Preparation and General Properties of Glucoamylase Bound to Halogenacetyl Cellulose†

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The coupling reaction of glucoamylase and halogenacetyl cellulose (HAC) without pretreating with organic solvent led to form large particles of immobilized glucoamylase and the activity and the specific activity of the preparation were very low. However, the coupling reaction with HAC pretreated with organic solvents allowed to form very fine particles and the activity was increased by five times. The latter contained 3~6% of enzyme protein and the specific activity to maltose reached to 80~90% of native glucoamylase. Since the specific activity of the preparation was presumed to be much influenced by the particle size, the specific activity and general properties of different particle sizes were compared with those of native enzyme. The specific activities of particles of 0~15 μ , 15~55 μ , 70~190 μ and 130~270 μ showed 82%, 33%, 27% and only 7% of native enzyme, respectively. K_m values of native form, 0~15 μ , 15~55 μ and 70~190 μ particles were 0.90×10^{-3} M, 1.35×10^{-3} M, 1.60×10^{-3} M and 2.15×10^{-3} M in the case of maltose as substrate, respectively. The other properties of particles of 0~15 μ were almost identical to those of native enzyme except for the effect of temperature on the reaction rate. However, pH activity, pH stability and urea stability of particles of 70~190 μ were much inferior to those of native enzyme and particles of 0~15 μ .

Many studies have been reported on the preparations and the properties of insoluble enzymes.^{1,2)} Glucoamylase was also immobilized by physical adsorption to activated carbon³⁾ and by ionic adsorption to DEAE-cellulose.⁴⁾ In the previous paper,⁵⁾ the authors reported the general properties of immobilized glucoamylase which was prepared by coupling the native form to carboxymethyl cellulose(CMC)-azide. Further, the authors reported that glucose was produced continuously from the column packed with CMC-glucoamylase and sawdust by passing 35% liquified starch solution.⁶⁾ However, the preparation of CMC-azide from CMC was complicated and the enzyme content of the resulted preparation was only 2%. The coupling reaction of glucoamylase to halo-

genacetyl cellulose was selected as a way to prepare insoluble glucoamylase with a simpler process and to get a preparation of higher enzyme content. This coupling reaction was reported by A. Patchornik⁷⁾ to prepare insoluble trypsin and chymotrypsin, but the detailed information has not yet been reported. In this paper, the authors report that the preparation of immobilized glucoamylase using HAC was improved to get higher activity of immobilized glucoamylase and that the specific activity and some general properties of the preparations were much influenced by its particle size.

MATERIALS AND METHODS

1. *Materials.* Glucoamylase originating from *Rhizopus niveus* was purchased from Seikagaku Kogyo Co., Ltd. It contained over 95% of protein and was homogeneous on Tiselius electrophoresis. The specific

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activity to maltose was 5.5 U/mg and that to amylose was 18 U/mg. These values of specific activity are often quoted as original activities in this paper. Crystalline trypsin was a product of Boehringer Co., Ltd and the specific activity to casein was 920 U/mg. The definition of enzyme unit will be described later. Microcrystalline cellulose preparations were used for thin-layer chromatography (Avicel TL) and column chromatography (Avicel CC). The former had particle size of $0\sim 50\ \mu$ in diameter and the latter had particle size of $20\sim 300\ \mu$ in diameter. The microcrystalline cellulose was purchased from Asahi Chemical Industry Co., Ltd.

2. *Fractionation of particles of microcrystalline cellulose.* Avicel TL was fractionated into equal weight of small and large particles by sedimentation in methanol. Avicel CC was fractionated into each particle size by sifting.

3. *Preparation of amylose.* Amylose was prepared by Azumi's method⁸⁾ to separate amylose as butanol complex.

4. *Assay of glucoamylase activity.* Standard condition of enzyme assay was carried out at 40°C by the method previously described.⁵⁾ One unit was defined as the amount of enzyme releasing 1 μ mole of glucose in one minute at pH 4.5 at 40°C . The effects of enzyme concentration of glucoamylase bound to bromoacetyl cellulose (BAC-glucoamylase) on the reaction rates to maltose and to amylose are shown in Fig. 1 and 2 respectively.

