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Thermal Properties of Partially Hydrolyzed Starch−Glycerophosphatidylcholine Complexes with Various Acyl Chains

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Complexes of starch and monoacyl-*sn*-glycerophosphatidylcholine (GPC) containing various acyl (myristoyl, palmitoyl, and stearoyl) chains were subjected to hydrolysis with glucoamylase (EC 3.2.1.3). The enzyme hydrolyzed ~40% of starch control and 20−28% of starch−GPC complexes. Among the GPCs examined, 1- and 2-monomyristoyl-*sn*-GPC showed the highest resistance to enzyme hydrolysis, and the hydrolysis rate of starch−GPCs was greater with longer chains. Enzymatic hydrolysis strongly affected the thermal properties of the starch. After enzymatic hydrolysis of starch−GPC complexes for 24 h, their thermograms had broader peaks with lower enthalpies than the corresponding starch without enzyme; however, the starch−GPC complexes showed little change. The surface of starch−GPC granules was less eroded. These results showed that the increasing amount of starch−GPC complexes could be more resistant to hydrolysis.

KEYWORDS: Starch; glucoamylase; enzymatic hydrolysis; glycerophosphatidylcholine; starch−lipid complex; differential scanning calorimetry

INTRODUCTION

Characteristics of enzyme action on starch granules have been the subject of numerous investigations and reports (*1*−*6*). These studies have shown that starches vary in their resistance to the action of α-amylase or glucoamylase. Starch susceptibility to enzyme attack is influenced by several factors, such as amylose and amylopectin contents (*7*−*10*), crystalline structure, particle size, and the presence of lipid such as in a starch−lipid complex (*11*). Among these factors, the starch−lipid complex is believed to be the most important. In general, the structure and amount of starch−lipid in foods depend on their botanical sources. Starch−lipid complexes show a relatively lower degree of hydrolysis than do the cereal starches. The complexed fatty acids (FA) and lysophosphatidylcholine (LPC) with amylose were hardly released from the complexes during hydrolysis by α-amylase (*1*, *3*). Between α-amylase and glucoamylase, the α-amylase breaks the α-1,4 linkages present in starch but cannot act on the α-1,6 links. However, the *exo*-splitting action of glucoamylase, which hydrolyzes both α-1,4 and α-1,6 linkages, could be considered in studies on the susceptibility of amylose complexes.

The formation of such complexes changes the properties of the glucan, decreases solubility, retards retrogradation, and increases the gelatinization temperature of starch (*12*, *13*). Early studies of thermal properties performed by differential scanning calorimetry (DSC) have shown that amylose−LPC complexes form more readily than amylose−monoglyceride complexes (*11*). However, little research has been done on the effect of the hydrolysis of starch on the thermal properties and susceptibility of starch−lipid complexes. From this point of view, the purposes of this study were to determine the thermal properties and susceptibility of glucoamylase hydrolyzed starch inclusion complexes having different acyl chains and *sn*-position of monoacyl-*sn*-glycerophosphatidylcholines (GPC). The relationships between thermal properties and the susceptibility of complex were also tested.

MATERIALS AND METHODS

Materials. The defatted starch samples were prepared from commercial wheat starches by refluxing with a hot aqueous solution containing 85% methanol for 1 h and re-extracted three times in a screw-capped tube. Then the defatted starch was dried at room temperature (*14*). High-grade glucoamylase (glucoamylase activity = 41.9 units/mg) obtained from *Rhizopus niveu*s and almost free of α-amylase was purchased from Amano Pharmaceutical Co., Ltd. (Nagoya, Japan). 1,2-Dimyristoyl (M)-*sn*-GPC, 1,2-dipalmitoyl (P)-*sn*-GPC, 1,2-distearoyl (S)-*sn*-GPC, and LPC were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan), checked for purity by thin-layer chromatography, and used as received. Phospholipase A_2 (phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4) from bee venom was obtained from Sigma Chemical Co. (St. Louis, MO). Lipase (triaclyglycerol acylhydrolase EC 3.1.1.3) from *Aspergillus niger* was provided by

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Table 1. Amount of Leached Starch during Incubation at 60 °C

	starch	starch–1-mono-*sn*-GPC			starch–2-mono-*sn*-GPC		
		M	P	S	M	P	S
soluble starch[a] (%)	0.22 ± 0.02c	0.11 ± 0.02a	0.14 ± 0.01b	0.21 ± 0.00c	0.11 ± 0.01a	0.18 ± 0.02b	0.21 ± 0.01c

[a] Percentage of total soluble starch of the sample; mean ± standard deviation ($n = 3$), means followed by the same letter are not significantly different according to Duncan's multiple-range test ($p < 0.05$).

