Binding to Chitosan of Multiple Forms of Glucoamylases from *Aspergillus saitoi* and *Rhizopus* sp.

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Multiple forms of glucoamylases [EC 3.2.1.3] from *Aspergillus saitoi* and *Rhizopus* sp. were studied for their chitosan binding. Two forms of *Aspergillus* enzyme, Gluc M_1 and Gluc M_2, were bindable to chitosan, whereas three forms of *Rhizopus* enzyme, $Gluc_1$, $Gluc_2$ and $Gluc_3$, exhibited no significant binding; of these enzyme forms, Gluc M_1 and $Gluc_1$ are bindable to raw starch and Gluc M_1 and Gluc M_2 (less strongly) bindable to chitin. Both Gluc M_1 (molecular weight (M.W.) 90000) and Gluc M_2 (M.W. 70000), lacking the C-terminal portion (20000 dalton) of Gluc M_1, bound to chitosan within the narrow pH range from 6.0 to 7.0, with pH optima of 6.5—6.7, and ionic strength-dependently. The binding constants K of Gluc M_1 and Gluc M_2 to chitosan at pH 6.5 and 4 °C were 1.5×10^6 and 1.6×10^6 M^{-1}, respectively. Upon denaturation and modification of Gluc M_1 with 6 M guanidine hydrochloride and water-soluble carbodiimide, respectively, Gluc M_1 almost completely lost the ability to bind to chitosan. A very low concentration of soluble starch (2.95×10^{-4} %) inhibited binding of Gluc M_1 to chitosan by 50%. Chitosan-bound Gluc M_1 showed little enzymatic activity on maltose. These results indicate that a chitosan-binding domain is not always identical with a chitin-binding domain or a raw starch-binding domain and that in Gluc M_1 the binding domains for raw starch, chitin and chitosan are located in this order in the direction from the C-terminal to the N-terminal; the latter two are also contained in Gluc M_2. The chitosan-binding domains of the enzymes seem to include or to reside near the active sites.

Keywords glucoamylase; chitosan; binding; *Aspergillus saitoi*; *Rhizopus* sp.; multiple form; raw starch; chitin

We have isolated three forms of glucoamylase [EC 3.2.1.3; α-D(1→4)-glucan glucohydrolase] of *Rhizopus* sp., called $Gluc_1$, $Gluc_2$ and $Gluc_3$,[1] as well as its two inactive fragments H and L,[2] and two forms of glucoamylase of *Aspergillus saitoi*, called Gluc M_1[3] and Gluc M_2,[4] for comparative characterization. The minor *Rhizopus* glucoamylases, $Gluc_2$ (molecular weight (M.W.) 58600) and $Gluc_3$ (M.W. 61400), are enzyme species derived from the most abundant enzyme, $Gluc_1$ (M.W. 74000), by the action of a certain proteinase(s) with concomitant liberation of its N-terminal glycopeptides of different sizes, fragments H (16700 dalton) and L (14400 dalton).[1,2,5] Similarly, the minor *Aspergillus* glucoamylase, Gluc M_2 (M.W. 70000), is an enzyme species produced by proteolysis of the C-terminal, but not N-terminal, part of the major enzyme, Gluc M_1 (M.W. 90000).[3,4]

Of the multiple forms of glucoamylases, only $Gluc_1$ and Gluc M_1 had high raw starch-binding and raw starch-digesting activities, whereas the smaller forms, $Gluc_2$ and $Gluc_3$ as well as Gluc M_2, had little raw starch-binding and much lower raw starch-digesting activities.[6,7] $Gluc_1$ and Gluc M_1 were concluded to possess raw starch-binding domains in the N-terminal and the C-terminal regions, respectively.[6,7] The raw starch-binding domains seemed to be located sterically near their active sites and, taken together with the results of the kinetic studies,[4,8] to interact not only with insoluble raw starch but also with soluble polysaccharides like soluble starch and glycogen.[6,7]

In a foregoing paper,[9] we demonstrated that Gluc M_1 as well as Gluc M_2, but not $Gluc_1$, $Gluc_2$ or $Gluc_3$, bound to chitin, an insoluble, unhydrolyzable polysaccharide with a β-D(1→4)-glucan structure and that the chitin-bound Gluc M_1 still retained almost the same soluble starch-hydrolyzing activity as free Gluc M_1. Thus, a chitin-binding domain was considered to be not always identical with a raw starch-binding domain, which also interacts with soluble polysaccharides such as soluble starch,[6,7] as well as with an active site.