5. *Determination of K_m .* Michaelis constant was determined by the method previously described.⁵⁾

6. *Assay of caseinolytic activity.* The enzyme reaction was carried out by incubating a mixture of 5 ml of 0.6% casein solution dissolved in 0.05 M phosphate buffer (pH 7.0) and 1 ml of native enzyme solution or suspension of insoluble enzyme at 30°C for 10 min. The incubation was static in the case of native enzyme and was shaken at 130 rpm in the case of insoluble enzyme. The enzyme reaction was stopped by adding 5 ml of the solution containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid. The absorbance of the supernatant at 275 $m\mu$ was measured. One unit was defined as the activity liberating the hydrolysate which was corresponded to absorbance of 1 μ g of tyrosine for one minute by the enzyme.

7. *Assay of protein content in insoluble enzyme.*

FIG. 1. Effect of Concentration of BAC-Glucoamylase on the Reaction Rate: Substrate; Maltose.

BAC-glucoamylase: Glucoamylase bound to bromoacetyl cellulose. The enzyme assay was carried out under standard conditions with varied amounts of enzyme. Figures in () are the amounts of bound enzyme.

○—○ BAC-glucoamylase of $0\sim 15\ \mu$.
 △—△ BAC-glucoamylase of $70\sim 190\ \mu$.

FIG. 2. Effect of Concentration of BAC-Glucoamylase on the Reaction Rate: Substrate; Amylose.

The enzyme assay was carried out under standard conditions with varied amounts of enzyme. Figure in () are the amounts of bound enzyme.

○—○ BAC-glucoamylase of $0\sim 15\ \mu$.

Five ml of the suspension of insoluble enzyme was added to the digestion flask and was condensed to 1~2 ml by heating. After the addition of 20 mg of copper sulfate, 180 mg of potassium sulfate and 3 ml of conc. sulfuric acid to it, the mixture was digested by heating vigorously for 3 hr. After digestion, the mixture was diluted with distilled water and was adjusted to pH 12~13 by sodium hydroxide solution. The copper hydroxide formed was removed by filtration and nitrogen in the filtrate was determined by Morita and Kogure's method.⁹⁾ The value multiplied the amount of nitrogen by 6.25 was regarded as the amount of bound enzyme.

In the case of the sample containing iodine, the pretreatment was carried out before digestion as follows. Five ml of the suspension and 0.5 ml of 20% sodium hydroxide solution were added to the digestion flask and the mixture was condensed to 1~2 ml by heating. After cooling, 1 ml of conc. sulfuric acid and several drops of 30% hydrogen peroxide solution were added to the mixture.

8. *Assay of bromine and iodine.* Bromine was determined by Ota's method using the combustion flask.¹⁰⁾ Iodine was determined by colorimetry,¹¹⁾ after 15 mg of iodoacetyl cellulose was boiled with 20 ml of 0.1 N sodium hydroxide solution in the beaker.

9. *Determination of particle size of insoluble enzyme.* One or two drops of uniform suspension of insoluble enzyme was taken on a slide glass and photographs of several hundred of particles were taken at random using microscopy. The number frequency was determined by the following method. The major diameter of particles in photograph was measured and particles were assorted at regular intervals, such as 5 μ , 10 μ or 20 μ . Secondly, the ratio of minor diameter to major diameter of particles in each sample was measured and its average was calculated. The result was 0.667 for glucoamylase bound to bromoacetyl cellulose of 0~15 μ , 0.5 for 15~55 μ , 0.333 for 70~190 μ and 0.667 for 130~270 μ , respectively. The weight frequency was determined as follows.

$$fw(\%/\mu) = \frac{Dp^3 \cdot (Sd/Ld)^2 \cdot fn}{\sum Dp^3 \cdot (Sd/Ld)^2 \cdot fn}$$

fw , the weight frequency at regular intervals.

fn , the number frequency at regular intervals.

Dp , mean major diameter of particles at regular intervals.

(Sd/Ld) , the ratio of minor diameter to major diameter.

10. *Preparation of halogenacetyl cellulose (HAC).*

1) *Preparation of Bromoacetyl cellulose (BAC).*

Particles below 350 mesh, 350~150 mesh and 150~100 mesh fractionated from Avicel TL and Avicel CC were bromoacetylated as follows, respectively. The mixture of 4.5 g of cellulose, 25 g of bromoacetic acid and 15 ml of dioxane was stirred overnight at room temperature. Then, 25 g of bromoacetyl bromide was added and the mixture was stirred for 8 hr at 10~20°C. This mixture was poured into ice water and the white precipitate formed was washed several times with distilled water, aqueous sodium bicarbonate solution and then again with water. The product was sifted out in water and was dried in vacuum. These bromine content was 8~16%.

