Incorporation of Glucoamylase into Some Polyelectrolyte Complexes

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Polyelectrolyte complexes (PEC) are formed in the reaction of polycations with polyanions in aqueous solutions, and the reaction is generally stoichiometric.1,2) The complexes are interesting not only for study on molecular interactions of biopolymers but also for their biological functions.3-5)

In the course of our study on the PEC of partially N-acylated chitosans6) [(1→4)-β-D-glucosaminan] with some acidic glycosaminoglycans,7) we noticed that glucoamylase was coprecipitated by mixing with both chitosan and some polyanions at appropriate mixing ratios to form PEC-enzyme complexes. Chitosan has been used for the immobilization of some enzymes and other biosubstances.6,8,9) We now wish to report an incorporation of glucoamylase (EC 3.2.1.3) into the PEC of chitosan-dextran sulfate, -polyvinylsulfate and -heparin. This incorporation may widely be used for a new method for the immobilization of enzymes and other biosubstances.

The polycationic component used in the present work was chitosan10) [α]18D-15.1° (c=1.0, 10% acetic acid), and the polyanionic components were dextran sulfate sodium salt (DS, Pharmacia Fine Chemicals Ltd., sulfur content=17±1%), polyvinylsulfate potassium salt (PVS, Wako Pure Chemical Industries Ltd., degree of esterification>90%) and heparin sodium salt (Hep, Fulka, more than 140 I.U./mg).

Each the polyelectrolytes (1 g) was dissolved with 0.1 M aqueous acetic acid solution. The pH values of those solutions were adjusted to 4.5 by the addition of 0.1 M sodium acetate, the resulting solutions were diluted with water at 0.2% of polyelectrolytes and kept as stock solutions. The mixing ratio (R) was defined as $R = PC/(PA + PC)$ where $PC$ and $PA$ were volumes of 0.2% stock solutions of polycation (chitosan) and polyanions (DS, PVS and Hep), respectively. Stock solutions (0.2%) of polyanions (1.4~0.4 ml) and chitosan (0.6~1.6 ml) were separately pipetted into test tubes, diluted to 2.0 ml with 0.05 M acetate buffer (pH 4.5), respectively, and the chitosan solutions were gently mixed to the polyanion solutions to make the solutions of various mixing ratios $(R=0.3~0.8)$. Thus, final amounts of both polyelectrolytes were fixed to 4 mg (0.1%) throughout experiments. The mixtures were allowed to stand at 5°C. The turbidities were measured at 575 nm after standing for 15 min and 24 hr, and the turbidity values were plotted against R as shown in Fig. 1-A. The $R_{\text{max}}$ value is defined as the mixing ratio of maximum turbidity after standing for 15 min, and the value agreed with the mixing ratio of minimum turbidity after standing for 24 hr. Such decrease in the turbidity was due to precipitation of the complex particles.

On the other hand, 0.5 ml of glucoamylase solution [0.05 M acetate buffer (pH 4.5), containing 500 µg of glucoamylase, Rhizopus niveus, Seikagaku Kogyo Co., Ltd. Pure preparation (22 unit/mg)] was added to each of the polyanion solutions, and the mixture was allowed to stand for 60 min at 5°C. No precipitates appeared at this step. The corresponding chitosan solutions were added at the various mixing ratios. The turbidity was measured after standing for 15 min and 24 hr. As shown in Fig. 1-A, B, no significant changes in the turbidity curves were observed by the addition of glucoamylase under the present conditions. Each suspensions were centrifuged at 1000 x g for 20 min after the second measurement of turbidity to afford the supernatant solution (S1), and an aliquot (0.5 ml)
FIG. 1-A, B. Turbidity Curves Plotted against the Mixing Ratio (R) for Chitosan-DS System. (A) in the absence of glucoamylase. (B) in the presence of glucoamylase. ---, turbidity produced after staining for 15 min; ---, turbidity after standing for 24 hr.

FIG. 1-C. Glucoamylase Activities of S₁, S₂ and P Using Maltose as Substrate in Chitosan-DS System. ---, glucoamylase activities of S₁ (supernatants collected by the centrifugation at 1000 × g for 20 min); ---, glucoamylase activities of S₂ (supernatants collected by the centrifugation at 25,000 × g for 30 min); ---, glucoamylase activities of P (precipitates collected by the centrifugation at 25,000 × g for 30 min, washed twice with 4 ml of 0.05 M acetate buffer, pH 4.5 and suspended in 4 ml of same buffer); ---, control.

