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Separation of Amyloglucosidase Using β-Cyclodextrin/ Chitosan

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ABSTRACT

The adsorption characteristics of commercial amyloglucosidase enzyme preparation from *Aspergillus niger* on the β-cyclodextrin–chitosan system were studied. The effects of pH, temperature, and ionic strength on the equilibrium partition coefficients were investigated in order to obtain the optimum process conditions for a possible AMG purification scheme. It was shown that the adsorption isotherm for this system at pH 4.5 at ambient temperature obeyed the Langmuir relationship, and the Langmuir parameters of q_m and K_p were estimated as 120.5 mg/mL hydrated gel and 1.4 mg/mL solvent, respectively. The complete elution of the enzyme from the matrix was achieved with an increase in pH by using 0.1 M borate buffer, pH 8.0.

INTRODUCTION

Amyloglucosidase (AMG) or glucoamylase (1,4-D-glucan glucohydrolase) is an exo-acting carbohydrase produced by molds and yeasts (1). Glucoamylases are commercially important bulk enzymes for food and beverage industries since they are involved in the complete hydrolyzation of starch to glucose.

When glucoamylases are produced, they are often contaminated by a number of other enzymes that are active on starches. Among these enzymes are α-amylase and transglucosidase which lower the yield of glucose and produces oligosaccharides; these activities are undesirable (2, 3). Other minor side activities, such as those of protease, cellulase, and

lactase, are also detected in fermentation broths (4). Therefore, enzyme preparations with a higher glucoamylase activity are in demand in the food industry.

Earlier studies showed that glucoamylases have an affinity site for raw starch and cyclodextrins. Cyclodextrins (CDs) are cyclic nonreducing D-glucopyranosyl polymers containing six (α-), seven (β-), or eight (γ-) residues joined by D-(1-4-)-linkages, so they have no reducing or nonreducing end groups. For that reason, CDs cannot be hydrolyzed by glucoamylases (5). Cyclic dextrins possess unusual complexing properties due largely to the hydrophobic nature of the central cavity of the cyclic molecules (6). The CD binding site of AMG also contains many hydrophobic amino acids and three hydrophobic regions. The insides of the cavities in both molecules are a hydrophobic environment in which the hydrophobic radicals help to form inclusion compounds.

Thus, AMG can be specifically adsorbed in a cavity of cyclodextrins through some hydrophobic radicals of the affinity site to form inclusion complexes. Most of the work done with glucoamylase enzymes and cyclodextrins has been to study the biochemical and genetic aspects of starch digestion and to characterize the binding domain (5, 7).

The biospecific binding of the AMG enzyme on cyclodextrins may be a valuable step in purification. Therefore, there is a need to explore the adsorption–desorption characteristics of glucoamylases on cyclodextrin-immobilized affinity supports to provide data for an alternative separation process design.

Therefore, the effect of the key process parameters (pH, ionic strength, temperature) on the phase equilibria of AMG/β-CD–chitosan system was investigated. The stability of AMG enzymes as well as the adsorption isotherms were investigated. Desorption experiments were also conducted.

EXPERIMENTAL

Materials

The adsorbent was β-cyclodextrin supported on chitosan, supplied from Japan Organo Co., in the hydrated form in distilled water.

Dry and hydrated pellet sizes of chitosan gels were determined by using a light microscope. The bead diameter in the dry form was found to be between 200 and 300 μm; the diameter of the hydrated pellets was in the 400–600 μm range. The hydrated volume for the chitosan gels used in the experiments was 12 mL/g dry resin. The pore size of chitosan was 0.1–0.2 μm, and the internal diameter of the β-CD cavity was 0.7 nm. The immobi-

lized β-CD concentration was 103 μmol/g dry resin [CD% (w/w) 12%], as given by the manufacturer.

The gels were filtered on glass filters and equilibrated with buffer solutions to the desired pH and ionic strength for use in the adsorption experiments.

Commercial AMG enzyme from *A. niger* was obtained from Novo Nordisk, Japan. Enzyme solutions were diluted in buffer solutions to the desired concentrations and pH values.