Recently we observed that Gluc M_1 also bound to chitosan, another insoluble, unhydrolyzable polysaccharide with a β-D(1→4)-glucan structure. To elucidate in more detail the structure–function correlations of the multiple forms of *Aspergillus* and *Rhizopus* glucoamylases, we studied their binding behavior towards chitosan. The present paper deals with chitosan binding of Gluc M_1 and Gluc M_2 as well as of $Gluc_1$, $Gluc_2$ and $Gluc_3$.

Materials and Methods

Chemicals Soluble starch for use as a substrate was purchased from Wako Pure Chemicals and used after exhaustive dialysis against distilled water. Crustacean chitosan was purchased from Tokyo Kasei Kogyo Co., Ltd. and used after successive washing with several changes each of distilled water and methanol, followed by drying over silica gel. Maltose, the D-glucose oxidase reagent (Glucose C-test Wako) and guanidine hydrochloride (GuHCl) were obtained from Wako Pure Chemicals; 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)carbodiimide metho *p*-toluenesulfonate (CMC) was from Sigma Chemical Co. All other chemicals were of analytical reagent grade.

Preparation of Gluc M_1 and Gluc M_2 as Well as $Gluc_1$, $Gluc_2$ and $Gluc_3$ Gluc M_1 and Gluc M_2 were purified from a commercial digestive from *A. saitoi*, Molsin (Seishin Pharm. Co., Ltd.), according to the respective methods reported previously.[3,4] $Gluc_1$, $Gluc_2$ and $Gluc_3$ were purified from a commercial digestive from *Rhizopus* sp., Gluczyme (Amano Pharm. Co., Ltd.), also by the reported method.[1]

Estimation of Protein Protein concentrations were determined from the absorbance at 280 nm taking A_{cm}^{280} (%) to be 14.97 and 14.18 for Gluc M_1 and Gluc M_2, respectively,[3,4] and 13.2, 13.7 and 13.4 for $Gluc_1$, $Gluc_2$ and $Gluc_3$.[1]

Determination of Glucoamylase Activity Glucoamylase activity was determined with soluble starch as a substrate at pH 5.0 and 37 °C according to the D-glucose oxidase method described.[1] 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 maltose as a substrate was determined as described.[8]

Denaturation of Gluc M_1 with 6 M GuHCl Gluc M_1 at 1 mg/ml of 0.05 M Tris–HCl buffer (pH 8.0) was treated with 6 M GuHCl at room temperature for 6 h and then dialyzed exhaustively against distilled water for 3 d.

Modification of Gluc M_1 with CMC Modification of Gluc M_1 with CMC at pH 4.5 and room temperature was carried out as described.[10] Concentrations of reactants were 12.4 μM for Gluc M_1 and 20 mM for CMC. Gluc M_1 was allowed to react with CMC up to for 74 min, where

almost complete inactivation of the enzyme was attained, and the modified Gluc M_1 was dialyzed exhaustively against distilled water for 3 d.

Estimation of Enzyme Bound to Chitosan Up to 100 μl of an enzyme solution was added to up to 50 mg of chitosan in 0.01 M acetate buffer (pH 6.5) containing 0.1 M NaCl to give a total volume of 1.1 ml. After incubation at 4 °C for 30 min with stirring, the suspension was transferred to a microfilter tube (Schleicher & Schuell, Inc.). with a filter paper and filtered by brief centrifugation at 4 °C and 3000 rpm for a few min. The resulting filtrate was used to estimate the amount of unbound enzyme, which was determined by measuring either enzymatic activity on soluble starch or the absorbance at 230 nm instead of 280 nm because of the small amount of enzyme used. The amount of bound enzyme was calculated as the difference between the total and unbound enzymes, and chitosan-binding activity of enzyme was expressed as the rate (%) of binding.

