Susceptibility of Amylose-Lipid Complexes to Hydrolysis by Glucoamylase from *Rhizopus niveus*

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ABSTRACT

The amylose-lipid complexes were prepared from potato amylose with fatty acids (FA) and lysophosphatidylcholine (LPC), and their susceptibilities to hydrolysis by glucoamylase (*Rhizopus niveus*) were examined. The prepared complexes had the respective amount of lipid in them, depending on the sort of lipid (0.91–2.93 μmol/10 mg of amylose), and showed no typical peaks of the crystallized V-form on X-ray diffractograms. The complexes showed the extent of hydrolysis ranging from 4.8% for LPC to 22.0% for linolenic acid by 83.4 nkat of the enzyme for 72 hr, which was lower than the hydrolytic extents of cereal starches. When the complexes in excess water were treated by heating at not more than the respective melting temperature for 2 hr, no change in the X-ray diffractogram was observed, but the extent of hydrolysis was decreased to 3.2–16.5%. During the hydrolysis of the heat-treated complexes, it was found that the complexed lipids were hardly released from them, except for unsaturated FA. Also, the ability of adsorption of the glucoamylase on the complex was determined. It was found that the residues after glucoamylase hydrolysis, glucoamylase-resistant complex, showed decreased ability of enzyme adsorption compared with the intact complex. These results indicate the complex-forming portion itself is strongly resistant to the action of glucoamylase.

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It is known that the glucoamylase (EC 3.2.1.3, 1,4-α-glucan glucohydrolase) is an *exo*-splitting enzyme that catalyzes hydrolysis of both α-1,4- and α-1,6-glucosidic linkages of starch and consecutively liberates the glucose units from the nonreducing end, but its action is blocked by a phosphorylated residue (Abe et al 1982). Although many reports on the susceptibility of amylose-lipid complex by amylolytic enzyme, especially α-amylases, have been reported (Vieweg and de Fekete 1976, de Fekete and Vieweg 1978, Hizukuri et al 1979, Holm et al 1983, Jane and Robyt 1984, Eliasson and Krog 1985, Biliaderis and Galloway 1989, Galloway et al 1989, Seneviratne and Biliaderis 1991), there are few reports concerning that by glucoamylases.

Cereal starches usually have lipids bound firmly in the granules (Morrison 1988). It has been generally considered that the bound lipids are firmly complexed with amylose in native starch granules. Previously, we examined loss of the bound free fatty acids (FFA) in corn starch upon hydrolysis by crude glucoamylase (Kitahara et al 1994). Contrary to our prediction that the amylose-lipid complexes resisted amylolysis, the result revealed that the bound FFA in corn starch were scarcely resistant to release from granules upon the hydrolysis. If the bound FFA exist as complexes with amylose in the native granules, the FFA would be resistant to being released from granules. It is, therefore, necessary to examine the resistance of model amylose-lipid complexes to hydrolysis by glucoamylase.

In this study, amylose-lipid complexes were prepared as an insoluble substrate, and their susceptibilities to hydrolysis by practically pure glucoamylase from *Rhizopus niveus* were examined.

MATERIALS AND METHODS

Amylose and Reagents

Amylose was prepared from potato starch according to the method of Hizukuri et al (1981). The amylose was recrystallized six times from 10% aqueous 1-butanol, and the final amylose was dehydrated in ethanol, followed by vacuum drying.

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In this study, we used two glucoamylase preparations originated from *R. niveus*. One was highly purified glucoamylase (glucoamylase activity: 1.22 Kat/kg) purchased from Seikagaku Kogyo Co., Ltd. (Tokyo). When γ-cyclodextrin was used as a substrate at 50-fold the usual enzyme concentration for assay, no increase of reducing power during 12 hr was detected. Also, no phosphatase activity using *p*-nitrophenylphosphoric acid as a substrate was detected, thus it was used without further purification. The other enzyme was a crude preparation (glucoamylase activity: 0.47 Kat/kg, total amylase activity: 0.63 Kat/kg) from Nagase Biochemicals., Ltd. (Kyoto). The activities of glucoamylase and total amylase were estimated based on the amount of liberated sugar measured by the glucose oxidase-catalase method (Ikawa and Obara 1965) and the Somogyi-Nelson method (Nelson 1944, Somogyi 1952), respectively. The reaction used 0.5% soluble starch, pH 5.0, as a substrate at 35°C.

