rate as the incubation time was increased. The D.H. was almost identical after 19-20 h of incubation for Promozyme® concentrations at 45-50 PUN/g of starch. A contour plot for D.H. as a function of a Promozyme® concentration and an incubation time is illustrated in Figure 3(b). The contour lines showed the isoresponse for D.H. that would enable us to select various combinations of Promozyme®

concentration and an incubation time to produce DBS of a desired D.H. It can be seen that D.H. was at the maximum (> 4.9) when the Promozyme® concentration was greater than approximately 45 PUN/g of starch and the incubation time was not less than 19 h. As a result, the optimum condition to produce DBS was the WRS concentration at 10% (w/w), the Promozyme® concentration at 45 PUN/ g starch and the incubation time at 19 h. From these results, DBS in the larger batches was prepared at the mentioned condition in order to evaluate the reliability of the model when the production scale was increased.

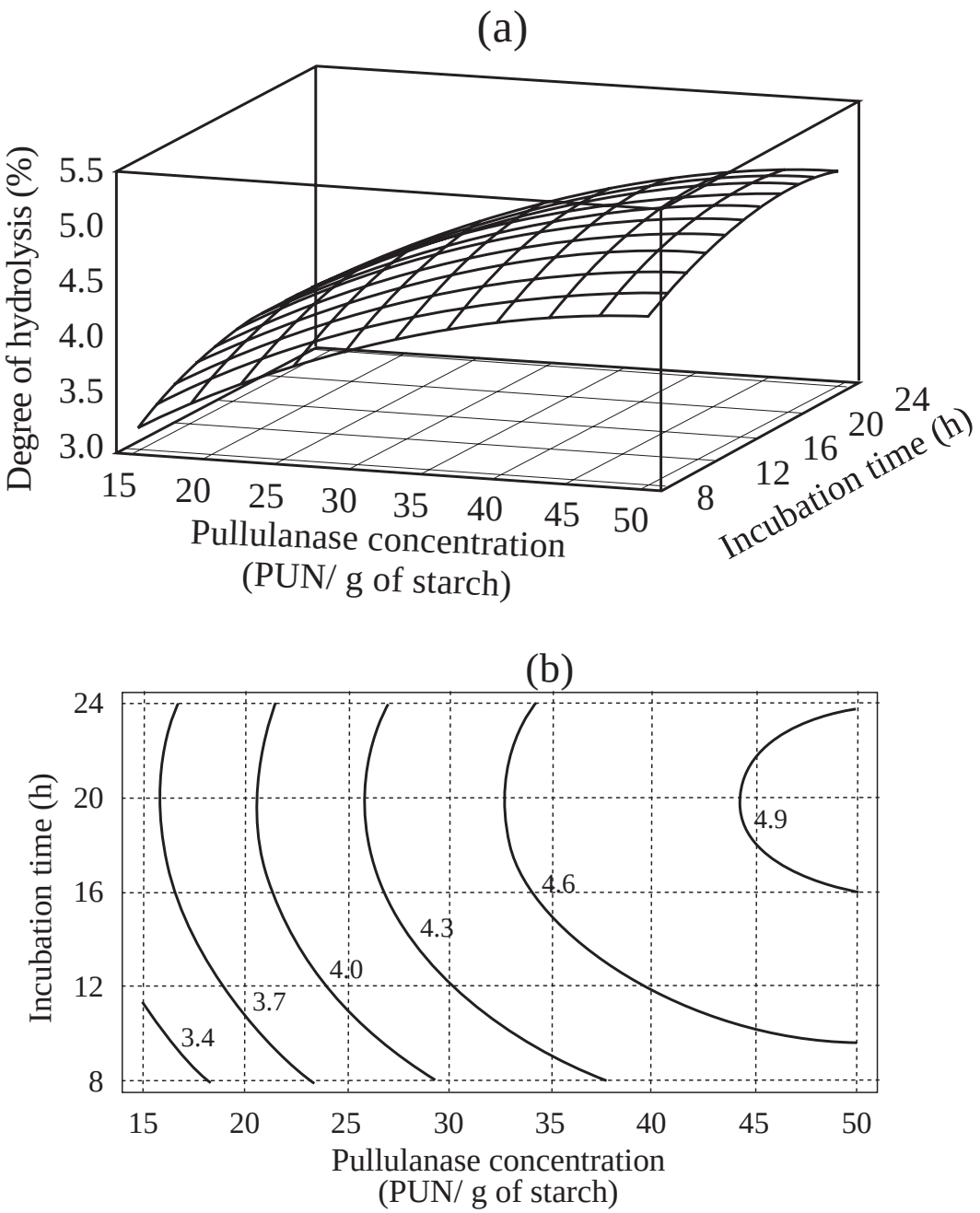

Figure 3. The three-dimensional response surface plot (a) and the contour plot (b) for the degree of hydrolysis of debranched waxy rice starch as a function of Promozyme® concentrations and incubation times when the starch concentration was 7.5% (w/w).