2) *Preparation of iodoacetyl cellulose (IAC).* The mixture contained 6 g of sodium iodide, 50 ml of ethanol and 2 g of BAC prepared from small particles in Avicel TL (abbreviated as BAC (small)) was stirred overnight at room temperature to replace bromine in BAC by iodine. The product was washed with ethanol, aqueous sodium bicarbonate solution and then again with ethanol and was dried in vacuum. The iodine content was 19% and the bromine content was below 1%.

11. *Standard preparation of insoluble enzyme.* As a carrier 300 mg of BAC or IAC was suspended in 2 ml of water or organic solvent. In the latter, a part or all of BAC or IAC was dissolved into organic solvents. Organic solvents and water thus used were simply referred to as solvent. Twenty ml of 0.1 M phosphate buffer (pH 8.4) and 18 ml of enzyme solution containing 30 mg of enzyme were added to the above suspension and the coupling reaction was carried out on a stirrer at 5°C overnight. The product was washed with distilled water, 0.5 M phosphate buffer (pH 6.0) and then again with distilled water and was stored as the aqueous suspension of water at 5°C. Glucoamylase bound to BAC was denoted as BAC-glucoamylase and glucoamylase bound to IAC as IAC-glucoamylase.

12. *Preparation of various particle size of BAC-glucoamylase*

1) *Particles of 0~15 μ .* BAC-glucoamylase of 0~15 μ was prepared under standard condition by using BAC (small) and dimethylsulfoxide (DMSO) as solvent and was fractionated by sedimentation in water. Particles of 0~15 μ could also be obtained by

the another method as follows. The BAC-glucoamylase was prepared under standard preparation by using water as solvent after crashing 300 mg of BAC with 300 mg of silica sand in a mortar and the particles of 0~15 μ were fractionated by sedimentation.

2) *Particles of 15~55 μ .* BAC-glucoamylase of 15~55 μ was prepared under standard preparation by using particles of BAC smaller than 350 mesh and water as solvent and was fractionated by sedimentation in water.

3) *Particles of 70~190 μ and 130~270 μ .* BAC-glucoamylases of 70~190 μ and 130~270 μ were prepared by the following method. The reaction mixture of 300 mg of 350~150 mesh of BAC or 150~100 mesh of BAC, 2 ml of glucoamylase solution dissolved 30 mg of enzyme and 1 ml of 0.1 M phosphate buffer (pH 8.4) was shaken overnight at 5°C after adjusting to pH 6.5~7.5. The product was washed as described in the standard preparation and was shifted in water to each particle size.

RESULTS

I. Preparation of BAC-glucoamylase

1. *Effects of organic solvents.* BAC-glucoamylase was prepared according to A. Patchornik's report⁷⁾ by using water as solvent, however, its activity was low and the protein content was only 2.5%. Accordingly, as a result of investigation for the purpose of increasing the activity and the protein content they could be increased by the procedure as follows. BAC was suspended in organic solvents instead of water and part or all of BAC was dissolved at first and the dissolved BAC was regenerated by adding buffer solution to it. The high activity of BAC-glucoamylase was prepared by coupling glucoamylase to the regenerated BAC. The results are shown in Table I. Among organic solvents, DMSO and methylcellosolve were very effective and the activities were increased by about four times and about five times, respectively, by using BAC regenerated from DMSO and methylcellosolve. The specific activities of these preparations increased by three times and

the protein contents could be also increased. They were stable in aqueous suspension for several months at 5°C.

2. *Effect of pH and enzyme concentration on the amount of bound enzyme.* Glucoamylase was immobilized at varying pH values in Table II by using BAC(small) and acetone as the solvent and the effect of pH was investigated. The preparation at pH 8.4 contained the highest amount of bound enzyme.

Glucoamylase was immobilized by using BAC (small) and from 10 mg to 40 mg of glucoamylase as shown in Table III and the effect of enzyme concentration was investigated. The amount of bound enzyme increased in proportion to the increase of enzyme concentration (as shown in Table III), but the remained enzyme in the reaction mixture also increased in proportion to it.