All reagents and solvents, unless otherwise specified, were obtained from Wako Pure Chemical Industries (Osaka) and were of the highest grade commercially available.

Preparation of Amylose-Lipid Complex

The amylose-lipid complexes were prepared by complexation of the amylose with five kinds of fatty acids (FA) (palmitic, stearic, oleic, linoleic, and linolenic acids, >99%, Nacalai Tesque Inc., Kyoto) and lysophosphatidylcholine (LPC) (>99%, from egg yolk, fatty acid composition: 16:0 69%, 18:0 25%, 18:1 4%, 18:2 1%, 18:3 trace, Nacalai Tesque Inc.), fundamentally according to the method of Karkalas and Raphaelides (1986).

Amylose (10 mg, dwb) was dissolved by adding 0.2 ml of 1*M* potassium hydroxide, and then 1.6 ml of distilled water in a screw-cap tube at 4°C, while the respective FA (15.6 μmol/ml) was dispersed in 0.01*M* potassium hydroxide with the aid of heating and sonication. FA solution (1 ml) was gradually added to the dissolved amylose under vortex-mixing. After letting it stand for a few minutes, the amylose-FA solution was neutralized by adding 0.21 ml of 1*M* hydrochloric acid dropwise under vortex-mixing. Finally, 3 ml of 50 m*M* sodium acetate buffer (pH 5.0) containing 0.02% sodium azide was added to the solution, and the precipitated complex stood overnight.

The LPC dispersed in water (15.6 μmol/ml) was added to the amylose solution after neutralization. In each case, more than 99% of amylose was precipitated as the complex. According to the experiments, the complex in the test tube was successively treated

with heating for 2 hr at 90°C for those with palmitic acid, stearic acid, oleic acid, and LPC; at 80°C for that with linoleic acid; at 70°C for that with linolenic acid, in which no melting of the complexes at the respective temperature was observed. The prepared complex is referred to as the heat-treated complex.

The preparation of the complexes and the following determinations were performed in triplicate.

Hydrolysis of Amylose-Lipid Complex by Glucoamylase

The required unit of glucoamylase in 50 m*M* sodium acetate buffer (pH 5.0) containing 0.02% sodium azide was added to the complex prepared as above, and it was finally increased to 7.5 ml with the same buffer. The reaction was kept at 35°C for various times with gentle stirring on a culture tube rotator at 8 rpm. After the reaction, the residual complex was collected by centrifugation at 4,500 × *g* for 10 min, and the glucose liberated in the supernatant was determined by the glucose oxidase-catalase method (Ikawa and Obara 1965). In the case of crude glucoamylase hydrolysis, the soluble carbohydrates in the supernatant was determined by the phenol-sulfuric acid method (Dubois et al 1956), the value of which agreed with that by the glucose oxidase-catalase method.

On the other hand, the collected residue was washed with diethyl ether for FA or with chloroform for LPC with vigorous stirring, and recollected by centrifugation. The residue was washed three times.