Table 2. Thermal Properties of Gelatinization of Starch and Starch–Lipid Complex

	starch		starch–lipid complex			
complex	T_o (°C)	T_p (°C)	ΔH_g (J/g)	T_o (°C)	T_p (°C)	ΔH_{s-l} (J/g)
starch	65.1a[a]	68.9a	7.8c	ND[b]	ND	ND
starch–1-M-*sn*-GPC	69.9b	73.5b	3.0a	94.7a	105.3a	8.2c
starch–1-P-*sn*-GPC	70.1bc	73.9bc	3.5a	95.6ab	106.0a	6.7ab
starch–1-S-*sn*-GPC	69.9b	74.0bc	4.7b	95.4ab	105.3a	5.8a
starch–2-M-*sn*-GPC	72.0d	76.1d	2.5a	96.6abc	106.3a	7.9bc
starch–2-P-*sn*-GPC	71.1cd	75.6d	2.6a	97.0bc	106.1a	6.5a
starch–2-S-*sn*-GPC	69.9b	75.0cd	5.4b	98.1c	106.4a	5.6a

[a] Values followed by the same letter in the same column are not significantly different according to Duncan's multiple-range test ($p < 0.05$). [b] Not detected.

Amano Pharmaceutical Co., Ltd. The 1- or 2-monoacyl-*sn*-GPC was prepared as reported by Siswoyo and Morita (*15*).

Preparation of Starch—Lipid Complexes. Complexes of GPC with starch were prepared according to the method of Eliasson et al. (*16*) with modification**.** Monoacyl-*sn*-GPCs did not readily disperse in water at room temperature, so they were suspended at 10 times of water, heated at 70 °C to give a lamellar liquid-crystalline phase, then allowed to cool to 60 °C, and kept at that temperature before the addition of the starch. Defatted starch (2 g) was dispersed in 3.4 mL of water, to which a GPC dispersed in water was added. The mixtures were stirred and completed under heating conditions for 1 h at 60 °C with stirring at 360 rpm and then immediately cooled to room temperature in a water bath. Because the starch−GPC complex preparation obtained by heating at 60 °C for 1 h is considered to involve the form of gelatinized granules, the partially gelatinized granules and also some considerably annealed granules are present. All lipids were added to the concentration of 2% (w/w) calculated on a dry starch basis. In the control, the same volume of water replaced the lipid suspension.

Preparation of Enzyme Solutions. The enzyme mixture was prepared as a 10-fold concentrate containing 200 units/mL of glucoamylase in 0.1 M acetate buffer, pH 4.8. In the standard method, the amount of reducing sugar liberated by glucoamylase hydrolyzed from the starch−GPC complexes was determined according to the glucose oxidase−peroxidase method (*17*).

Enzymatic Hydrolysis of Starch—GPC Complexes. Five hundred milligrams of a sample was immersed in 5 mL of acetate buffer and treated with the enzyme solution, which was at a final concentration of 200 units/mL. In the control, the same volume of buffer replaced the enzyme solutions. The samples were incubated at 37 °C for various times with gentle stirring in a shaker bath at 8 rpm, and the reaction was stopped by the addition of 0.2 N HCl. The residual sample was separated by centrifugation at 2500*g* for 10 min. The reducing sugar concentration of the supernatant was assayed according to the glucose oxidase−peroxidase method. The separated insoluble residue was washed several times with deionized water and dried overnight at room temperature. This sample was used for DSC and scanning electron microscopy (SEM).

Calculation of Degree of Hydrolysis (DH). The DH was calculated as follows:

$$\mathrm{DH} = \frac{\text{reducing sugar produced by enzymatic hydrolysis}}{\text{reducing sugar produced by acid hydrolysis}} \times 100\% \quad (1)$$

Reducing sugar was assayed according to the glucose oxidase−peroxidase method using glucose as a standard. Starch (1 g) was hydrolyzed with 1 N HCl (200 mL) at 100 °C for 2 h.