Subsequently, glucoamylase was immobilized by decreasing the volume of the reaction mixture, keeping constantly the amount of added enzyme as follows. After 300 mg of BAC (small) was dissolved into 1 ml of DMSO, 1 ml of acetone was added to the solution. After cooling at 5°C, 1 ml of 0.5 M phosphate buffer (pH 8.4) and 5 ml of enzyme solution containing 30 mg of glucoamylase were added to the suspension and then incubated and stirred overnight at 5°C. The product was washed according to the standard procedure. A yield of 285 mg of BAC-glucoamylase was obtained. The protein content of the preparation increased to 5.9% and the total activity to maltose was 55 U. The specific activity was 3.4 U/mg and was 62% of original activity. The protein content could be increased to 5.9% by this procedure.

II. Preparation of IAC-glucoamylase

Glucoamylase was immobilized according to the standard procedure by using IAC as a carrier and the preparations were com-

TABLE I. PREPARATION OF BAC-GLUCOAMYLASE AND EFFECT OF ORGANIC SOLVENTS

Solvent	Yield (mg)	Bound protein (mg)	Protein content (%)	Activity to maltose			Activity to amylose		
				Total activity (U)	Specific activity (U/mg)	% of original activity (%)	Total activity (U)	Specific activity (U/mg)	% of original activity (%)
BAC-GA	234	5.9	2.5	9.4	1.6	29	4.2	0.7	4
BAC-GA	271	7.0	2.6	23.3	3.3	60	7.9	1.1	6
BAC-GA	210	8.4	4.0	40.5	4.8	87	21.0	2.5	14
BAC-GA	275	6.8	2.5	28.2	4.2	76	18.6	2.8	16
BAC-GA methyl cellosolve	254	9.4	3.9	48.7	4.9	90	23.8	2.4	13

BAC-GA: Glucoamylase bound to bromoacetyl cellulose. BAC-GA was prepared by using 300 mg of BAC and 28.5 mg of glucoamylase (specific activity: 5.5 U/mg in the case of maltose, 18 U/mg in the case of amylose (M.W. 1,950,000)). BAC was prepared from Avicel TL.

pared with those from BAC. As for the protein content and the activity, the data of IAC-glucoamylase were almost identical to those of BAC-glucoamylase as shown in Table IV. Considering the organic solvents, acetone and DMSO were also effective and DMSO was superior in this case.

III. Effect of particle size on the activity

Since the particle size of BAC-glucoamylase prepared by using water as the solvent was observed to be much larger than that of BAC-glucoamylase prepared by using organic solvents, the particle size was presumed to be closely related to the specific activity. Although it was ideal to fractionate particles to every size from the same sample of BAC-glucoamylase by any method, it is very difficult in practice. Accordingly, each particle of BAC-glucoamylase was prepared as described in experimental methods and the relation between particle size and the specific activity was investigated. The results are shown in Table V and the descriptions of the particle size used in this work are shown in Table VI and Table VII. The data in Table V indicate that the specific activities of particles were much influenced by their own particle size. Especially, the specific activity of particles of 0~15 μ showed over 60% of original activity. Whereas the specific activity was only 7% of original activity in the case of particles of 130~270 μ . However, in the case of particles of 0~15 μ , the specific activity seemed to be slightly influenced by their protein content, therefore the protein content might be a factor to influence the specific activity.

IV. General properties of BAC-glucoamylase

The general properties of BAC-glucoamylase of 0~15 μ prepared by using DMSO as the solvent and BAC-glucoamylase of 70~190 μ

TABLE II. THE pH DEPENDENCE OF THE BINDING OF GLUCOAMYLASE ON BROMOACETYL CELLULOSE

Exp. No.	pH in the mixture	BAC-(small)-GA (mg)	Bound enzyme (mg)	Protein content (%)	Activity to maltose		
					Total activity (U)	Specific activity (U/mg)	% of original activity (%)
1	pH 8.4	280	7.3	2.6	38.6	5.3	96
2	pH 7.0	302	6.3	2.1	26.6	4.3	78
3	pH 5.5	287	6.4	2.2	27.6	4.3	78
4	pH 4.0	275	4.8	1.7	23.4	4.9	89

BAC(small)-GA: Glucoamylase bound to bromoacetyl cellulose (small). BAC(small)-GA was prepared by using 300 mg of BAC(small), 28.5 mg of glucoamylase and acetone as the solvent. BAC(small) was prepared from small particles in Avicel TL. The reaction mixtures were buffered at pH shown by 0.1 M phosphate buffer (pH 8.4 and pH 7.0) and 0.2 M acetate buffer (pH 5.5 and pH 4.0).