Estimation of Binding Constant of Enzyme to Chitosan The binding constant K of an enzyme–chitosan complex is expressed by Eq. 1 as follows:

$$K=\frac{B}{F(B_{max}-B)} \quad (1)$$

thus

$$\frac{1}{B}=\frac{1}{B_{max}\cdot K\cdot F}+\frac{1}{B_{max}} \quad (2)$$

where B and F stand for the molar concentrations of bound and free enzymes, respectively, and B_{max} stands for the maximum molar concentration of enzyme bindable to the total amount of chitosan, which is equivalent to the total molar concentration of enzyme-binding domain of chitosan. On the basis of Eq. 2, the value of K was estimated graphically from a plot of $1/B$ *versus* $1/F$.

Results

The bindability to chitosan of the multiple forms of glucoamylases from *Rhizopus* sp. and *A. saitoi* was preliminarily explored under the same conditions as for chitin binding[9] of the enzymes. When binding of each enzyme (50—70 μg) to chitosan (50 mg) was examined at pH 6.5 (I=0.1) and 4 °C with stirring for 20 min, only *Aspergillus* enzymes, Gluc M_1 and Gluc M_2, tightly bound to the chitosan, whereas neither *Rhizopus* enzymes, $Gluc_1$, $Gluc_2$ nor $Gluc_3$, bound significantly. With $Gluc_1$, which is the only raw starch-bindable one of the three *Rhizopus* enzymes,[6] chitosan binding was reexamined at various pH values from 5.5 to 11.0 but, again, no detectable amounts of the enzyme were observed to bind over the pH range tested; chitosan binding test at lower pH values was not performed because of possible gelation of chitosan.[11] These results indicate that a raw starch-binding domain in a glucoamylase does not always correspond to chitosan binding, as was the case with chitin binding.[9] Thereafter, we examined chitosan binding with *Aspergillus* enzymes, Gluc M_1 and Gluc M_2, in more detail and quantitatively.

To determine the optimal conditions for chitosan binding of the enzymes, the time course of the binding was followed with Gluc M_1 at pH 6.5 and 4 °C (Fig. 1). Gluc M_1 bound to chitosan more slowly than to raw starch[7] or chitin,[9] taking 20 to 23 min for its nearly complete binding, as compared with about 10 min for its complete binding to the latter two polysaccharides.

The effect of pH on chitosan binding of the enzymes was tested at 4 °C for 20 min using various buffers (I=0.1) of from pH 6.3 to 11.0. The binding of the enzymes occurred sharply within the narrow pH range from 6.0 to 7.0. The binding pH range was slightly narrower for Gluc M_2 than for Gluc M_1 and maximum binding of both enzymes occurred at pH around 6.5—6.7 (Fig. 2).

The effect of ionic strength on chitosan binding of the enzymes was examined by changing the ionic strength from 0.05 to 1.0 at pH 6.5 and 4 °C (Fig. 3). Lower ionic strength up to 0.4 for Gluc M_1 and up to 0.2 for Gluc M_2 had little effect on the binding, whereas at higher ionic strength the enzymes scarcely bound to chitosan. Thus, as the optimal conditions for chitosan binding of both enzymes, we chose

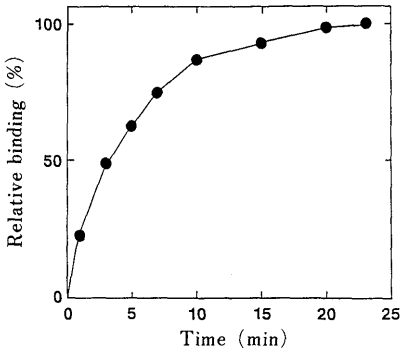

Fig. 1. Time Course of Binding of Gluc M_1 to Chitosan

Binding of Gluc M_1 (100 μg) to chitosan (25 mg) was measured as described in the text, except that various incubation times from 1—23 min were used at pH 6.5. The binding is expressed as a percentage of the maximum binding (81% binding).