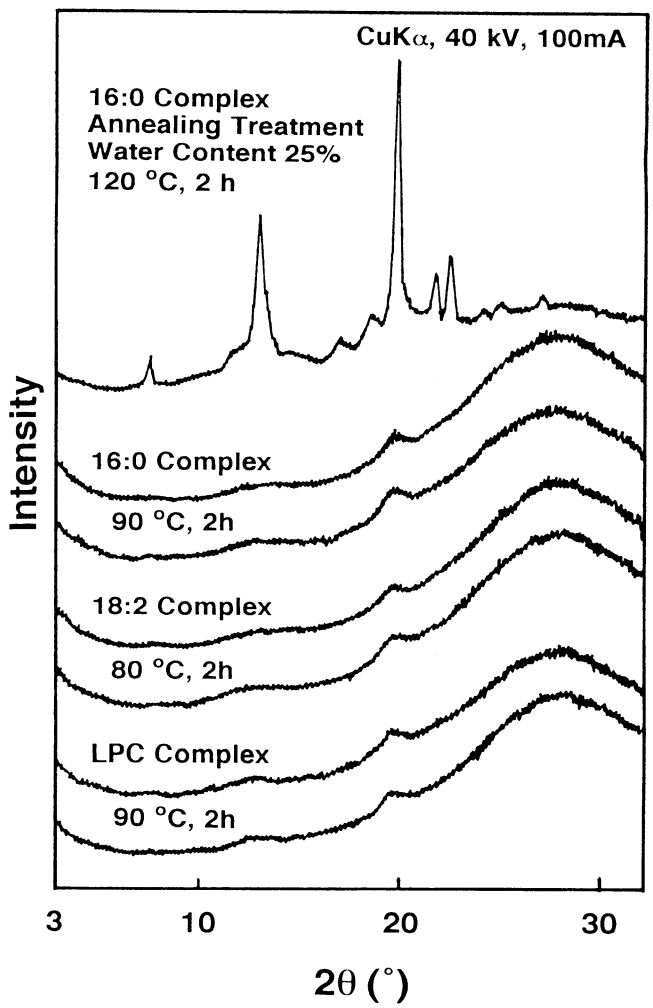

Fig. 1. X-ray diffractograms of amylose-lipid complexes. Samples include: annealed 16:0 complex, 16:0 complex, heat-treated 16:0 complex, 18:2 complex, heat-treated 18:2 complex, LPC complex and heat-treated LPC complex.

Determination of Complexed FA and LPC with Amylose

The complexed FA with amylose was determined by a NEFA-C test kit after liquefaction of the complex by thermostable α-amylase at 100°C as described previously (Kitahara et al 1994). The complexed LPC was converted into inorganic phosphate by wet ashing of the whole complex with 60% perchloric acid (1 ml) and with the aid of four drops of nitric acid. The phosphate was determined by the Fiske-Subbarow method (Fiske and Subbarow 1925).

Adsorption of Glucoamylase on Amylose-Lipid Complex

The heat-treated complexes prepared in a scale of 25 mg of amylose were lyophilized and used for this experiment. The complex was incubated with 83.4 nkat of the pure glucoamylase in 1 ml of 50 m*M* sodium acetate buffer (pH 5.0) at 5°C for 20 min. After centrifugation at 7,000 × *g*, 5°C for 5 min, the adsorption of the enzyme (%) was calculated on the basis of the residual activity in the supernatant.

The residues after glucoamylase hydrolysis, glucoamylase-resistant complex, were prepared, and the adsorption experiment was also performed. The glucoamylase-resistant complex was prepared as follows. The heat-treated complex was hydrolyzed by 167 nkat of the pure glucoamylase at 35°C for 48 hr. The glucoamylase-resistant complex was washed successively with diethyl ether for FA or with chloroform for LPC, 1*M* sodium chloride and then with distilled water three times each, and then lyophilized.

X-Ray Diffraction Analysis

The amylose-lipid complexes were collected by centrifugation at 4,500 × *g* for 10 min. After removing the supernatant, the wet precipitate was deposited 1 mm thick on aluminum holder. X-ray diffraction pattern was obtained using an X-ray diffractometer (RIGAKUDENKI RAD-RB) under the following conditions. Radiation: CuKα (counter monochromator), voltage: 40 kV, current: 100 mA, angle (2θ): 3°~32°, scan speed: 1°/min.