Methods

In this study, experiments were conducted in the batch mode to investigate the optimum process conditions, and the adsorption capacity and desorption behavior of the enzyme. A bulk/liquid (v/v) ratio of 0.1 was used in all experiments.

pH Stability of the Enzyme. The glucoamylase enzyme solution (1.8 units) was incubated for 24 hours at 25°C in 3 mL of various buffer solutions (KCl–HCl, glycine–HCl, phosphate, borate, glycine–NaOH, acetate, citrate, Tris–HCl) in a pH range of 1.5 to 10.0. After the incubation period, the residual activities were measured under standard assay conditions.

Effect of pH on Partitioning. AMG enzymes (4.0 mg/mL) were used to study the effect of pH on partition coefficients. Therefore, gels were equilibrated in appropriate 0.1 M buffer solutions, pH 2.5–7.0. Adsorption experiments at each pH were carried out in a shaking water bath at 25°C for 24 hours. Then the samples were assayed and the partition coefficients were calculated at different pH values.

Effect of Temperature on Partitioning. This experimental procedure was conducted using 4.0 mg/mL AMG enzyme in 0.1 M acetate buffer, pH 4.5. Samples were placed in shaking water baths at different temperatures (between 5 and 40°C). Samples (1 mL) were taken, centrifuged, and assayed for protein concentration after 24 and 48 hours. Then the partition coefficients were calculated from the differences in the concentrations.

Effect of Ionic Strength on Partitioning. 0.5 mL (4.0 mg/mL) AMG enzyme samples were prepared in 30 mL of different ionic strength (0–2.0 M NaCl) acetate buffers at a pH value of 4.5 and added to 3 mL gel. These samples were kept at 25°C in a shaking water bath for 24 hours. After adsorption was complete, samples were taken, assayed, and the partition coefficients were calculated as described earlier.

Determination of Adsorption Isotherms. Adsorption isotherm experiments were conducted in the batch mode in stirred solutions (total

volume about 30 mL) at 25°C in a shaking water bath for 24 hours. Samples were taken, centrifuged, and analyzed. The initial and equilibrium concentrations of the protein were determined for the enzyme solutions in sodium acetate 0.1 M buffers, pH 4.5.

Desorption of the Enzyme from the Matrix. Desorption experiments were conducted using samples of gel with adsorbed protein. These were prepared under the same conditions described above. The adsorbed sample (protein + gel) was mixed with different pH and ionic strength elution buffers (acetate, borate, etc.) and placed in a shaking water bath at 25°C. After equilibrium was reached, the supernatant solution was assayed for protein concentration and enzyme activity.

Analytical Methods

Protein Measurements. Protein concentrations were measured using the Lowry protein method with bovine serum albumin as a standard. The kit produced by Sigma Chemicals (catalog no. P 5656) was used to determine protein concentrations. This procedure is based on Peterson's modification of the micro-Lowry method (8) and the method of Lowry et al. (9). AMG adsorbed in the gel was estimated from the difference between the initial and final concentrations of free protein.

Enzyme Activity Determinations. AMG activities were measured using the method of Novo Nordisk (10). Enzyme solutions (1 mL) were incubated with 1 mL preheated substrate solution (2% maltose substrate in 0.1 M acetate buffer, pH 4.3) at 25°C for 30 minutes. After stopping the reaction with 3 mL of 1.66 M Tris buffer, pH 7.6, the reducing sugar formed was assayed using the glucose kit produced by Boehringer Mannheim (catalog no. 124036), and the degree of hydrolysis was calculated by Novo's analytical method. One unit of glucoamylase activity was defined as the amount of enzyme which at standard conditions splits 1 μmol maltose per minute at 25°C and at pH 4.30.