TABLE III. RELATION BETWEEN THE AMOUNT OF GLUCOAMYLASE AND BOUND GLUCOAMYLASE

Exp. No.	Native enzyme added (A) (mg)	BAC(small)-GA (mg)	Bound enzyme(B) (mg)	(B)/(A) (%)	Activity to maltose		
					Total activity (U)	Specific activity (U/mg)	% of original activity (%)
1	10	275	5.6	56	26.1	4.7	85
2	20	277	7.4	37	33.5	4.5	82
3	30	285	10.9	36	39.6	3.6	65
4	40	273	11.7	27	50.0	4.3	78

BAC(small)-GA was prepared by using 300 mg of BAC(small), acetone as the solvent and each amount of glucoamylase.

were compared with those of native glucoamylase.

1. *K_m*. *K_m* of native glucoamylase and BAC-glucoamylase of 0~15 μ , 15~55 μ and 70~190 μ was determined. In the case of maltose as substrate, *K_m* of native one, BAC-glucoamylase of 0~15 μ , 15~55 μ and 70~190 μ was 0.90×10^{-3} M, 1.35×10^{-3} M, 1.60×10^{-3} M and 2.15×10^{-3} M, respectively. In the case of amylose as substrate, *K_m* values of native one and BAC-glucoamylase of 0~15 μ were 1.00×10^{-7} M and 1.50×10^{-7} M, respectively. Activities to amylose of particles of 15~55 μ and 70~190 μ were so low that their *K_m* could not be estimated exactly. *K_m* of BAC-glucoamylase increased a little more than that

of native enzyme and *K_m* of the large particles was recognized to have a tendency to increase a little, as compared with that of the small particles.

2. *Effect of temperature on the reaction rate*. Effect of temperature on the reaction rate of BAC-glucoamylase was almost identical to that of native enzyme in the case of maltose as the substrate as shown in Fig. 5. However, in the case of amylose as the substrate, the action of BAC-glucoamylase was inferior to that of native enzyme as shown in Fig. 6.

3. *Heat stability*. Their heat stability was almost identical to it of native enzyme as shown in Fig. 7.

TABLE IV PREPARATION OF IAC-GLUCOAMYLASE AND EFFECT OF ORGANIC SOLVENTS.

Solvent	Yield (mg)	Bound protein (mg)	Protein content (%)	Activity to maltose			Activity to amylose		
				Total activity (U)	Specific activity (U/mg)	% of original activity (%)	Total activity (U)	Specific activity (U/mg)	% of original activity (%)
IAC-GA	294	4.7	1.6	11.6	2.5	45	4.7	1.0	6
IAC-GA	278	7.2	2.6	25.6	3.5	64	6.1	0.8	5
IAC-GA	291	11.9	4.1	67.4	5.7	104	36.1	3.0	17

IAC-GA: Glucoamylase bound to iodoacetyl cellulose. IAC-GA was prepared by using 300 mg of IAC and 28.5 mg of glucoamylase. IAC was prepared from small particles in Avicel TL.

FIG. 3. Hofstee Plots of BAC-Glucoamylase: Substrate; Maltose.

The enzyme assay was carried out under standard assay of K_m . In the case of native glucoamylase, BAC-glucoamylase of 0~15 μ , BAC-glucoamylase of 15~55 μ and BAC-glucoamylase of 70~190 μ , 79 μ g, 8.0 mg (bound enzyme 348 μ g), 15.1 mg (bound enzyme 270 μ g) and 35.4 mg (bound enzyme 638 μ g) were used for the enzyme assay, respectively.

x—x Native glucoamylase
o—o BAC-glucoamylase of 0~15 μ .
●—● BAC-glucoamylase of 15~55 μ .
Δ—Δ BAC-glucoamylase of 70~190 μ .

FIG. 4. Hofstee Plots of BAC-Glucoamylase: Substrate; Amylose.

The enzyme assay was carried out under standard assay of K_m . In the case of native glucoamylase and BAC-glucoamylase of 0~15 μ , 79 μ g and 10.7 mg (bound enzyme 464 μ g) were used for the enzyme assay, respectively.

x—x Native glucoamylase; o—o BAC-glucoamylase of 0~15 μ .