RESULTS AND DISCUSSION

Preparation of Amylose-Lipid Complex

Several structurally distinct forms for the amylose-lipid complexes in the aggregated structure have been identified and characterized by differential scanning calorimetry, X-ray diffractometry, and solid-state nuclear magnetic resonance analysis (Biliaderis et al 1985; Biliaderis et al 1986; Raphaelides and Karkalas 1988; Biliaderis and Galloway 1989; Galloway et al 1989; Biliaderis and Seneviratne 1990a,b; Karkalas et al 1995). The susceptibilities of the complexes in polymorphic forms to α-amylase were different from one another, in which highly crystallized complex showed lower extent of hydrolysis (Seneviratne and Biliaderis 1991).

Figure 1 shows X-ray diffractograms of the prepared complexes for palmitic acid, linoleic acid, and LPC. For the comparison, the complex with palmitic acid was annealed at a water content of 25%, 120°C, for 2 hr. The diffractogram is shown topmost in Fig-

TABLE I
Amount of Lipid in Complex and Molar Ratio of Lipid to Amylose

Complex	Complexed Lipid[a] μmol/10 mg amylose	Molar Ratio Lipid/Amylose[b]
Palmitic acid (16:0)	2.00 ± 0.06	0.032
Stearic acid (18:0)	2.47 ± 0.04	0.040
Oleic acid (18:1)	2.24 ± 0.04	0.036
Linoleic acid (18:2)	1.23 ± 0.07	0.020
Linolenic acid (18:3)	0.91 ± 0.08	0.015
Lysophosphatidylcholine (LPC)	2.93 ± 0.08	0.047

[a] Mean ± standard deviation, $n = 3$.
[b] Using 162 as the molecular weight of amylose.

ure 1. The complexes used in this study showed no typical peaks of crystallized V-form of amylose on the X-ray diffractograms, such as annealed complex. Also, the heat treatment at the respective temperature for 2 hr did not alter the diffractograms. Results were the same as with the complexes with stearic, linoleic, and linolenic acids. The feature of the diffractograms corresponded to form I complex (Biliaderis and Seneviratne 1990a), which was morphologically described by a random distribution of helices being in very little crystallographic register with one another. Thus, these complexes are considered to exist predominantly in an amorphous state even after the heat treatment.

Table I shows the amounts of complexed FA or LPC with amylose and the respective molar ratio of lipid and amylose. The molecular weight of amylose was taken as that of glucose unit (162). The amount of complexed lipid varied from 0.91 μmol/10 mg of amylose for the complex with linolenic acid to 2.93 μmol/10 mg of amylose for that with LPC, corresponding to 0.015 and 0.047 of the respective molar ratio. As the respective molar ratio obtained here was lower than the values calculated for the saturation molar ratio (Karkalas and Raphaelides 1986, Raphaelides and Karkalas 1988), the amylose-FA complexes prepared in this study, especially with unsaturated FA, might be not fully saturated with the FA inside the amylose. In the case of LPC, when the monopalmitoyl phosphatidylcholine molecule exists in the extended linear state, the length is estimated to be about 3.36 nm on the basis of computer modeling (using Chem3D on a Macintosh computer) and the length of palmitic acid. The saturation molar ratio is calculated to be 0.040, which was lower than 0.047 from the experimental data. At present, the reason for this is unclear, but the assumption that the polar ends of the lipid exist in the amylose helix is questionable (Godet et al 1993a,b; Morrison 1995).

