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Properties of *Rhizopus* sp. Glucoamylase Polymerized by Crosslinking with Glutaraldehyde

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A major glucoamylase [EC 3.2.1.3] from a *Rhizopus* sp., $Gluc_1$, was polymerized by crosslinking with glutaraldehyde. The polymerized $Gluc_1$ (named poly-$Gluc_1$) showed a drastic decrease (55%) in lysine content as well as a slight decrease (11%) in tyrosine content as compared with $Gluc_1$ and had an apparent molecular weight higher than 10^6 daltons as estimated by gel filtration on Sepharose 6B. The circular dichroism spectrum, together with the ultraviolet and fluorescence spectra, indicated that poly-$Gluc_1$ was different from $Gluc_1$ not only in the states of aromatic amino acid side chains but also in the protein backbone conformation. The stabilities of poly-$Gluc_1$ to pH and heat were not enhanced appreciably as compared with those of $Gluc_1$, though the heat stability showed a slight enhancement at 40—45 °C followed by a sharper decline at higher temperatures. For low-molecular-weight substrates, poly-$Gluc_1$ had similar kinetic parameters and activities to those of $Gluc_1$, whereas for high-molecular-weight substrates, poly-$Gluc_1$ showed 2—10 times higher K_m and 2—4.5 times lower V_{max} values and thus 2—4.5 times lower activities. Poly-$Gluc_1$ exhibited 25 times lower activity than $Gluc_1$ towards raw starch but was still tightly bound to it, suggesting that the lysine residues are not involved in the binding to raw starch.

Keywords——glucoamylase; *Rhizopus*; glutaraldehyde; crosslinking; polymerization; kinetic parameter; raw starch binding; raw starch digestion

Glutaraldehyde,[1] a bifunctional crosslinking reagent, has often been used to prepare enzyme-conjugated antibodies in enzyme immunoassay studies.[2] The conjugation of enzyme to antibody by this reagent does not always give only a 1 : 1 enzyme–antibody conjugate, but also gives a variety of conjugates containing variously polymerized forms of enzyme. Such polymerized forms of enzyme seem to differ more or less from the monomer form in enzymatic as well as physico-chemical properties. We made an attempt to conjugate a major glucoamylase [EC 3.2.1.3 α-D-(1→4)-glucan glucohydrolase] from a *Rhizopus* sp., $Gluc_1$,[3] as a label enzyme to antibodies by using glutaraldehyde and preliminarily found that a part of the conjugated product exhibited considerably low activity, as compared with $Gluc_1$ monomer, towards high-molecular-weight substrates, especially raw starch, but not towards low-molecular-weight substrates. Various enzymes have also been shown to be greatly stabilized by crosslinking with glutaraldehyde to form insoluble aggregates.[4a-d]

In the present work, therefore, we treated $Gluc_1$ with glutaraldehyde and examined the effect of this treatment on the physico-chemical and enzymatic properties of $Gluc_1$, including the kinetic properties, in some detail. For simplicity, $Gluc_1$ alone was treated with glutaraldehyde to obtain soluble polymerized $Gluc_1$, without any addition of antibodies. The polymerized $Gluc_1$ was also examined as regards behavior towards raw starch, and the results suggested no involvement of the lysine residues in the binding to raw starch.

Materials and Methods

Chemicals——Gluczyme (a commercial digestive from a *Rhizopus* sp.), used as a source of glucoamylase, was

generously supplied by Amano Pharmaceutical Co. Soluble starch and raw corn starch as substrates were purchased from Wako Pure Chemicals; the former was used after exhaustive dialysis against distilled water and the latter after successive washing with several changes each of distilled water and methanol, followed by drying over silica gel. Other substrates used were as follows: maltose (Wako Pure Chemicals), *p*-nitrophenyl α-D-glucopyranoside (PNPG) (Koch-Light Lab. Ltd.), amylopectin (Tokyo Kasei Co., Ltd.), and glycogen (E. Merck). The D-glucose oxidase reagent (Glucose C-Test Wako) and glutaraldehyde as a 25% aqueous solution were obtained from Wako Pure Chemicals; Sepharose 6B and Blue Dextran 2000 were from Pharmacia Fine Chemicals. All other chemicals were of analytical reagent grade.