The larger-scale production of debranched waxy rice starch at the optimum condition

DBS was prepared in a larger scale at the optimum condition. WRS slurry (800 g of starch in 7,200 g of 0.05 M acetate buffer solution) was heated and stirred in a boiling water bath for 30 min, and starch paste was cooled down to 55°C before Promozyme® 400L (36,000 PUN, 45 PUN/g of starch) was added. The sample was incubated at 55°C for 19 h to produce DBS, and the enzymatic reaction was stopped by boiling the hydrolysate for 30 min. After the enzyme hydrolysis was terminated, the hydrolysate solutions were analyzed for D.H. and % β-amylolysis limits. The D.H. of the DBS samples was 5.07±0.22%, which was only 1.4% different from that of the predicted D.H. value (5.00%), calculated from the model equation. This result indicated that the model equation could be used to predict the condition to prepare the DBS in a larger scale. The % β-amylolysis limit of the DBS was 97.47±1.66%. This value was identical to that of DBS prepared by hydrolysis of WRS with Promozyme® 50 PUN/g of starch at 55°C for 24 h, which was suggested to be completely debranched. The hydrolysate solutions were incubated at 4°C for 24 h in order that DBS would crystallize and precipitate. After separation and drying, the yield of DBS precipitant obtained in larger batches was 78.4±3.3% of the total WRS used. The loss of some DBS was thought to occur in the preparation processes, particularly during the precipitation of DBS. The $\overline{DP}_n$ of the hydrolysate solutions was 17.6±0.6, whereas that of the DBS precipitant was 21.3±0.3, indicating that some short-chain DBS did not precipitate during the incubation at 4°C. The chain length distribution of debranched amylopectin in DBS precipitant is shown in Figure 4. It consisted of 76% short and medium chains with DP less than 34 and of 24% long chains with DP equal to or greater than 34.

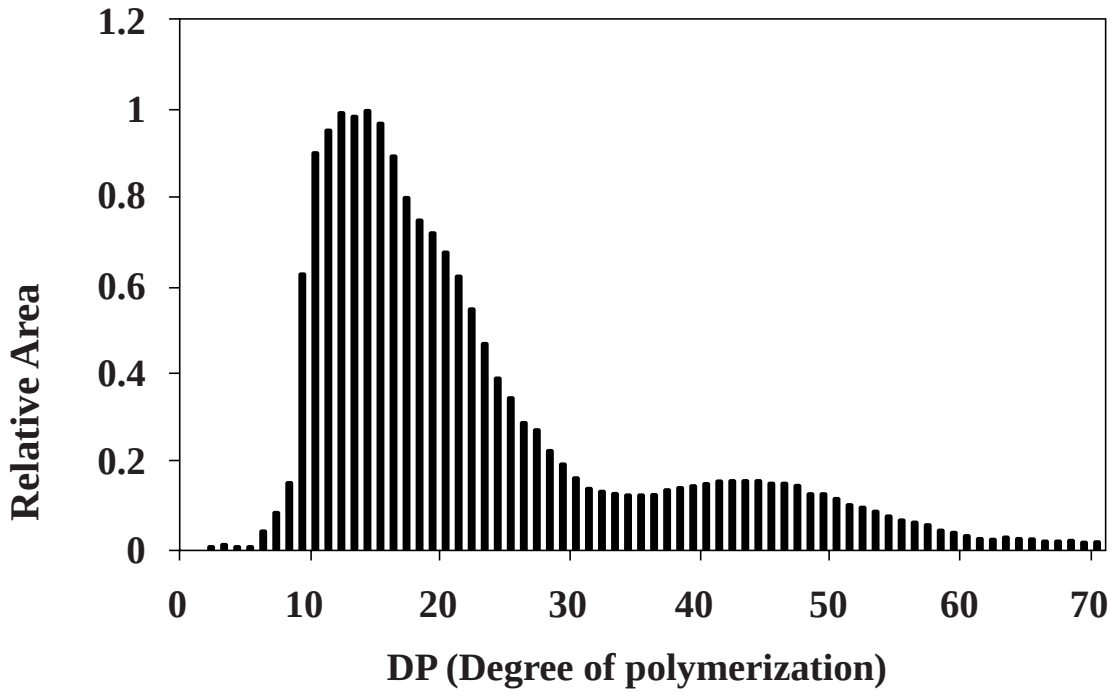

Figure 4. Chain length distribution of debranched amylopectin in debranched waxy rice starch precipitant by high-performance anion-exchange chromatography.

CONCLUSION

The complete central composite design study revealed that a Promozyme® concentration and an incubation time were factors that influenced the debranching of WRS, while a starch concentration was not a significant factor. The optimum Promozyme® concentration and incubation time were 45 PUN/g of starch and 19 h, respectively. The optimum condition obtained from the complete central composite design could be applied successfully for the preparation of DBS in a larger scale. The D.H. and % β-amylolysis limit of DBS obtained at the optimum condition in the larger scale indicated that WRS was completely debranched. The yield of DBS precipitant obtained was about 78%. The debranched amylopectin in DBS precipitant consisted of 76% short and medium chains with DP less than 34 and of 24% long chains with DP equal to or greater than 34.

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