SEM. For SEM, starch granule samples were sprinkled onto double-sided tape on the surface of silver paste on SEM metal stubs. Samples were coated with a thin layer (~150 μm) of palladium/platinum, viewed at 10 kV, and photographed at a speed of 100 s/picture at a 17 mm working distance in a Hitachi scanning electron microscope model S-800.

DSC. The DSC measurements were done using a Shimadzu DSC apparatus (model DSC-60, Kyoto, Japan), controlled by TA-60 WS software and connected to a thermal analyzer. The calorimeter was calibrated with indium (melting point = 156.7 °C; $\Delta H = 27.6$ J/g), and the reference used was liquid paraffin (*15*). Starch−lipid complexes (4−5 mg) in aluminum DSC pans were weighed, and deionized water was added to the dry starch sample to give a 2:1 ratio of water to starch. After sealing, the pan was left for 1 h to allow the sample to mix and equilibrate at room temperature before heating. The pans were scanned at a rate of 10 °C/min from 30 to 125 °C under nitrogen gas. Onset temperature (T_o) and peak temperature (T_p) of starch and starch−lipid complexes and enthalpy values for starch (ΔH_g) and starch−lipid complexes (ΔH_{s-l}) were measured to characterize the thermal properties of starch.

Statistical Analysis. Values were obtained as the means ± standard deviation of three determinations, following ANOVA, and analyzed by Duncan's multiple-range test. Differences among samples were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

Leached Starch during Complexing at 60 °C. The amount of leached starch, determined by total sugar analysis using the glucose oxidase−peroxidase method, is shown in **Table 1**. Starch complexed with 1- or 2-monomyristoyl-*sn*-GPCs showed the lowest amylose leaching (0.11%), and all complexed samples showed less amylose leaching than the control (0.22%). The chain lengths of fatty acids of the starch−GPC complexes obviously affected the degree of amylose leaching from the granule at 60 °C and related to the ability of the starch−GPC to form complexes. The formation of amylose−lipid complexes would inhibit granular swelling; therefore, amylose leaching is reduced. Similar phenomena were observed for wheat and potato starches (*1*, *18*).

Characteristic Starch—GPC Complex Formation before Enzymatic Hydrolysis. Enthalpy values of starch complexes with different chain lengths in the GPCs are shown in **Table 2**. The increase of fatty acid chain lengths of GPC caused a significant increase in gelatinization enthalpies (ΔH_g). As the

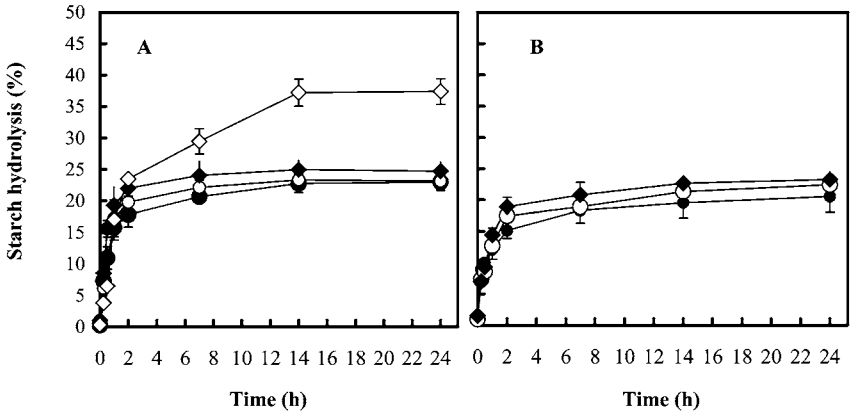

Figure 1. Susceptibility of starch–GPC complexes to hydrolysis of starch by glucoamylase at 37 °C: (A) 1-monoacyl-*sn*-GPC; (B) 2-monoacyl-*sn*-GPC; (◇) starch; (◆) stearoyl; (○) palmitoyl; (●) myristoyl. Bars indicate standard deviation ($n = 3$).