Preparation of $Gluc_1$——The major glucoamylase, $Gluc_1$, from a *Rhizopus* sp. was purified from Gluczyme according to the method reported previously.[3]

Estimation of Protein——Protein contents were estimated by the method of Lowry *et al.*[5] with bovine serum albumin as a standard, or by measuring the absorbance at 280 nm; an $A_{cm}^{280\,nm}$ (1%) value of 13.2 was used for $Gluc_1$.[3]

Determination of Glucoamylase Activity——For routine assay, glucoamylase activity was determined with soluble starch as a substrate according to the D-glucose oxidase method described previously.[6] One unit of glucoamylase activity was defined as the amount of enzyme liberating 1 μmol of glucose per min under the specified conditions.

The enzymatic activity with raw starch as a substrate was determined as described previously.[7]

Determination of Kinetic Parameters——Kinetic parameters, K_m and V_{max}, were measured at pH 5.0 (0.1 M acetate buffer) and 37 °C, as described previously.[6] The substrates used were soluble starch, glycogen and amylopectin (0.03—0.2% for these three), maltose (1.0—7.0 mM), and PNPG (1.0—20 mM). The values of K_m and V_{max} were estimated from Hofstee plots for the former three substrates of high molecular weight and Lineweaver-Burk plots for the latter two of low molecular weight.

Estimation of Enzyme Bound to Raw Starch——Binding of the enzyme to raw starch was measured at pH 5.0 (0.01 M acetate buffer containing 0.1 M NaCl) and 4 °C and the percentage of bound enzyme was calculated, as described previously.[7]

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis——SDS–Polyacrylamide gel electrophoresis in 7% gel containing 1% SDS was carried out as described by Shapiro *et al.*[8] Denatured proteins were prepared by incubating proteins in boiling water for 5 min in a solution containing 1% SDS, 2% mercaptoethanol and 25% glycerol. Electrophoresis was performed at room temperature for 4 h at 8 mA per tube. After an electrophoretic run, protein in the gel was detected by staining with 0.05% Coomassie brilliant blue G-250 solution in 9% acetic acid containing 45% methyl alcohol.

Amino Acid Analysis——Samples of 0.2—0.3 mg protein were hydrolyzed with 6 N HCl in evacuated, sealed tubes at 110 °C for 24 h. The hydrolysates were analyzed by the method of Spackman *et al.*[9] with a Nihondenshi JLC-6AH amino acid analyzer.

Spectroscopic Measurements——Circular dichroism (CD) spectra were measured at 25 °C with a JASCO J-40 spectropolarimeter in cells of 0.2-cm and 0.5-cm light path for the wavelength regions of 200—250 and 250—320 nm, respectively; the protein concentrations used were 0.02% at 200—250 nm and 0.1% at 250—320 nm. All data are expressed as molar ellipticity ($[\theta]$).

Ultra violet (UV) absorption and fluorescence emission spectra were measured at 25 °C with a Shimadzu UV 200 spectrophotometer and a Shimadzu RF-502 spectrofluorometer, respectively.

Results

Preparation of Poly-$Gluc_1$

$Gluc_1$ (0.5%) was allowed to react with 0.2% glutaraldehyde in 0.01 M phosphate buffer, pH 7.0, at room temperature for 2 h; under these conditions $Gluc_1$ gave only a soluble product that had 57% of the original enzymatic activity. At the end of the reaction, the solution was subjected to gel filtration on Sepharose 6B. The elution profile of glutaraldehyde-treated $Gluc_1$ is shown in Fig. 1. A sharp and large peak appeared at the void volume of the column, followed by a small amount of slowly eluting materials, demonstrating heterogeneity of the reaction product. All the crosslinked enzyme forms, eluting at various positions from the void volume to that for $Gluc_1$ monomer, exhibited considerable activity. The fast-eluting material was pooled, dialyzed against distilled water, and stored at 4 °C until used as poly-$Gluc_1$. Freezing was unsuitable for poly-$Gluc_1$ since insoluble material tended to appear.