RESULTS AND DISCUSSION

In this section, experimental results on the applicability of β-cyclodextrin–chitosan to adsorptive separation are presented together with the Langmuir model parameters. Data obtained from the desorption experiments are also given below.

pH Stability of Amyloglucosidase Enzymes

This experiment determined the stability range of the AMG enzyme. Figure 1 shows the stability of AMG enzymes produced from *A. niger*

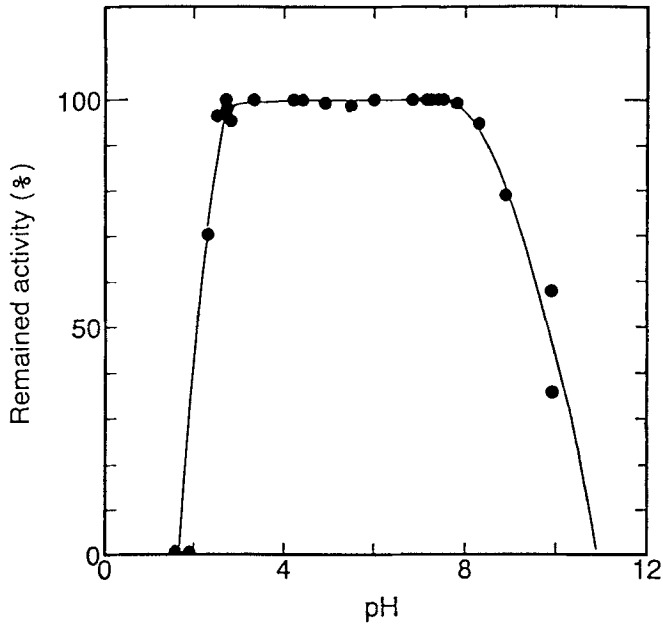

FIG. 1 pH stability of AMG enzymes.

over a wide pH range in different buffer solutions. As seen from the graph, AMG is almost unaffected by pH in the 2.8 to 8.0 range. Below pH 2.8, the enzyme rapidly loses its activity due to acid denaturation. AMG enzyme preparation seems to be very stable above the pH values of its isoelectric point.

The stability of the enzyme is expressed as the percent activity remaining after 24 hours at a particular pH at 25°C, based on the original activity. Experimental details are given in the Experimental Section.

Effect of pH on the Partition Coefficients

Here, the pH and partition coefficient curve was determined for AMG enzyme. Partition coefficients were calculated from the initial and adsorbed fraction of enzymes on β-CD–chitosan.

Partition coefficients indicate the fraction of adsorbed protein at equilibrium which is given by the equation described in detail earlier (11):

$$f = q/p \tag{1}$$

where q and p are the equilibrium concentrations of adsorbed protein and free protein, respectively.

Figure 2 shows that the partition coefficient of AMG adsorption on β-CD–chitosan is affected by pH. As can be seen, at low and high pH values the partition coefficient decreases sharply. A peak was obtained in the partitioning behavior. There is an optimal pH range for AMG enzymes and the CD–chitosan system.

The minimum value for the partition coefficients was obtained near pH 7.0. Below this value the partition coefficient increases and reaches its peak between 3.3 and 4.5. This value also corresponds to the isoelectric point of AMG enzymes (p*I* at around 3.5–3.6). Below this value the partition coefficient also decreases, and very little adsorption takes place at pH 3.0. The pH effect may be due to electrostatic repulsions among the enzyme molecules, and since these effects are minimum at low pI values, maximum adsorption may be obtained (1).

Our findings on the effect of pH are not in agreement with Fukuda et al. (5) who did not observe any pH effect in the 2.0 to 9.0 range in their studies of glucoamylase adsorption onto CD–Sepharose 6B. This may be due to the different chromatographic support they used in their experiments. Nevertheless, studies conducted with raw starch showed a pH effect on the adsorption system (5, 12). The highest solid phase concentra-

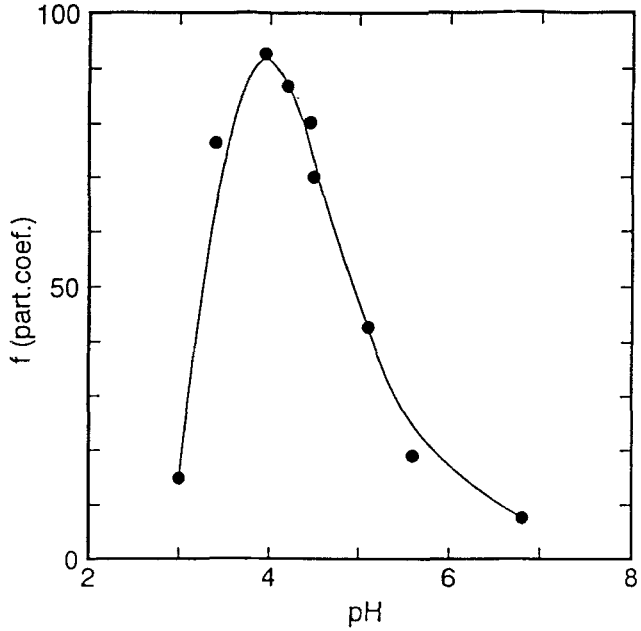

FIG. 2 Effect of pH on partition coefficient of *A. niger* AMG adsorption on β-CD–chitosan.