chain length of the 1- or 2-monoacyl-*sn*-GPC added to starch became longer, the enthalpy of gelatinization of starch granules increased, whereas that of the starch−GPC complexes (ΔH_{s-l}) decreased. The shorter chain length of 1- or 2-monoacyl-*sn*-GPC suppresses the dissociation of starch−GPC complexes, because the lipid of the shorter chain is more easily accommodated into the amylose helix than is a longer chain. The gelatinization enthalpies of starch−GPCs were significantly lower than the 7.8 J/g of the starch control (ΔH_g), as follows: 3.0 (M), 3.5 (P), and 4.7 (S) J/g for 1-monoacyl-*sn*-GPC; and 2.5 (M), 2.6 (P), and 5.4 (S) J/g for 2-monoacyl-*sn*-GPC. The decrease in gelatinization enthalpy might be due to further complex formation during the starch gelatinization process. Morrison et al. (*19*) reported that if lipids capable of forming complexes with amylose are present during starch gelatinization, the exothermic heat of complex formation partially offsets the endothermic heat of starch gelatinization.

Similar changes during starch gelatinization were caused by the presence of a monoglyceride or LPC (*11*, *20*). Such phenomena might be caused by structural changes in starch granules when they complex with lipids. The acyl chain length of a monoglycerol was limited to enter into the amylose helix. For example, monopalmitoyl-*sn*-GPC with a chain of ∼2.2 nm is accommodated to enter into the amylose helix with a requirement of ∼16.5 glucose units (*20*). Shorter monoacyl-*sn*-GPCs are more easily accommodated into the amylose helix. The melting enthalpy of the amylose−monoacyl-*sn*-GPC complexes is therefore influenced by the ease with which the monoacyl-*sn*-GPC can be accommodated into the helix. The significant difference in the melting behaviors of starch and amylose−monoacyl-*sn*-GPC is probably due to the structural differences. Our results showed that 1- or 2-monomyristoyl-*sn*-GPC formed complexes more easily than palmitoyl- or stearoyl-*sn*-GPC.

Effects of Chain Length of GPC on Susceptibility to Hydrolysis of Starch—GPC Complexes. Figure 1 shows the susceptibility of starch−GPC complexes to hydrolysis of starch by glucoamylase. About 40% of starch control in solution was hydrolyzed by the enzyme, and hydrolysis did not proceed further. The limitation of hydrolysis may be due to the existence of phosphate groups attached to the glucosyl residue (*21*). The wheat starch prepared in this study contained ∼44.3 μg of phosphorus/100 mg of starch.

Hydrolysis of the complexes proceeded rapidly during the early stages of the reaction and then was nearly stationary after 7 h. At 14 h of the reaction, the extent of hydrolysis ranged from 18.3 to 21.2% for 1- or 2-M-*sn*-GPC and from 23 to 26% for 1- or 2-S-*sn*-GPC. Among the GPC complexes, enzyme susceptibilities of these samples rank in the order control > S > P > M. Complexes with 1- or 2-monomyristoyl-*sn*-GPC hydrolyzed with glucoamylase had lower degrees of hydrolyzed starch than other complexes with palmitoyl or stearoyl. The extent of hydrolysis was correlated positively with GPC in complexes with the chain lengths of fatty acids ($r^2 = 0.95-0.98$), suggesting that the extent of hydrolysis of the complexes primarily depends on the chain lengths of the complexed GPC with starch rather than the *sn*-position of acyl chain.

Amylose−GPC complexes affected enzyme susceptibility of starch at two levels: first, starch granules are restricted to swell during incubation at 60 °C; second, amylose−GPC complexes are more resistant to enzyme digestion than free amylose. Such behavior probably arises from structural characteristics that limit the access of the enzyme to glycosidic bonds (*22*). Susceptibility to enzymes may be related to granule surface characteristics that affect accessibility to enzymes. Morrison et al. (*19*) suggested that resistant wheat−starch granules have a more rigid crystalline layer than nonresistant wheat starch granules at the granular surface; therefore, only the external glucosyl chain residues of amylopectin are accessible. During mild heating in water, starch granules swell somewhat at amorphous zones, so pores on the granule surface become soluble. Subsequently, amylose leaches from starch granules, and an enzyme can access the granule interior. In the presence of GPC, amylose−GPC complexes are formed and the complexes inhibit the swelling of starch granules during heating. Subsequently, less amylose leaches out than with free starch. Amylose−GPC complexes are much more resistant to amylolysis than uncomplexed amylose.