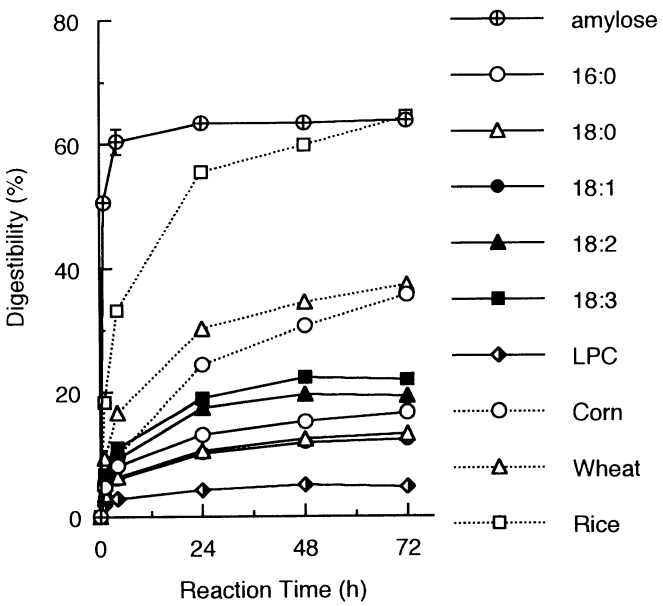

Fig. 2. Susceptibility of amylose-lipid complexes to hydrolysis by pure glucoamylase. Bar indicates standard deviation (n = 3). The complex prepared in the scale of 10 mg of amylose and the starches (10 mg) were hydrolyzed by 83.4 nkat of glucoamylase.

Susceptibility of Amylose-Lipid Complex to Hydrolysis by Glucoamylase

Figure 2 shows the susceptibility of the complexes to hydrolysis by the pure glucoamylase, and also shows the susceptibility of raw starch granules. The amylose alone in solution was hydrolyzed by the enzyme to the extent of 64%, and the hydrolysis did not proceed further with the addition of more enzyme. The limit of hydrolysis may be due to the existence of phosphate groups attached to the glucosyl residues (Abe et al 1982), but the extent was lower than 90% from previous results (Marshall and Whelan 1970) and lower than 89% in the case of glucoamylase from *Aspergillus niger* (Abe et al 1982). The extent of hydrolysis of the amylose is considered to be sensitively affected by the amount or location of the phosphate group. In practice, the potato amylose prepared in this study had a larger amount of phosphorus (32 ppm) than that (8 ppm) used in the above study (Abe et al 1982).

The hydrolysis of the complexes proceeded rapidly during the early stages of the reaction, and then nearly leveled off after 48 hr. At 72 hr of the reaction, the extent of hydrolysis ranged from 4.8% for LPC to 22.0% for linolenic acid by 83.4 nkat of the enzyme. The extent of hydrolysis of the cereal starches was higher than those of the complexes, and they were gradually hydrolyzed as the reaction progressed. As seen in Table II, no marked increase in hydrolysis of the complexes was observed at a higher enzyme concentration, while the starches were gradually increased with

TABLE II
Hydrolysis (%) of Amylose-Lipid Complexes by Glucoamylase at Different Enzyme Concentrations[a]

	Enzyme Units (nkat)		
	41.7	83.4	250
Complex			
16:0	11.9 ± 0.1	13.2 ± 0.2	15.9 ± 0.1
18:0	9.9 ± 0.1	10.6 ± 0.3	13.0 ± 0.1
18:1	8.7 ± 0.2	10.2 ± 0.1	11.9 ± 0.2
18:2	14.6 ± 0.6	17.5 ± 0.7	17.2 ± 0.2
18:3	17.3 ± 0.6	19.0 ± 0.5	19.0 ± 0.5
LPC	3.7 ± 0.1	4.3 ± 0.1	4.5 ± 0.1
Starch			
Corn	21.5 ± 0.1	24.5 ± 0.2	30.7 ± 0.4
Wheat	27.0 ± 0.5	30.3 ± 0.2	37.5 ± 0.3
Rice	53.0 ± 0.1	55.5 ± 0.5	65.4 ± 0.5

[a] Mean ± standard deviation, n = 3. The complex prepared in the scale of 10 mg amylose and the starches (10 mg) were hydrolyzed by the respective enzyme unit for 24 hr, respectively.