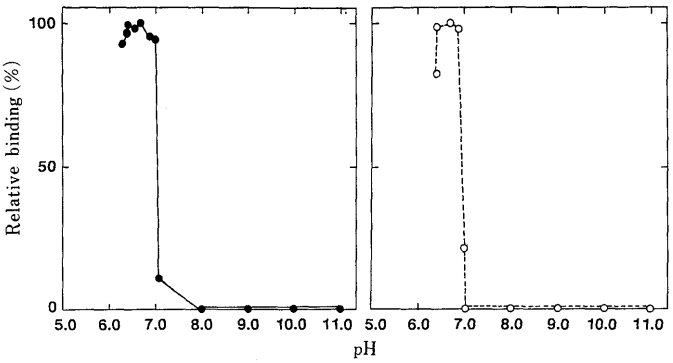

Fig. 2. Effect of pH on Chitosan Binding of Gluc M_1 and Gluc M_2

Binding of Gluc M_1 (100 μg) and Gluc M_2 (78 μg) to chitosan (20 mg) was measured as described in the text, except that the pH was changed from 6.3—11.0. The buffers (0.01 M, I=0.1) used were acetate buffer for pH 6.3—6.5, phosphate buffer for pH 6.6—6.9, borax–HCl buffer for pH 7.0—9.0 and borax–NaOH buffer for pH 10.0—11.0. For each enzyme, the binding is expressed as a percentage of the maximum binding (79% and 80% binding for Gluc M_1 and Gluc M_2, respectively). ●, Gluc M_1; ○, Gluc M_2.

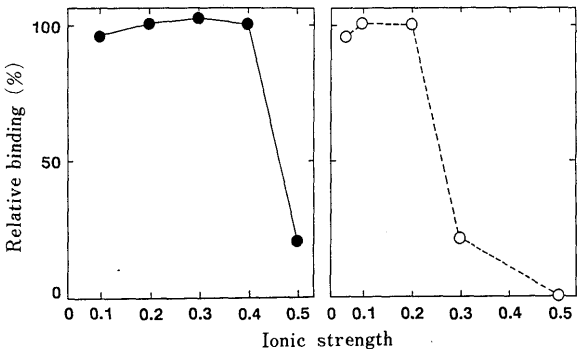

Fig. 3. Effect of Ionic Strength on Chitosan Binding of Gluc M_1 and Gluc M_2

Binding of Gluc M_1 (90 μg) and Gluc M_2 (70 μg) to chitosan (20 mg) was measured at pH 6.5 as described in the text, except that the ionic strength was changed from 0.05—1.0. Only the results obtained at ionic strength from 0.05—0.5 are depicted. For each enzyme, the binding is expressed as a percentage of the maximum binding (82% and 84% binding for Gluc M_1 and Gluc M_2, respectively). ●, Gluc M_1; ○, Gluc M_2.

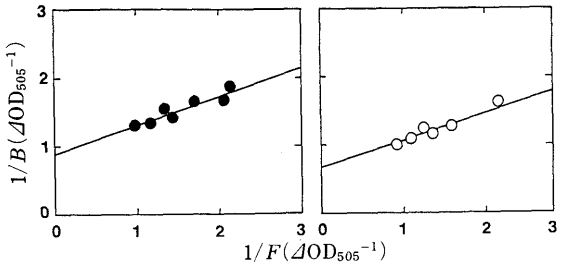

Fig. 4. Estimation of the Binding Constants of Gluc M_1 and Gluc M_2 to Chitosan

Binding of various amounts of Gluc M_1 (150—280 μg) and Gluc M_2 (90—200 μg) to a fixed amount of chitosan (10 mg) was measured at pH 6.5 and 4 °C as described in the text. The amounts of free (F) and bound (B) enzymes as estimated by enzymatic activity are expressed in terms of ΔOD_{505}. ●, Gluc M_1; ○, Gluc M_2.

TABLE I. Chitosan Binding of GuHCl-Denatured Gluc M_1 and CMC-Modified Gluc M_1

Enzyme	Binding activity (%)	Enzymatic activity (%)
Gluc M_1	86.0	100
6 M GuHCl-denatured Gluc M_1	0	2
CMC-modified Gluc M_1	3.2	0

Binding of each enzyme (75 μg) to chitosan (20 mg) was measured as described in the text. Enzymatic activity was determined with soluble starch as a substrate and the activity is expressed as a percentage of that of Gluc M_1.