Effects of Hydrolyzed Starch—GPC Complexes on Thermal Properties of Starch. The susceptibility to hydrolysis of starch−GPC complexes with various acyl chain lengths to glucoamylase was investigated in terms of gelatinization properties. The DSC curves for starch−GPC complexes showed a main endothermic peak gelatinization (T_p) at ∼68.1−73.5 °C without enzyme and at ∼69.1−82.9 °C in enzyme hydrolysate (**Table 3**). The T_p of starch−GPC complexes was higher than without the enzyme (**Figure 2A**). The rate of increase in T_p was rapid during the early reaction. After 2 h, the hydrolysis rate increased more slowly than in the previous step, until 24 h. The results without the enzyme and with enzyme hydrolysis showed that $T_p - T_o$ of 1- or 2-M-*sn*-GPC increased slightly during hydrolysis time, but that of the control tended to decrease slightly during the hydrolysis (**Figure 2B**). The enthalpy value (ΔH_g) of 1- or 2-M-*sn*-GPC starch complex decreased rapidly

Table 3. Comparison of Thermal Properties of Starch Gelatinization Hydrolyzed by Glucoamylase at 37 °C for 24 h

	gelatinization						
	without enzyme			with enzyme			
sample	T_o (°C)	T_p (°C)	ΔH_g (J/g)	T_o (°C)	T_p (°C)	ΔH_g (J/g)	DE[a] (%)
starch	65.1 ± 0.1[b]	68.1 ± 0.1	7.7 ± 0.1	64.3 ± 0.3	68.3 ± 0.3	4.6 ± 0.3	39
starch–1-M-*sn*-GPC	69.9 ± 0.4	73.5 ± 0.5	3.0 ± 0.1	77.3 ± 0.3	83.1 ± 0.4	2.4 ± 0.1	20
starch–1-P-*sn*-GPC	70.1 ± 0.1	73.9 ± 0.1	3.5 ± 0.1	77.9 ± 0.4	81.9 ± 0.8	2.4 ± 0.2	31
starch–1-S-*sn*-GPC	69.9 ± 0.7	74.0 ± 0.4	4.7 ± 0.1	78.4 ± 0.5	82.1 ±0.6	3.1 ± 0.1	34
starch–2-M-*sn*-GPC	72.0 ± 0.4	76.1 ± 0.8	2.5 ± 0.2	75.9 ± 0.6	82.8 ± 0.2	1.9 ± 0.3	24
starch–2-P-*sn*-GPC	71.1 ± 1.1	75.6 ± 1.3	2.6 ± 0.2	75.6 ± 0.8	82.5 ± 0.6	1.8 ± 0.1	30
starch–2-S-*sn*-GPC	69.9 ± 0.3	75.0 ± 1.3	5.4 ± 1.1	76.1 ± 0.3	82.7 ± 0.3	3.3 ± 0.2	38

[a] DE, degree of enthalpy, calculated as $\{(\Delta H_{no-enz} - \Delta H_{enz})/\Delta H_{no-enz}\} \times 100\%$. [b] Mean ± standard deviation.