When poly-$Gluc_1$ was examined by SDS–polyacrylamide gel electrophoresis, it did not enter a 7% polyacrylamide gel at all, but formed a sharp band on the top of the gel (Fig. 2). Together with this result, the above elution profile from a Sepharose 6B column indicates that

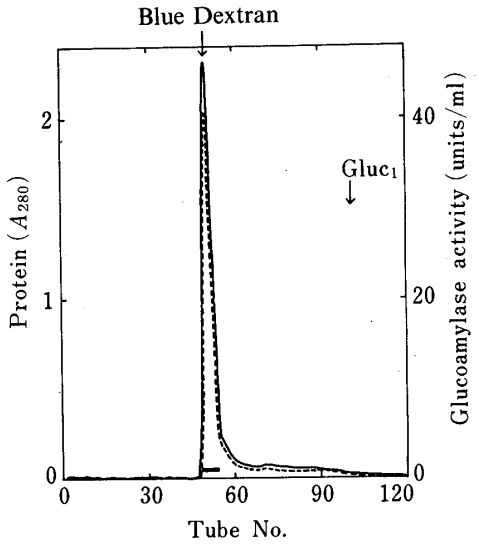

Fig. 1. Gel Filtration on Sepharose 6B of Glutaraldehyde-Treated $Gluc_1$

Twenty mg of $Gluc_1$ was allowed to react with 0.2% glutaraldehyde in 0.01 M phosphate buffer (pH 7.0) in a total volume of 4 ml. After incubation at 25 °C for 2 h with stirring, the reaction solution was applied to a column (2 × 110 cm) of Sepharose 6B equilibrated with 40 mM phosphate buffer (pH 6.8) containing 0.15 M NaCl and the column was eluted with the buffer. Fractions of 2.5 ml each were collected. The arrows indicate the positions expected for the elution of Blue Dextran 2000 and $Gluc_1$. The contents of the tubes designated by the bar were pooled for further studies.

——, $A_{280\,nm}$; ----, glucoamylase activity.

TABLE I. Amino Acid Composition of Poly-$Gluc_1$

Amino acid	Residues per molecule (nearest integer)	
	Poly-$Gluc_1$ [a]	$Gluc_1$ [b]
Trp	—	13
Lys	14	31
His	4	4
Arg	15	14
Asp	77	78
Thr	59	62
Ser	77	79
Glu	34	34
Pro	29	28
Gly	55	54
Ala	65	65
Cys 1/2	—	3
Val	34	35
Met	10	11
Ile	29	31
Leu	36	37
Tyr	33	37
Phe	25	26

a) No analyses for tryptophan and half-cystine were carried out. Values, which are per monomer equivalent, are not corrected for losses on hydrolysis. *b*) Cited from T. Takahashi *et al.*[3]

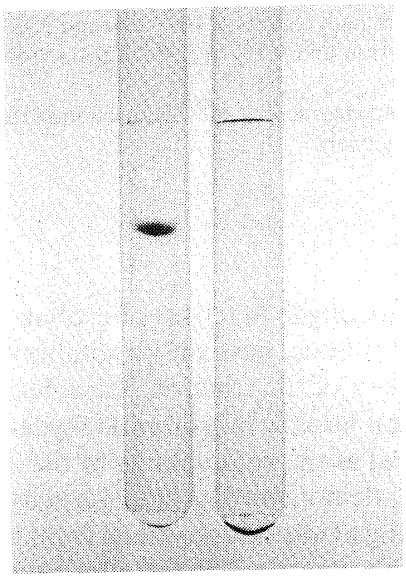

Fig. 2. SDS–Polyacrylamide Gel Electrophoresis of Poly-$Gluc_1$ in 7% Gel

About 10 μg of poly-$Gluc_1$ (right) and 10 μg of $Gluc_1$ (left) were applied.

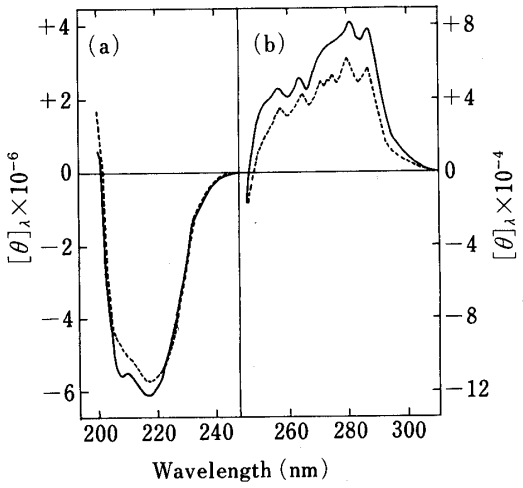

Fig. 3. CD Spectrum of Poly-$Gluc_1$

CD measurement was done for poly-$Gluc_1$ and also $Gluc_1$ in 0.01 M acetate buffer (pH 5.0) containing 0.1 M KCl. (a) CD spectra in the short wavelength region. (b) CD spectra in the long wavelength region. ----, poly-$Gluc_1$; ——, $Gluc_1$.

poly-$Gluc_1$ has an apparent molecular weight higher than 10^6 daltons.