TABLE III
Hydrolysis (%) of Heat-Treated Complexes by Glucoamylases[a]

	Pure Glucoamylase			Crude Glucoamylase		
	Reaction Time (hr)			Reaction Time (hr)		
Complex	24	48	72	24	48	72
16:0	6.4 ± 0.1	7.1 ± 0.1	7.6 ± 0.1	93.4 ± 2.2	98.5 ± 1.0	95.9 ± 0.3
18:0	6.1 ± 0.1	6.7 ± 0.1	7.3 ± 0.1	94.0 ± 4.0	97.7 ± 2.2	97.4 ± 0.5
18:1	5.7 ± 0.1	6.4 ± 0.1	6.9 ± 0.1	95.0 ± 2.3	97.6 ± 2.0	95.6 ± 1.6
18:2	8.7 ± 0.1	10.6 ± 0.3	11.7 ± 0.4	95.2 ± 1.6	97.7 ± 1.4	98.4 ± 3.4
18:3	12.5 ± 0.2	14.3 ± 0.5	16.5 ± 0.2	96.4 ± 2.7	99.5 ± 1.6	97.4 ± 2.1
LPC	2.7 ± 0.1	3.0 ± 0.1	3.2 ± 0.1	89.3 ± 1.4	96.5 ± 1.8	97.2 ± 1.6

[a] Mean ± standard deviation, n = 3. The complex prepared in the scale of 10 mg amylose was hydrolyzed by 83.4 nkat of the respective enzyme.

increasing enzyme concentration. Among the complexes with FA, the complexes with stearic and oleic acids showed less hydrolysis when compared with the other complexes, which was similar to the result from hydrolysis of amylose-monoglyceride complexes by the combination of α-amylase and glucoamylase (Eliasson and Krog 1985). It was found that the extent of hydrolysis was negatively correlated to the amount of lipids in the complexes ($r = -0.947$), suggesting that the extent of hydrolysis of the complexes primarily depends on the amount of complexed lipids with amylose rather than type of lipid.

Table III shows the effect of heat treatment on the hydrolysis. The hydrolysis of complexes by the pure glucoamylase was decreased by the heat treatment. Interestingly, only 3.2% of the LPC-complex was hydrolyzed during 72 hr. In spite of the addition of the same unit of amylase activity, the extent of hydrolysis by pure and crude glucoamylase differed greatly from each other. On the hydrolysis by crude glucoamylase, all complexes reached more than 95% hydrolysis, in which the hydrolysate was almost glucose, and the free lipids were liberated from the complexes. It was considered that the high extent of hydrolysis was due to the contamination of α-amylase in the crude glucoamylase because the α-amylase can fully hydrolyze amylose-lipid complex (Seneviratne and Biliaderis 1991). In a previous article (Kitahara et al 1994), we used a crude glucoamylase preparation and reported the release of bound FFA from hydrolyzed corn starch. The use of crude preparation may confound the results; this subject will be reported later.