FIG. 5. Effect of Temperature on the Reaction Rate: Substrate; Maltose.

The enzyme assay was carried out under standard conditions except for each incubation temperature. The reaction rate obtained at 40°C was taken as 100%. Respectively, 79 μ g of native glucoamylase, 4.0 mg (bound enzyme 174 μ g) of BAC-glucoamylase of 0~15 μ and 16.4 mg (bound enzyme 290 μ g) of BAC-glucoamylase of 70~190 μ were used for the enzyme assay.

x—x Native glucoamylase; O—O BAC-glucoamylase of 0~15 μ ; Δ — Δ BAC-glucoamylase of 70~190 μ .

FIG. 6. Effect of Temperature on the Reaction Rate: Substrate; Amylose.

The enzyme assay was carried out under standard conditions except for each incubation temperature. The reaction rate obtained at 40°C was taken as 100%. In the case of native glucoamylase, 16 μ g was used for the enzyme assay. In the case of BAC-glucoamylase, 2.0 mg (bound enzyme 87 μ g) was used in range of 25°C to 45°C and 1.0 mg (bound enzyme 44 μ g) was used in range of 50°C to 60°C.

x—x Native glucoamylase; O—O BAC-glucoamylase of 0~15 μ .

TABLE V. EFFECT OF PARTICLE SIZE ON THE ACTIVITY

	Particle size (μ)	Protein content (%)	Activity to maltose			Activity to amylose		
			Activity (U/g)	Specific activity (U/mg)	% of original activity (%)	Activity (U/g)	Specific activity (U/mg)	% of original activity (%)
BAC-GA (A)	0~15	4.3	194	4.5	82	91.0	2.1	12
BAC-GA (B)	0~15	3.0	115	3.8	70	63.6	2.1	12
BAC-GA (C)	0~15	1.2	39.8	3.3	60	22.9	2.0	11
BAC-GA	15~55	1.8	32.4	1.8	33	13.1	0.73	4
BAC-GA	70~190	1.8	26.5	1.5	27	traces	—	—
BAC-GA	130~270	2.3	9.6	0.36	7	—	—	—

The weight frequency distribution of particle size of the above BAC-GA and their preparations will be described in Table VI and Table VII.

FIG. 7. Heat Stability.

A mixture of 1 ml of enzyme solution or suspension and 4 ml of 0.1 M acetate buffer (pH 4.5) was preincubated for 15 min at each temperature, rapidly cooled and 5 ml of 2% maltose solution was added for the enzyme assay. Respectively, 79 μ g of native glucoamylase, 4.0 mg (bound enzyme 174 μ g) of BAC-glucoamylase of 0~15 μ and 8.7 mg (bound enzyme 156 μ g) of BAC-glucoamylase of 70~190 μ were used for the enzyme assay.

×—× Native glucoamylase; ○—○ BAC-glucoamylase of 0~15 μ ; Δ—Δ BAC-glucoamylase of 70~190 μ .

4. *pH activity and pH stability.* Their optimum pH values were almost identical to it of native enzyme as shown in Fig. 8, however the reaction rate of BAC-glucoamylase of 70~190 μ in the range of pH 2~4 was inferior to them of native one and BAC-glucoamylase of 0~15 μ .

Figure 9 showed that pH stability of BAC-glucoamylase of 70~190 μ was much inferior to those of native enzyme and BAC-glucoamylase of 0~15 μ in the range of pH 2~4 and was slightly inferior in the range of pH 8~10.

5. *Urea stability.* The reaction rates in the presence of urea were assayed and the

FIG. 8. pH-Activity Curves.

The enzyme assay (maltose as substrate) was carried out under standard conditions except for buffer employed. Respectively, 79 μ g of native glucoamylase, 4.0 mg (bound enzyme 174 μ g) of BAC-glucoamylase of 0~15 μ and 8.7 mg (bound enzyme 156 μ g) of BAC-glucoamylase of 70~190 μ were used for the enzyme assay. At pH 2.20~3.0, pH 4.0~5.5 and pH 6.0~8.0, tartarate buffer, acetate buffer and phosphate buffer of 0.5 of ionic strength were used, respectively.