30-min incubation (to assure binding) at pH 6.5 (0.01 M acetate buffer–0.1 M NaCl) and 4 °C with stirring.

When a constant amount of enzyme (104 μg of Gluc M_1 or 81 μg for Gluc M_2) was mixed with various amounts of chitosan (5—40 mg) at pH 6.5 and 4 °C, binding of the enzymes occurred hyperbolically against the amount of chitosan (data not shown). To estimate the binding constants K to chitosan of Gluc M_1 and Gluc M_2, binding of various amounts of each enzyme to a fixed amount of chitosan was measured at pH 6.5 and 4 °C. A plot of $1/B$ against $1/F$ was made according to Eq. 2; the linear slope, which is equal to $1/B_{max} \cdot K$, was calculated by least squares analysis (Fig. 4). Based on the plot, the K values for Gluc M_1 and Gluc M_2 were estimated to be 1.5×10^6 and $1.6 \times 10^6\ M^{-1}$, respectively.

Gluc M_1 is denatured by the presence of 6 M GuHCl.[4] Chemical modification of Gluc M_1 with CMC at pH 4.5 results in the inactivation with the incorporation of about 12 CMC moieties, but without any gross change in its peptide backbone conformation.[10] It is interest to examine whether or not GuHCl-denatured Gluc M_1 and CMC-modified Gluc M_1 are still chitosan-bindable. Thus Gluc M_1 was denatured with 6 M GuHCl at pH 8 and room temperature for 6 h. Gluc M_1 was also allowed to react with 20 mM CMC at pH 4.5 for up to 74 min. Seventy five μg each of the GuHCl-denatured and CMC-modified Gluc M_1 were incubated with 20 mg of chitosan at pH 6.5 and 4 °C with the results listed in Table I. It was found that neither the GuHCl-denatured Gluc M_1 nor CMC-modified Gluc M_1 any longer retained the ability to bind to chitosan, with concomitant loss of enzymatic activity.

Various saccharides which are substrates or analogs of glucoamylase showed inhibitory effects on raw starch binding of $Gluc_1$[6] and chitin binding of Gluc M_1.[9]

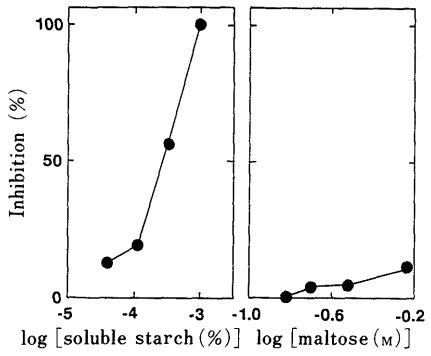

Fig. 5. Inhibition of Chitosan Binding of Gluc M_1 by Soluble Starch and Maltose

Binding of Gluc M_1 (90 μg) to chitosan (20 mg) was measured at pH 6.5 and 4 °C in the absence and presence of the indicated concentrations of soluble starch and maltose.

TABLE II. Enzymatic Activity of Chitosan-Bound Gluc M_1 towards Maltose

	Enzymatic activity on maltose (%)
Gluc M_1 + chitosan	10.2
Bound Gluc M_1 94.6%	(4.8)
Free Gluc M_1 5.4%	
Gluc M_1	100.0

Enzymatic activity on maltose was determined as described in the text, except that the buffer used was 0.01 M acetate buffer (pH 6.5) containing 0.1 M NaCl. Chitosan-bound Gluc M_1 was prepared by preincubating 6.6 μg of Gluc M_1 and 20 mg of chitosan in 0.5 ml of the buffer at 37 °C for 20 min. Maltose in 0.5 ml of the buffer was then added to give a final concentration of 5 mM and the enzymatic reaction was started. The enzymatic activity is expressed as a percentage of that of Gluc M_1. In a separate experiment, binding of Gluc M_1 to chitosan was measured under the same conditions as for the preincubation to estimate the net amount of chitosan-bound Gluc M_1. The figures in parenthesis express an estimated value.