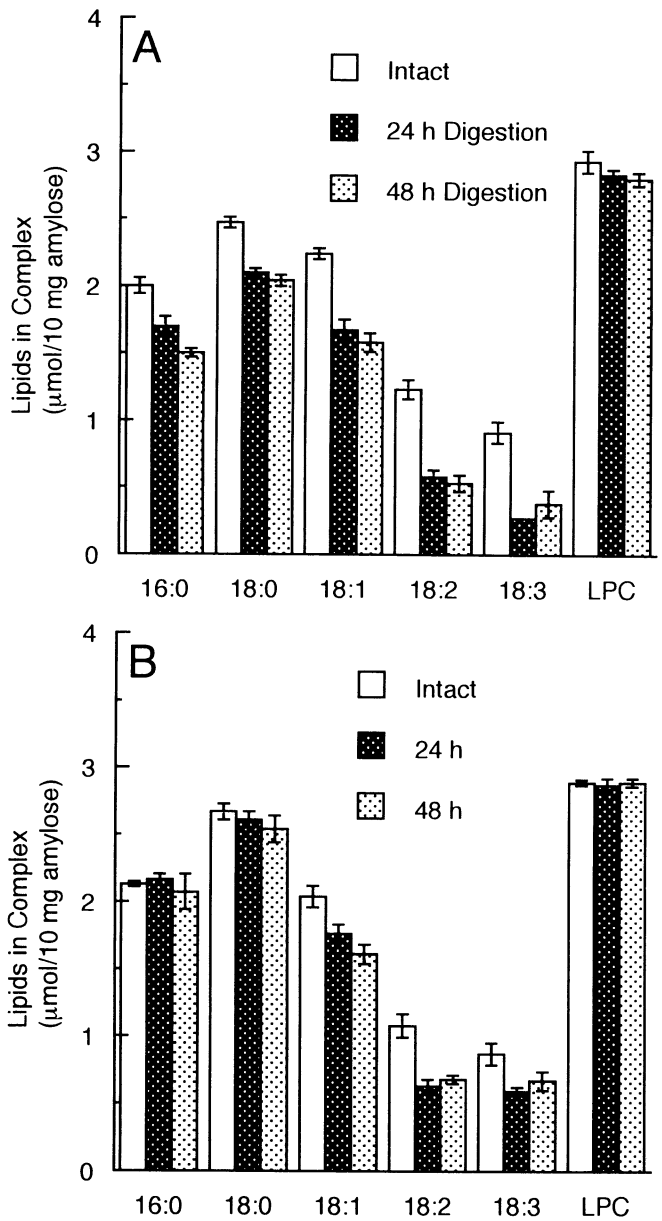

Fig. 3. Remaining lipid in residual complex after hydrolysis by pure glucoamylase. **A,** nontreated complex. **B,** heat-treated complex. Bar indicates standard deviation ($n = 3$).

Remaining Lipid in the Residue after Glucoamylase Hydrolysis

The amylose-lipid complexes were hydrolyzed more slowly by glucoamylase than were the cereal starches. However, on comparison of the hydrolysis kinetics in the solid state, the slower hydrolysis of the complexes should be apparent resistance because the forms of solid substrate, such as particle size and porosity, affect the kinetics. Therefore, we examined the resistance in view of the amount of remaining lipid in the residue after the hydrolysis.

As seen in Figure 3, the complexed lipid in every nontreated complex was released from the complex during the 24-hr hydrolysis, but the loss was smaller during the following 24 hr. The losses after heat treatment further decreased, especially the complex with saturated FA, and LPC hardly released lipids. However, one interesting result was that even heat-treated complexes released a part of the unsaturated FA during 24 hr, but there was no loss during subsequent hydrolysis. Therefore, in the cases of palmitic and stearic acids and LPC, it can be concluded that those complexes are hardly hydrolyzable by glucoamylase, and the hydrolyzed fraction may not participate in the formation of the complex.

It is interesting to note that the extent of hydrolysis was lowered, and the release of complexed lipids was restricted by the heat treatment without development of the crystallinity (Fig. 1). In this study, the amylose-lipid complexes were prepared by way of neutralization of the alkaline solution of amylose-FA mixture at ambient temperature, and most of precipitates occurred instantaneously. It is likely that the incomplete complexation occurs to some extent during such rapid insolubilization. It is satisfactory to consider the reason for strong resistance to glucoamylase caused by the heat treatment as improvement of the complexation of amylose with lipids, which means a change from loose to complete complexes (six glucosyl residues per turn).