Amino Acid Composition of Poly-$Gluc_1$

The amino acid composition of poly-$Gluc_1$ is shown in Table I. A drastic decrease in lysine content from 31 to 14 residues was noted on polymerization of $Gluc_1$; a small but significant decrease in tyrosine content from 37 to 33 residues was also seen. The variations for all other amino acids are probably within the range of error of the analyses. It is evident that glutaraldehyde reacted predominantly with the ε-amino groups of lysine in $Gluc_1$, and also partially with the phenolic rings of tyrosine residues, to form inter- and possibly also intra-molecular crosslinkages.

Spectroscopic Properties of Poly-$Gluc_1$

In order to detect the conformational changes accompanying the reaction with glutaraldehyde, the spectroscopic properties of poly-$Gluc_1$ were studied. The CD spectrum of poly-$Gluc_1$ in the short wavelength region (200—250 nm), which is indicative of the backbone

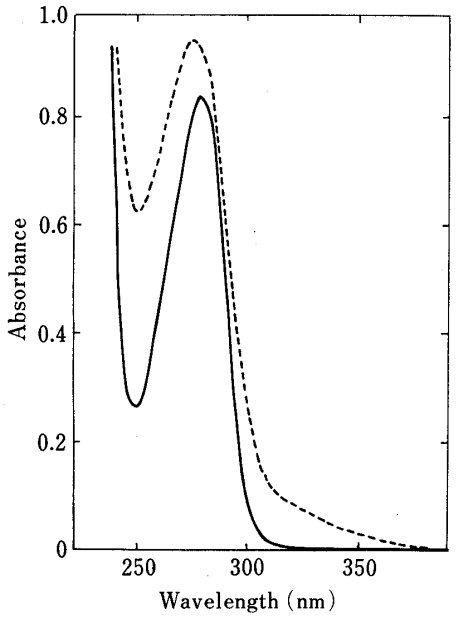

Fig. 4. UV Absorption Spectrum of Poly-$Gluc_1$

UV absorption spectra of poly-$Gluc_1$ (0.50 mg/ml) and $Gluc_1$ (0.63 mg/ml) were measured in 0.01 M acetate buffer (pH 5.0) containing 0.1 M KCl.
----, poly-$Gluc_1$; ——, $Gluc_1$.

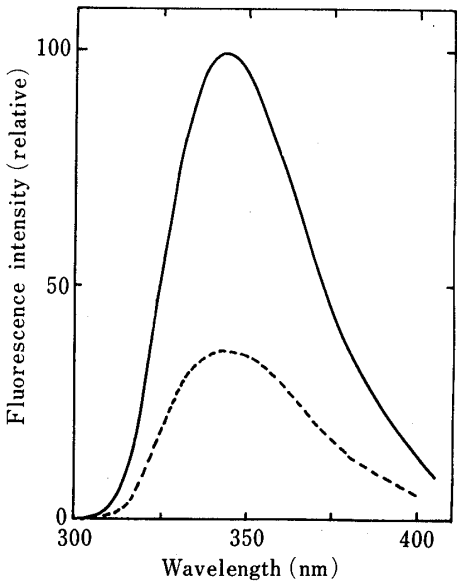

Fig. 5. Fluorescence Emission Spectrum of Poly-$Gluc_1$

Fluorescence emission spectra of poly-$Gluc_1$ and $Gluc_1$ (0.05 mg/ml for both) were measured in 0.01 M acetate buffer (pH 5.0) containing 0.1 M KCl. The excitation wavelength was 295 nm.
----, poly-$Gluc_1$; ——, $Gluc_1$.