TABLE I. Rmax, Amax AND Enzyme ACTIVITIES OF S₁, S₂ AND P IN CHITOSAN-DS, -PVS AND -HEP SYSTEMS

The glucoamylase activity was expressed against the control (100%), and the amounts of D-glucose formed were proportional to the amounts of enzyme under the present conditions (amounts of glucoamylase were less than 56 μg or 33 μg). n.d., not determined.

<table>
<thead>
<tr>
<th>Systems</th>
<th>Rmax in the absence of glucoamylase</th>
<th>Rmax in the presence of glucoamylase</th>
<th>Amax</th>
<th>Activity at Amax (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S₁</td>
</tr>
<tr>
<td>Chitosan-DS</td>
<td>0.45</td>
<td>0.43</td>
<td>0.43</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n.d. (3.8)</td>
</tr>
<tr>
<td>Chitosan-PVS</td>
<td>0.45</td>
<td>0.43</td>
<td>0.43</td>
<td>3.3</td>
</tr>
<tr>
<td>Chitosan-Hep</td>
<td>0.48</td>
<td>0.48</td>
<td>0.43</td>
<td>44</td>
</tr>
</tbody>
</table>

a 0.5 ml of S₁, S₂ and P were added to 4.5 ml of substrate solution (buffered with 0.05 M acetate buffer, pH 4.5) containing 1.2% maltose and incubated at 40°C for 30 min (a standard assay condition). The reaction was terminated by heating on a boiling water bath for 10 min. D-Glucose resulting from the reaction was determined by the glucostat method.1)

b Similarly glucoamylase activity to soluble starch was determined as follows: To 4.5 ml of substrate solution (buffered with same buffer) containing 1% soluble starch, 0.5 ml of S₁, S₂ and P was added, incubated at 40°C for 15 min and the reducing sugar formed was determined by the Somogyi-Nelson method. Results were shown in parentheses.

was withdrawn from S₁ and centrifuged again at 25,000 × g for 30 min to afford the supernatant solution (S₂). The precipitates were twice washed with 4.0 ml of 0.05 M acetate buffer (pH 4.5) by centrifugation (25,000 × g, for 30 min) and suspended in 4.0 ml of same buffer (P). All the procedures were carried out at 5°C.
Incorporation of Glucoamylase

Glucoamylase activities of S₁, S₂ and P were assayed and plotted against the mixing ratios (Fig. 1-C). The presence of both polycationic and polyanionic components in P were evident by IR absorptions at ca. 1600 (–NH₃⁺ in chitosan) and 1150~1240 cm⁻¹ (S=O in polyanions).

Results obtained from each systems are shown in Table I. Aₘₐₓ was defined as the mixing ratio of the maximum activity of glucoamylase of P. At all the systems, the mixing ratio of minimum activity of S₁ and S₂ agreed with Aₘₐₓ (Fig. 1-C). A significant decrease in residual activities of S₁ and S₂ exhibits the precipitation of the enzyme molecules with PEC. As shown in Table I, glucoamylase activities of P were ca. 100, 84 and 46 % using maltose as substrate in chitosan-DS, chitosan-PVS and chitosan-Hep systems, respectively, and 76 % using soluble starch as substrate in chitosan-DS system. About 80 % of the enzymic activity retained in chitosan-DS system after repeated use of six times. The activities of S₁ were almost equal to those of S₂ at Aₘₐₓ in chitosan-DS and -PVS systems and this indicate that the PEC-enzyme complex precipitated almost completely after the centrifugation at 1000xg for 20 min. The Aₘₐₓ values agreed with the mixing ratio of minimum turbidity after standing for 24 hr in the presence of enzyme. However, the Aₘₐₓ shifted clearly from 0.48 of Rₘₐₓ to 0.43~0.45 at chitosan-Hep system. This shift indicates that the effective incorporation of glucoamylase is achieved under the excessive condition of Hep at chitosan-Hep system.

REFERENCES

7) S. Hirano, C. Mizutani, R. Yamaguchi and O. Miura, Biopolymers, in press.