Therefore, the effects on chitosan binding of Gluc M_1 of two substrates of different sizes, soluble starch and maltose, were tested (Fig. 5). Both saccharides were found to be inhibitory towards the binding, regardless of whether they were added before or after the formation of the complex of Gluc M_1 with chitosan. The inhibitory effect of soluble starch on chitosan binding of Gluc M_1 was very much higher than those on chitin binding of Gluc M_1 and on raw starch binding of $Gluc_1$. The concentration ($I_{50\%}$) of soluble starch causing 50% inhibition was as low as 2.95×10^{-4} % for chitosan binding of Gluc M_1, as compared with 1.2% for chitin binding of Gluc M_1[9] and 6.9×10^{-2} % for raw starch binding of $Gluc_1$.[6] In contrast, the value of $I_{50\%}$ of maltose was too high to estimate; maltose inhibited the binding by only 12% even at a concentration as high as 0.6 M.

It was shown that chitin-bound Gluc M_1 retained almost the same soluble starch-hydrolyzing activity as and about 3 times higher maltose-hydrolyzing and 3 times lower raw starch-hydrolyzing activities than free Gluc M_1.[9] Therefore chitosan-bound Gluc M_1 was examined as to its enzymatic activity, but only for maltose-hydrolyzing activity, because the $I_{50\%}$ value of soluble starch for chitosan binding of Gluc M_1 was much too low for measurement of the soluble starch-hydrolyzing activity of chitosan-bound Gluc M_1; the concentration of soluble starch as a substrate is 1% in the standard enzyme assay,[1] and an unusualy low concentra-

tion of soluble starch substrate (0.05%) was used in the assay with chitin-bound Gluc M_1,[9] even the latter concentration being much higher than the $I_{50\%}$ value (2.95×10^{-4} %) of soluble starch obtained above. Chitosan-bound Gluc M_1 was prepared by preincubating Gluc M_1 (6.6 μg) and chitosan (20 mg) at pH 6.5 and 37 °C, but not 4 °C, for 20 min and then the enzymatic activity of the chitosan-bound Gluc M_1 was determined with maltose as a substrate under the same conditions, instead of at usual pH 5.0,[8] to assure the binding of Gluc M_1 to chitosan (Table II). Since free Gluc M_1, although only a trace (5.4% of the total enzyme), was detectable in the preparation of chitosan-bound Gluc M_1 under the present assay conditions, the net maltose-hydrolyzing activity of chitosan-bound Gluc M_1 was estimated, by subtracting the activity of the free enzyme present, to be 4.8% of that of Gluc M_1. Thus Gluc M_1, upon binding to chitosan, was found to lose almost completely the enzymatic activity towards maltose.

Discussion

As herein described, of the multiple forms of glucoamylases from *A. saitoi* and *Rhizopus* sp., only *Aspergillus* enzymes, Gluc M_1 and Gluc M_2, were bindable to chitosan, whereas *Rhizopus* enzymes, $Gluc_1$, $Gluc_2$ and $Gluc_3$, were not. Considering that both Gluc M_1 and Gluc M_2 are bindable to chitin while $Gluc_1$, $Gluc_2$ and $Gluc_3$ are not,[9] it was initially expected that the chitin-binding domains in the enzymes might also be responsible for chitosan binding. Contrary to expectation, the binding behavior of the enzymes to chitosan was considerably different from that to chitin,[9] as well as from that to raw starch,[7] indicating that a chitosan-binding domain is not always identical with a chitin-binding domain or a raw starch-binding domain. This is also not incompatible with the finding reported by other investigators that glucoamylase I of *A. awamori* var. *kawachi*, the largest of the three enzyme forms produced by the organism, was not chitosan-adsorbable at all although the enzyme was chitin-adsorbable,[12,13] as well as being raw starch-adsorbable and raw starch-digestible.[14]