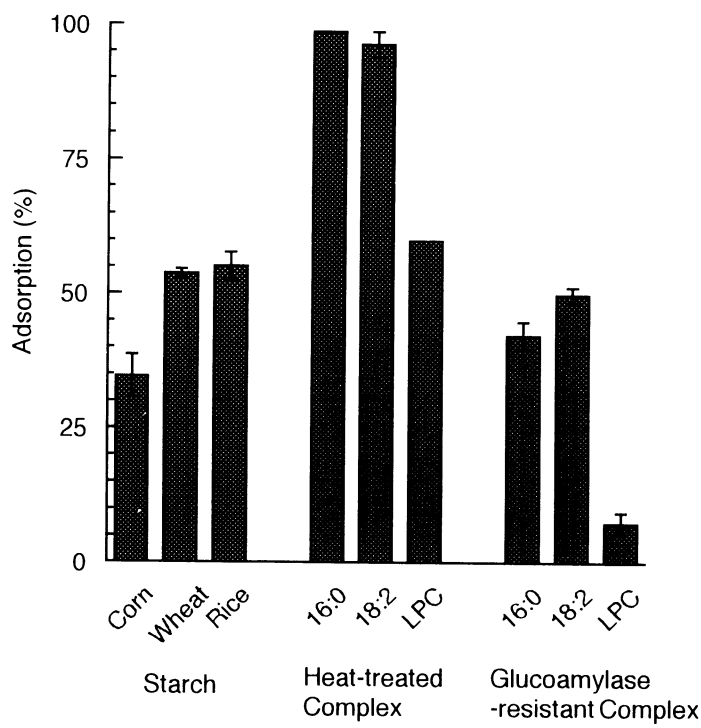

Fig. 4. Adsorption of glucoamylase on amylose-lipid complex. Bar indicates standard deviation ($n = 3$).

Adsorption of Glucoamylase on Amylose-Lipid Complex

The binding of glucoamylase on raw starch granules is important for extensive hydrolysis (Ueda et al 1988). The ability of glucoamylase to adsorb onto the heat-treated complex (palmitic acid, linoleic acid, and LPC complexes) was determined. As seen in Figure 4, 25 mg of the cereal starches adsorbed 34.6–55.1% of the added enzyme (83.4 nkat). In spite of the low hydrolytic extent of the complex, the enzyme was more strongly adsorbed by the initial heat-treated complexes than by starches, which amounted to 98.6% for that with palmitic acid, 96.2% with linoleic acid, and 59.9% with LPC.

Furthermore, the adsorption ability on the glucoamylase-resistant complex prepared by progressive glucoamylase hydrolysis was examined. On the preparation of glucoamylase-resistant complexes, the remaining enzyme in the residual complex is somewhat troublesome to use for the adsorption experiment. Washing with 1% maltose for three times followed by washing with distilled water for three times was insufficient because the extract with sodium acetate buffer (50 m*M*, pH 5.0) at 35°C from the final complex contained 2.0 nkat of enzyme activity. Instead of using maltose solution, 1*M* sodium chloride effectively washed out the enzyme from the complex. In comparison with the added enzyme (167 nkat) for the preparation, a negligible amount of enzyme activity (0.15 nkat) was detected in the same extract.

The abilities of adsorption on the glucoamylase-resistant complexes were reduced to about one-half of the initial values for the complexes with FA (Fig. 4). It was interesting that the complex with LPC could adsorb only 7.4% of the enzyme.

In this study, it was observed that the complexed saturated FA and LPC were not released from the complexes by the glucoamylase. The result suggests that the glucoamylase action is prevented by the complex-forming portion of the complex. Thus, it is probable that the extensive action of the glucoamylase on the amylose-lipid complexes might expose the complex-forming portion near the nonreducing end. Therefore, the results from adsorption experiments indicate that the glucoamylase has a low affinity for such complex-forming portions.

CONCLUSION

The action of glucoamylase (*R. niveus*) on the amylose-lipid complexes was examined. The complexes showed low extent of hydrolysis by the glucoamylase even after prolonged treatment or at high enzyme concentrations compared with cereal starches. The hydrolytic extent of complexes was decreased by the heat treatment without development of the crystallinity. During the hydrolysis of the heat-treated complex, it was found that the complexed lipids were hardly released, except for unsaturated FA, and the glucoamylase-resistant complex showed decreasing ability to adsorb enzyme compared with the intact complex. The results indicate that, in contrast to α-amylase (Seneviratne and Biliaderis 1991), the complex-forming portion itself is strongly resistant to the action of glucoamylase.

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