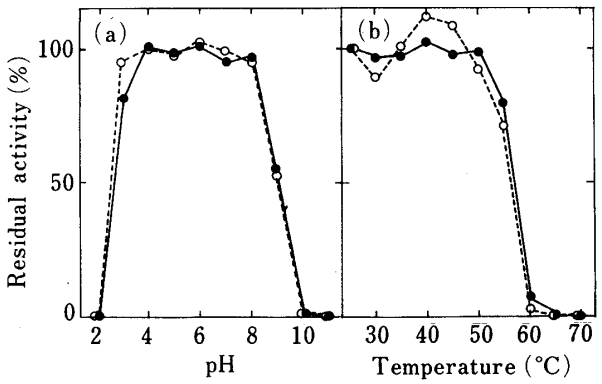

Fig. 6. pH and Heat Stabilities of Poly-$Gluc_1$

(a) pH Stability: Poly-$Gluc_1$ as well as $Gluc_1$ (each at 0.2 mg/ml) were incubated for 1 h at 37 °C in 0.01 M buffer (I=0.1) of various pH values. The solutions were immediately adjusted to pH 5.0 and their residual activity was assayed. The buffers used were glycine–HCl buffer for pH 2.0—3.0, acetate buffer for pH 4.0—6.0, borax–HCl buffer for pH 7.0—9.0 and borax–NaOH buffer for pH 10.0—11.0.

(b) Heat Stability: Poly-$Gluc_1$ as well as $Gluc_1$ (each at 0.2 mg/ml) were heated in 0.1 M acetate buffer (pH 5.0) for 5 min at various temperatures. The solutions were immediately cooled in an ice bath and their residual activity was assayed.
----, poly-$Gluc_1$; ——, $Gluc_1$.

conformation of protein, was superimposed on that of $Gluc_1$ at 225—250 nm, but at 200—225 nm the negative values of $[\theta]$ for poly-$Gluc_1$ were consistently lower than those for $Gluc_1$, with the disappearance of a shoulder shown by $Gluc_1$ at 207 nm (Fig. 3a). The CD spectrum of poly-$Gluc_1$ in the long wavelength region (250—320 nm), which reflects the states of the aromatic amino acid side chains in protein, was similar in shape to that of $Gluc_1$ but had decreased $[\theta]$ values, as compared with those of $Gluc_1$, over the whole wavelength region (Fig. 3b).

The UV absorption spectrum of poly-$Gluc_1$ showed a shift in the absorption maximum wavelength from 278 nm for $Gluc_1$ to 274 nm (Fig. 4). There was also a marked increase in absorbance over the whole wavelength region.

The fluorescence emission spectrum of poly-$Gluc_1$ excited at 295 nm again showed a shift in the maximum wavelength from 344 nm for $Gluc_1$ to 346 nm, with a 65% decrease in intensity at 344 nm (Fig. 5).

Stability of Poly-$Gluc_1$

To test the effect on pH stability of the crosslinking and its attendant conformational changes, poly-$Gluc_1$ was incubated for 1 h at 37 °C in 0.01 M buffer ($I=0.1$) of various pH values from 2.0—11.0 (Fig. 6a). Little difference was observed between the pH-stabilities of poly-$Gluc_1$ and $Gluc_1$.

On heating for 5 min at pH 6.0 and various temperatures from 25—100 °C, both poly-$Gluc_1$ and $Gluc_1$ again showed generally similar heat stability (Fig. 6b). It was noted, however, that the heat stability of poly-$Gluc_1$ showed a slight enhancement at 40—45 °C followed by a sharper decline at higher temperatures, as compared with that of $Gluc_1$.

Kinetic Parameters and Specific Activities of Poly-$Gluc_1$ for Various Substrates

It is of interest to see whether or not the polymerization of $Gluc_1$ causes any change in kinetic properties and activity. The kinetic parameters, K_m and V_{max} values, and the specific activities of poly-$Gluc_1$ for three high-molecular-weight substrates and two low-molecular-weight substrates are listed in Table II; those of $Gluc_1$ are also included for comparison.

For the large substrates, both the K_m and V_{max} values of poly-$Gluc_1$ differed markedly from the corresponding values of $Gluc_1$, whereas the kinetic parameters of poly-$Gluc_1$ for the small substrates differed little; the K_m values of poly-$Gluc_1$ for amylopectin, soluble starch and glycogen were about 2.3, 6.6 and 9.7 times higher and the V_{max} values were 4.5, 1.8 and 3.3 times lower, respectively.