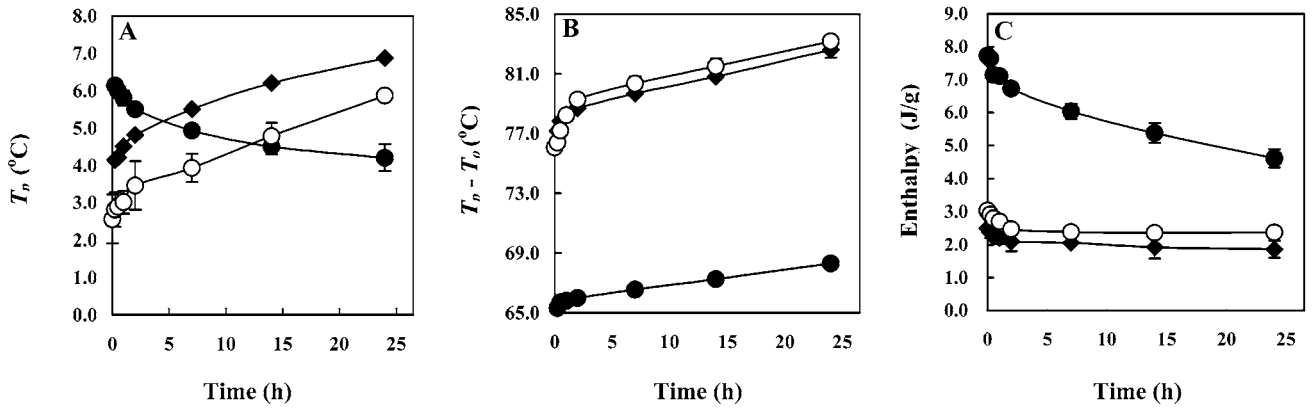

Figure 2. Effect of monoacyl-*sn*-GPC on thermal properties of gelatinization of starch after hydrolysis with glucoamylase at 37 °C: (A) peak temperature (T_p); (B) width of endotherm peak ($T_p - T_o$); (C) entalhpy value [(●) starch; (○) 1-monomyristoyl-*sn*-GPC; (◆) 2-monomyristoyl-*sn*-GPC].

Table 4. Comparison of Thermal Properties of Starch–GPC Complexes Hydrolyzed by Glucoamylase at 37 °C for 24 h

	starch–lipid						
	without enzyme			with enzyme			
sample	T_o (°C)	T_p (°C)	ΔH_{s-l} (J/g)	T_o (°C)	T_p (°C)	ΔH_{s-l} (J/g)	DE[a] (%)
starch	ND[b]	ND	ND	ND	ND	ND	
starch–1-M-*sn*-GPC	94.7 ± 1.0[c]	105.3 ± 0.1	8.2 ± 0.1	97.6 ± 0.2	109.7 ± 0.5	8.1 ± 0.9	1
starch–1-P-*sn*-GPC	95.6 ± 0.5	106.0 ± 0.7	6.7 ± 0.1	99.3 ± 0.2	110.3 ± 0.3	5.7 ± 0.1	15
starch–1-S-*sn*-GPC	95.4 ± 0.2	106.3 ± 0.2	5.8 ± 0.2	101.8 ± 0.2	110.8 ± 0.8	4.3 ± 0.4	25
starch–2-M-*sn*-GPC	96.6 ± 0.5	106.3 ± 0.6	7.9 ± 0.1	104.6 ± 0.7	111.3 ± 0.4	7.4 ± 0.1	6
starch–2-P-*sn*-GPC	97.0 ± 1.6	106.1 ± 1.8	6.5 ± 0.3	105.0 ± 0.6	109.0 ± 0.7	5.4 ± 0.3	17
starch–2-S-*sn*-GPC	98.1 ± 1.6	106.4 ± 0.6	5.6 ± 0.5	106.0 ± 0.4	114.3 ± 2.3	4.5 ± 0.2	20

[a] DE, degree of enthalpy, calculated as $\{(\Delta H_{no-enz} - \Delta H_{enz})/\Delta H_{no-enz}\} \times 100\%$. [b] Not detected. [c] Mean ± standard deviation.

during the early stages of the hydrolysis and then became nearly stationary after 2 h. The ΔH_g of the starch control gradually decreased during hydrolysis (**Figure 2C**). Thermal properties of starch gelatinization (G) after hydrolysis with glucoamylase for 24 h showed somewhat broader peaks with lower enthalpies than without the enzyme. However, peaks of starch−GPC complexes (SL) slightly changed in enthalpy values as shown in **Figure 3**. The thermal properties of starch−GPC after hydrolysis with glucoamylase at 37 °C for 24 h are shown in **Table 4**. The enthalpy of the starch−GPC complex was lower than that obtained from the starch−GPC complex without hydrolysis of glucoamylase, and the degree of enthalpy change (DE) of starch−GPC complexes with shorter chains was smaller than those with longer chains. It can be summarized that the enthalpies of starch−GPC decreased in the order M > P > S; the DE change decreased in the order S > P > M. This indicates that a distinct relationship exists between the thermal properties and susceptibilities to glucoamylase attack. Zhang and Oates (*23*) reported that crystalline arrangement of the starch granules plays an important role in their susceptibility to glucoamylase attack, with the high gelatinization temperature of starch−GPC being less susceptible to enzyme attack. The amount of starch−GPC complexes was critical to the gelatinization temperature.

Hydrolysis Pattern. The modes of hydrolysis by glucoamylase of starch−GPC complexes were investigated by SEM, and the samples showed different hydrolysis patterns (**Figure 4**). The surface of starch control granules hydrolyzed with the enzyme was eroded with many holes. The surface of enzyme-hydrolyzed starch−GPC granules was rough with limited erosion and few holes. Hydrolysis of glucoamylase apparently formed tunnels into the granules prior to the hydrolysis of the interior granules.