×—× Native glucoamylase; ○—○ BAC-glucoamylase of 0~15 μ ; Δ—Δ BAC-glucoamylase of 70~190 μ .

results are shown in Fig. 10. It was found that the stability of BAC-glucoamylase of 0~15 μ in the presence of urea was almost identical to that of native enzyme, however the stability of BAC-glucoamylase of 70~190 μ against urea was much inferior to the former.

DISCUSSION

The preparation of immobilized trypsin and chymotrypsin by coupling with halogenacetyl cellulose (HAC) was reported by A. Patchornik,⁷⁾ however the preparation has not been investigated in detail up to this time. The authors prepared immobilized glucoamylase by HAC according to A. Patchornik's method. However, the activity and the protein content

FIG. 9. pH Stability.

A mixture of 1 ml of enzyme solution or the suspension and 1 ml of 0.2 of ionic strength of buffer was incubated for 48 hr at 30°C and after adjusting to about pH 4.5, the enzyme assay (maltose as substrate) was carried out under standard conditions. At pH 1.4, pH 2.4~3.0, pH 3.8~5.0, pH 6.0~8.0 and pH 8.4~10.1, hydrochloric acid-acetic acid buffer, tartarate buffer, acetate buffer, phosphate buffer and ammonium buffer were used, respectively. Respectively, 79 μ g of native glucoamylase, 8.0 mg (bound enzyme 348 μ g) of BAC-glucoamylase of 0~15 μ and 13.3 mg (bound enzyme 340 μ g) of BAC-glucoamylase of 70~190 μ were used for the enzyme assay.

×—× Native glucoamylase; ○—○ BAC-glucoamylase of 0~15 μ ; Δ—Δ BAC-glucoamylase of 70~190 μ .

of the BAC-glucoamylase were low. Accordingly, as a result of investigation of the improvement to increase the activity and the protein content, the authors improved A. Patchornik's method as follows. HAC was at first suspended in organic solvents, such as DMSO and methylcellosolve, and was plenti-

FIG. 10. Effect of Urea Concentration on the Reaction Rate.

The enzyme assay (maltose as substrate) was carried out under standard conditions in presence of each urea concentration. Respectively, 79 μ g of native glucoamylase, 5.1 mg (bound enzyme 230 μ g) of BAC-glucoamylase of 0~15 μ and 13.3 mg (bound enzyme 340 μ g) of BAC-glucoamylase were used for the enzyme assay.

×—× Native glucoamylase; ○—○ BAC-glucoamylase of 0~15 μ ; Δ—Δ BAC-glucoamylase of 70~190 μ .

TABLE VI. PARTICLE SIZE OF BAC-GLUCOAMYLASE (1)

BAC-glucoamylase of 0~15 μ (A)

Particle size		The number frequency distribution (number)	The weight frequency distribution (%)
Range of diameter (μ)	Mean diameter (μ)		
0~5	2.5	988	14
5~10	7.5	108	38
10~15	12.5	28	48
total		1124	100

The above BAC-glucoamylase was prepared under standard preparation by using DMSO as solvent and was fractionated.

BAC-glucoamylase of 0~15 μ (B)

Particle size		The number frequency distribution (number)	The weight frequency distribution (%)
Range of diameter (μ)	Mean diameter (μ)		
0~5	2.5	2208	22
5~10	7.5	253	68
10~15	12.5	6	7
15~20	17.5	1	3
total		2473	100

The above BAC-glucoamylase was prepared under standard preparation after crashing 300 mg of BAC with 300 mg of silica sand in mortar and was fractionated. Organic solvent was not used.

BAC-glucoamylase of 0~15 μ (C)

Particle size		The number frequency distribution (number)	The weight frequency distribution (%)
Range of diameter (μ)	Mean diameter (μ)		
0~5	2.5	3433	21
5~10	7.5	272	44
10~15	12.5	39	29
15~20	17.5	3	6
total		3747	100

The above BAC-glucoamylase was prepared under standard preparation except for using 20 mg of glucoamylase after crashing as described the above and was fractionated. Organic solvent was not used.

TABLE VII. PARTICLE SIZE OF BAC-GLUCOAMYLASE (2)

BAC glucoamylase of 15~55 μ .

Particle size		The number frequency distribution (number)	The weight frequency distribution (%)
Range of diameter (μ)	Mean diameter (μ)		
0~10	5	81	0
10~20	15	149	18
20~30	25	56	31
30~40	35	18	27
40~50	45	6	19
50~60	55	1	6
total		311	101

The above BAC-glucoamylase was prepared under standard preparation without organic solvent and was fractionated.