As regards the specific activities, a similar situation to the above was observed. Although the specific activities of poly-$Gluc_1$ towards the small substrates did not differ appreciably

TABLE II. Kinetic Parameters and Specific Activities of Poly-$Gluc_1$ for Various Substrates

Substrate	K_m		V_{max} (units $\times 10^{-2}/\mu$mol)		Specific activity (units/mg)[a]	
	Poly-$Gluc_1$	$Gluc_1$	Poly-$Gluc_1$[b]	$Gluc_1$	Poly-$Gluc_1$	$Gluc_1$
Soluble starch	0.046%	0.0070%	26.6	47.3	34.4	63.4
Glycogen	0.039%	0.0040%	12.6	41.2	17.1	52.8
Amylopectin	0.0058%	0.0025%	11.5	51.7	16.2	66.4
Maltose	2.86 mM	2.38 mM	4.2	5.3	3.6	4.2
p-Nitrophenyl α-D-glucoside	2.90 mM	2.78 mM	0.16	0.19	0.13	0.15
Raw starch	—	—	—	—	0.99	24.7

a) All enzyme assays were carried out at pH 5.0 (0.1 M acetate buffer) and 37 °C. The substrate concentrations used were: 1% for soluble starch, glycogen and amylopectin, 4 mM for maltose and PNPG, and 20 mg/ml for raw starch. *b*) The V_{max} values for poly-$Gluc_1$ are expressed in terms of monomer equivalent.

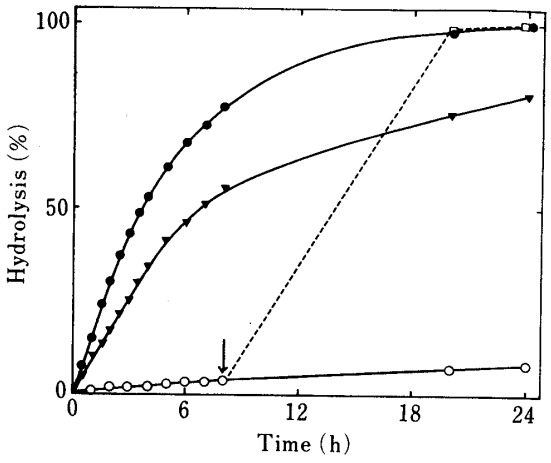

Fig. 7. Digestion of Raw Starch with Poly-$Gluc_1$

Four mg of raw starch was digested with 87 μg (3 units) of poly-$Gluc_1$ in 3.4 ml of 0.1 M acetate buffer (pH 5.0) at 37 °C with stirring. For comparison, raw starch was also digested with either an equal amount or equal number of units of $Gluc_1$ under the same conditions as for poly-$Gluc_1$. Portions (0.25 ml) of the digestion mixtures were taken at various times with a pipette having a wide nozzle and heated at 100 °C for 1 min to terminate the reaction. The heated suspensions, after being cooled to room temperature, were centrifuged at 3000 rpm for 10 min, and then the liberated glucose in the resulting supernatants was determined as described in the text. The arrow indicates the time when a further 2.8 mg (97 units) of poly-$Gluc_1$ was added to the digestion mixture.

○, 87 μg (3 units) of poly-$Gluc_1$; □, 2.8 mg (97 units) of poly-$Gluc_1$; ●, 87 μg (5.5 units) of $Gluc_1$; ▲, 47 μg (3 units) of $Gluc_1$.

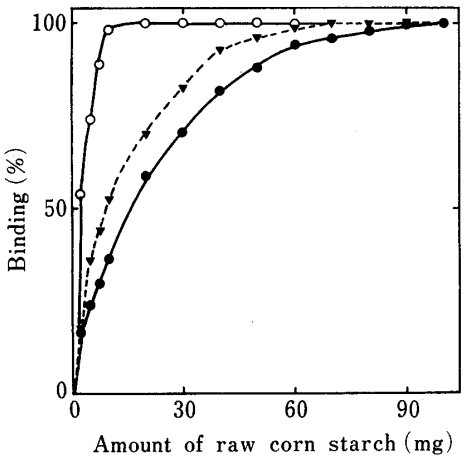

Fig. 8. Binding of Poly-$Gluc_1$ to Raw Starch

Binding of a fixed amount of poly-$Gluc_1$ (1 mg, 34 units) to various amounts of raw starch (1—100 mg) was measured at pH 5.0 and 4 °C as described previously.[6] For comparison, binding to raw starch of an equal amount or equal number of units of $Gluc_1$ was also measured.