Binding of Gluc M_1 and Gluc M_2 to chitosan was more sharply pH-dependent than that to chitin,[9] while maximum binding of the enzymes occurred at similar pH values (around 6.5—6.7 for chitosan and 6.5 for chitin). The pH-dependences of chitosan binding of the enzymes were considerably different from those of raw starch binding of Gluc M_1[7] and $Gluc_1$[6] with pH optima of 3.0 and 4.5—5.5, respectively. Chitosan binding of Gluc M_1 and Gluc M_2 was largely affected by ionic strength, whereas raw starch binding of $Gluc_1$[6] (and possibly of Gluc M_1 although not tested) was little affected; the ionic strength-dependences of chitin binding of Gluc M_1 and Gluc M_2 were not examined. Thus, in chitosan binding of Gluc M_1 and Gluc M_2, unlike at least in raw starch binding of $Gluc_1$, the electrostatic interactions between the enzymes and chitosan are considered to play important roles. In any event, the differences in the pH- and ionic strength-dependences of binding to the different insoluble polysaccharides of the enzymes seem to result from the different structures of the respective binding domains for the polysaccharides.

Both Gluc M_1 and Gluc M_2 tightly bound to chitosan with binding constants K of $1.5—1.6 \times 10^6$ M^{-1} at pH 6.5 and 4 °C; these are the first reported K values of the chitosan–glucoamylase complexes. The K values were similar to that of the chitin complex with Gluc M_1 (1.8×10^6 M^{-1})[9] and about 10 times larger than those of the raw starch complexes with Gluc M_1 (1.6×10^5 M^{-1})[7] and $Gluc_1$ (1.2×10^5 M^{-1}),[6] indicating tighter binding to chitosan of the enzymes, similar to chitin binding of Gluc M_1, than the binding to raw starch of Gluc M_1 and $Gluc_1$.

Gluc M_2 lacking the C-terminal portion (about 20000 dalton) of Gluc M_1, which failes to bind to raw starch but retains a part of the ability of Gluc M_1 to bind to chitin,[15] bound to chitosan as tightly as Gluc M_1. This, together with the previous results,[4,7,9] implies that the chitosan-binding domain of Gluc M_1 is located much farther inside the C-terminal region than the chitin-binding domain, which is followed by the raw starch-binding domain in the C-terminal region, and that Gluc M_2 preserves the chitosan-binding domain completely and the chitin-binding domain partially. However, the chitosan-binding domain of Gluc M_2 appears to be not always identical in tertiary structure with that of Gluc M_1, so as to be reflected in the observed differences of the enzymes in the pH- and ionic strength-dependences of chitosan binding. The alternation of Gluc M_2 induced by lacking the C-terminal 20000-dalton portion of Gluc M_1 is also observed in the former's susceptibility to such denaturants as urea and GuHCl, as well as in the CD spectrum in the longer wavelength region (250—320 nm), but not in the shorter wavelength region (200—250 nm).[4]

Chitosan-bound Gluc M_1 no longer retained any enzymatic activity towards even maltose, which is the smallest substrate of glucoamylase and is most easily accessible to the active site, with the steric hindrance of chitosan bound, if any. It is believed that chitosan-bound Gluc M_1 may show no enzymatic activity towards soluble starch or raw starch, although such enzymatic activity was not measured because of practical difficulty. This result is in striking contrast to the previous finding[9] that chitin-bound Gluc M_1 retained enzymatic activity not only towards maltose but also towards soluble starch and raw starch. In addition, an extremely low concentration of soluble starch (2.95×10^{-4} %) was enough for 50% inhibition of chitosan binding of Gluc M_1, as compared with 1.2% and 6.9×10^{-2} % of soluble starch necessitated for 50% inhibition of chitin binding of Gluc M_1[9] and of raw starch binding of $Gluc_1$,[6] respectively. From these results, it is concluded that the chitosan-binding domain of Gluc M_1 is located in the region including the active site, which consists of about 7 subsites,[16] or near the active site.

So far as we know, Gluc M_1 is the only glucoamylase that binds to the three insoluble polysaccharides, chitosan, chitin and raw starch. The reason chitosan and chitin, neither of which are substrates of glucoamylase, tightly bind with Gluc M_1 (and Gluc M_2) and in the separate domains is unknown.

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15) Gluc M_2 has as small a K value as $3.2 \times 10^3\ M^{-1}$, with practically no binding to raw starch[7]; the K value of Gluc M_2 for chitin is $0.33 \times 10^6\ M^{-1}$.[9]
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