The pattern of enzymatic attack could influence the susceptibility of starch granules. Starch granules of the control were attacked greatly on the surface with less intermolecular association. However, enzyme-hydrolyzed starch granules in the starch−GPC complexes showed little erosion. This could be due to the differences in the granule structure. The amount of

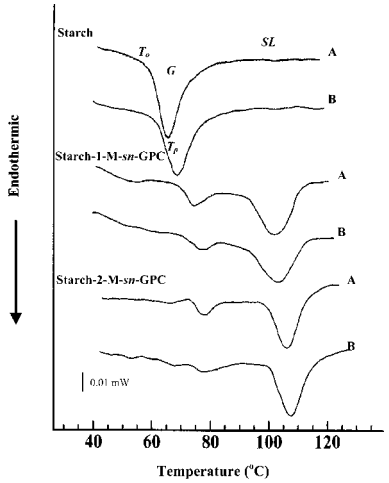

Figure 3. DSC thermograms of starch–1- or 2-monoacyl-*sn*-GPC after hydrolysis with glucoamylase at 37 °C for 24 h: (A) without enzyme; (B) with enzyme; G, gelatinization; SL, starch–GPC complexes.

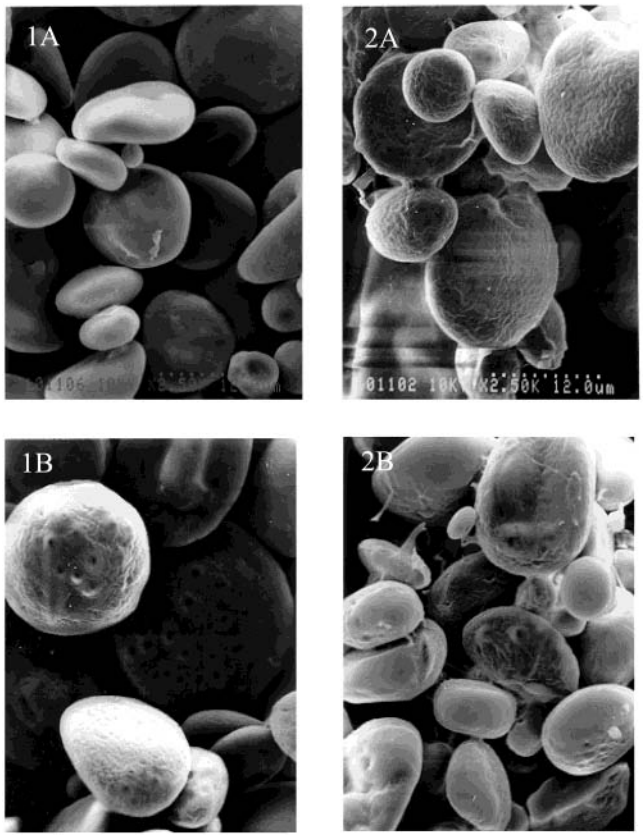

Figure 4. SEM photograph of starch–GPC complex samples hydrolyzed by glucoamylase at 37 °C for 24 h: (1) starch; (2) starch–1-monomyristoyl-*sn*-GPC; (A) without enzyme; (B) with enzyme.

starch−GPC complexes and endothermic characteristics are critical factors to the hydrolysis pattern, and all of these factors influence the susceptibility to enzyme attack.

Conclusion. The results indicated that starch−GPC complexes retarded the enzymatic hydrolysis of starch, and no significant differences in the susceptibility of starch−GPC complexes were observed between 1- and 2-monoacyl-*sn*-GPCs. 1- or 2-monomyristoyl-*sn*-GPC showed both the highest gelatinization temperature and complexing abilities compared with those of palmitoyl- or stearoyl-*sn*-GPC. These properties were correlated with the susceptibility of glucoamylase hydrolyzed starch−GPC complex.

ABBREVIATIONS USED

GPC, glycerophosphatidylcholine; M, myristoyl; P, palmitoyl; S, stearoyl; DSC, differential scanning calorimetry; ΔH_g, gelatinization enthalpy; ΔH_{s-l}, enthalpy of the melting of starch−lipid complexes; T_o, onset temperature; T_p, peak temperature.

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