○, 1 mg (34 units) of poly-$Gluc_1$; ●, 1 mg (63 units) of $Gluc_1$; ▲, 0.55 mg (34 units) of $Gluc_1$.

from those of $Gluc_1$, the specific activities of poly-$Gluc_1$ towards the large substrates, amylopectin, glycogen and soluble starch, were 4.1, 3.1 and 1.8 times lower, respectively, than the corresponding values of $Gluc_1$.

Behavior of Poly-$Gluc_1$ towards Raw Starch

Among the three forms of glucoamylase from a *Rhizopus* sp., $Gluc_1$, $Gluc_2$ and $Gluc_3$,[3,10-12] only $Gluc_1$ has strong raw starch-digesting and raw starch-binding activities, while both $Gluc_2$ and $Gluc_3$ show weak (22—25 times less) raw starch-digesting and little raw starch-binding activities.[7] To examine the effect of glutaraldehyde treatment on raw starch-digesting activity, 4 mg of raw starch was digested with equal amounts or equal numbers of units of poly-$Gluc_1$ and $Gluc_1$ (Fig. 7): 87 μg (3 units) of poly-$Gluc_1$ digested the raw starch only partially even after a 72-incubation (not shown here), whereas the same amount of $Gluc_1$ digested it almost completely within 24 h; with an equal number of units of $Gluc_1$, the digestion was about 80% complete after 24 h. When a large amount (2.8 mg, 97 units) of poly-$Gluc_1$ was further added at 10 h to the digestion mixture, the raw starch digestion was completed after 24 h. Therefore, it was concluded that the degree of hydrolysis of raw starch by poly-$Gluc_1$ was similar (near 100%) to that by $Gluc_1$, although the rate was quite slow. The specific raw starch-digesting activity of poly-$Gluc_1$ as determined by the standard assay method[7] was estimated to be 0.99 unit/mg (see Table II); this value is 25 times lower than the corresponding value of $Gluc_1$ and 13.4 times lower if compared in terms of units/unit of soluble starch-hydrolyzing activity.

Although poly-$Gluc_1$ showed such a low raw starch-digesting activity, its raw starch-

binding activity appeared to be rather strong (Fig. 8). The amount of raw starch required for 50% binding was 2.25 mg for poly-$Gluc_1$ (1 mg, 34 units) as compared with 15.7 mg for an equal amount of $Gluc_1$ or 9.5 mg for an equal number of units of $Gluc_1$. Poly-$Gluc_1$ did not show any pH dependency in the binding to raw starch, in contrast with $Gluc_1$, which bound pH-dependently with a pH optimum of 4.5—5.5,[7] indicating a tight binding of poly-$Gluc_1$ to raw starch.

Discussion

Various enzymes, such as trypsin, chymotrypsin and catalase, all give insoluble aggregates when treated with glutaraldehyde.[4] Crosslinking of $Gluc_1$ with glutaraldehyde under the conditions described here predominantly formed highly polymerized but soluble $Gluc_1$. This polymerized $Gluc_1$ (named poly-$Gluc_1$) had an apparent molecular weight higher than 10^6 daltons as estimated by gel filtration on Sepharose 6B; this indicates that more than 14 molecules of $Gluc_1$ (M_r 74000) are inter-molecularly crosslinked. On glutaraldehyde treatment of $Gluc_1$, 55% of the lysine residues as well as 11% of the tyrosine residues were irreversibly modified to form inter- and possibly also intra-molecular crosslinkages (Table I). Although the tryptophan and half-cystine residues in poly-$Gluc_1$ were not determined, it seems unlikely that these residues were affected by glutaraldehyde treatment because of the reported unreactivity of tryptophan residues in other proteins[13] with this reagent and because the 1 SH group contained in $Gluc_1$ is buried.[11] Histidine residue, as well as SH group, is somewhat reactive with glutaraldehyde,[13] but such products, in contrast to the cases of lysine and probably tyrosine residues, regenerate the original amino acids on acid hydrolysis with 6 N HCl.[1,4a,14] Amino acid residues other than the lysine and tyrosine residues in $Gluc_1$ might have been affected very little, if at all.