BAC-glucoamylase of 70~190 μ and BAC-glucoamylase of 130~270 μ .

Particle size		The number frequency distribution (number)		The weight frequency distribution (%)	
Range of diameter (μ)	Mean diameter (μ)	70~190 μ of BAC-glucoamylase	130~270 μ of BAC-glucoamylase	70~190 μ of BAC-glucoamylase	130~270 μ of BAC-glucoamylase
0~20	10	22	—	—	—
20~40	30	127	1	1	—
40~60	50	114	8	2	—
60~80	70	88	18	5	—
80~100	90	87	28	11	1
100~120	110	76	42	17	2
120~140	130	50	59	19	6
140~160	150	22	69	13	10
160~180	170	13	86	11	18
180~200	190	4	79	5	23
200~220	210	6	36	9	14
220~240	230	4	24	8	12
240~260	250	—	9	—	6
260~280	270	—	6	—	5
280~300	290	—	3	—	3
total		613	468	101	100

The above BAC-glucoamylases were prepared under standard preparation.

TABLE VIII. CASEINOLYTIC ACTIVITY OF TRYPSIN BOUND TO HALOGENACETYL CELLULOSE

	Solvent	Yield (mg)	Protein content (%)	Total activity (U)	Specific activity (U/mg)	% of original activity
BAC (small)-T	water	288	2.8	751	93	10
	acetone	283	4.5	1,190	93	10
	DMSO	278	4.7	2,230	171	19
IAC-T	water	294	2.5	765	104	11
	acetone	278	4.2	1,123	96	10
	DMSO	291	5.1	2,521	170	19

BAC(small)-T; Trypsin bound to BAC(small). IAC-T; trypsin bound to IAC. The above immobilized trypsin was prepared by using 300 mg of BAC or IAC and 30 mg of trypsin (specific activity: 920 U/mg in the case of casein as substrate)

fully dissolved in organic solvents in large amount. Buffer solution was added to this solution and the dissolved HAC was regenerated. Glucoamylase solution was added to the suspension and the coupling reaction between glucoamylase and the regenerated HAC was started. Since the regenerated HAC was brittle, it was easily ground up to fine particles by

stirring during the coupling reaction and very fine particles of HAC-glucoamylase were prepared. Total activity of HAC-glucoamylase prepared by this method increased by about five times and the specific activity increased by about three times, as compared with the preparation by A. Patchornik's method.

Optimum pH in this coupling reaction was

observed at pH 8.4 and higher enzyme concentration in the reaction mixture allowed to prepare higher enzyme content of the preparation.

As a result of investigating the cause of effect of organic solvents on the activity of the resulted preparation, the authors presumed that the activity of BAC-glucoamylase was much influenced by the particle size. In fact, particles of $0\sim 15\mu$ had much higher specific activity than that of particles over 15μ as shown in Table V. The close relation between the particle size and the specific activity was presumed by the authors in the previous paper relating to the preparation of CMC-glucoamylase and P. Bernfeld *et al.*,¹²⁾ however its relation could be proved more clearly by this experiment. The effect of organic solvent on the activity of BAC-glucoamylase was developed to the preparation of BAC-trypsin and IAC-trypsin. The results were shown in Table VIII. The effect of organic solvents on the activity and the protein content was also observed in this case.

General properties of BAC-glucoamylase of $0\sim 15\mu$ and $70\sim 190\mu$ were compared with those of native glucoamylase. K_m of BAC-glucoamylase increased slightly, as compared with that of native enzyme and K_m of large particles had a tendency to increase than it of the fine particles. Accordingly, it was concluded that the absolute K_m of BAC-glucoamylase could hardly be determined. The other general properties of BAC-glucoamylase of $0\sim 15\mu$ were almost identical to

those of native enzyme except for the effect of temperature on the reaction rate to amylose. However, pH activity, pH stability and urea stability of BAC-glucoamylase of $70\sim 190\mu$ were much inferior to those of native one and BAC-glucoamylase of $0\sim 15\mu$. Although the authors can not clearly explain the reason at present, it is very interesting phenomena that the specific activity and general properties of BAC-glucoamylase are influenced by the particle size of the preparation.

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