Spectroscopic measurements of poly-$Gluc_1$, especially its CD spectrum, apparently indicated that poly-$Gluc_1$ differed from $Gluc_1$ not only in the states of aromatic amino acid side chains but also in the protein backbone conformation. The blue shift as well as the hyperchroism exhibited in the UV spectrum of poly-$Gluc_1$ may be due partly to such conformational changes and to the partial modification of tyrosine residues, but must be due at least partly to the reaction of lysine residues with glutaraldehyde, which gives 265-nm absorbing products.[14] The fluorescence spectrum of poly-$Gluc_1$ indicates a change in the state of tryptophan residues, but not modification of the residues.

Crosslinking with glutaraldehyde stabilizes some proteins and enzymes,[4,13,15] especially through their insolubilization. $Gluc_1$ was also expected to be stabilized by crosslinking with glutaraldehyde, but little enhancement in the stabilities to pH and heat resulted. On preheating at 40—45 °C for 5 min, poly-$Gluc_1$ exhibited a slightly enhanced activity, but showed greater inactivation at higher temperatures as compared with $Gluc_1$ (Fig. 6b). This enhanced heat stability may be due to a conformational change in poly-$Gluc_1$ caused by heating, so as to make the active sites more available to the substrate.

Poly-$Gluc_1$ differed little from $Gluc_1$ in the K_m and V_{max} values as well as the specific activities towards small substrates; however, it differed markedly in both the K_m and V_{max} values and thus in the specific activities towards large substrates (Table II). Not only increased K_m values but also decreased V_{max} values of poly-$Gluc_1$ for the large substrates are suggestive of reduced availability of active sites in poly-$Gluc_1$, through steric hindrance to the approach of the large substrate molecules to the active site. In addition, the increased K_m values of poly-$Gluc_1$ may arise from conformational change or chemical modification at or near the large substrate-interacting site of poly-$Gluc_1$, leading to decreased affinity for the large substrates; $Gluc_1$ is considered to have an additional site(s) interacting only with large substrates, not with small substrates (the large substrate-interacting site), besides the active site, within the N-

terminal region of about 15000 daltons.[11] The K_m values of poly-$Gluc_1$ for the large substrates (0.006—0.046%) were, although high as compared with those of $Gluc_1$, still rather low in relation to the substrate concentration (1%) used in the enzyme assay, so that the specific activities of poly-$Gluc_1$ towards the large substrates appeared to be related rather to the V_{max} values, not to both the V_{max} and K_m values.

$Gluc_1$ also contains a separate raw starch-binding site(s), different from the active site, within the N-terminal region and this raw starch-binding site is considered to be the same as or near the large substrate-binding site mentioned above.[7] As a result of crosslinking with glutaraldehyde, poly-$Gluc_1$ appeared to acquire rather strong raw starch-binding activity as compared with $Gluc_1$ on an equal amount or equal unit basis (Fig. 8). Considering that poly-$Gluc_1$ was composed of at least 14 monomer molecules, however, we do not know at present whether or not the binding constants of the available raw starch-binding sites in poly-$Gluc_1$ are still higher than that of the raw starch-binding site in $Gluc_1$. On the other hand, the raw starch-digesting activity of poly-$Gluc_1$ became 25 times lower than that of $Gluc_1$. It seems likely that the active sites in poly-$Gluc_1$ become much less available for raw starch, a very large insoluble substrate, than for such large soluble substrates as soluble starch and glycogen. In addition, similarly to the case of the large substrate-interacting sites in poly-$Gluc_1$, lower availability and/or lower binding activity of the raw starch-binding sites in poly-$Gluc_1$ may be involved. It is interesting that the specific activity of poly-$Gluc_1$ towards raw starch is as low (22—25 times lower than that of $Gluc_1$) as those of $Gluc_2$ and $Gluc_3$[7] both of which lack the N-terminal portions of $Gluc_1$,[10] containing the raw starch-binding site. It seems likely that the lysine residues in $Gluc_1$ are not involved in the binding to raw starch, because poly-$Gluc_1$, in which 55% of the lysine residues are chemically modified and many of the remainder may be buried or otherwise inaccessible, still binds tightly to raw starch to form an insoluble complex.

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