tions of glucoamylase at various protein concentrations were obtained at pH 3.5, the isoelectric point of the enzyme.

Effect of Temperature

In this study the influence of temperature on the partition coefficient for AMG enzymes was investigated at certain temperatures between 4 and 40°C. Figure 3 shows the effect of temperature on partitioning (data for temperatures below 20°C are not shown). As can be seen, temperature slightly affects the phase equilibria. As the temperature value increases, the partition coefficient decreases. Nevertheless, at temperatures below 293 K the time to reach equilibrium was found to be longer (i.e., 48 hours).

Adsorptivity is expected to decrease with increasing temperature, since adsorption is an exothermic process. Operations at high temperatures with proteins are unfavorable because protein denaturation and enzyme inactivation may occur. Room temperature is usually selected for large-scale protein purifications.

Effect of Ionic Strength

This experiment was conducted to investigate the effect of ionic strength on adsorption. Figure 4 shows the effect of ionic strength on the partition

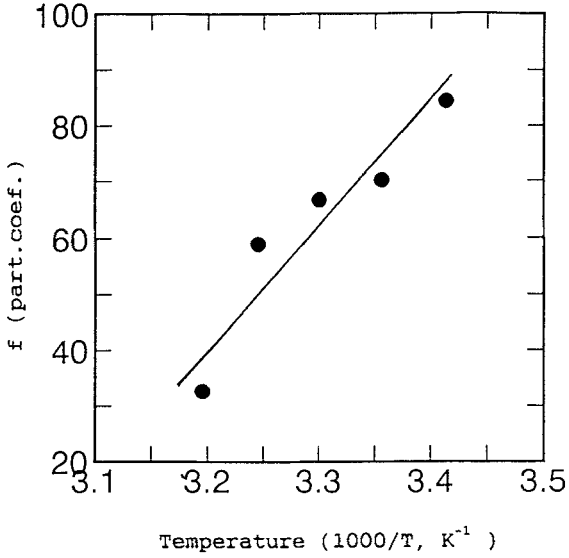

FIG. 3 Effect of temperature on partition coefficient.

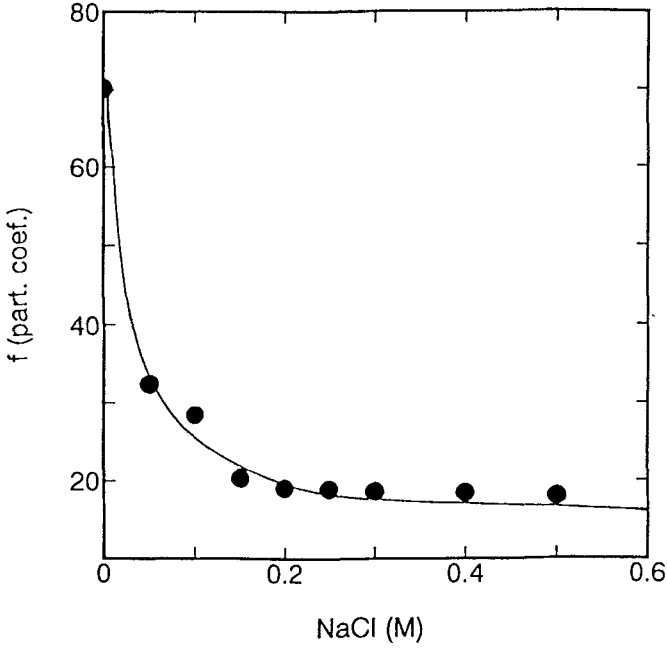

FIG. 4 Effect of ionic strength on partition coefficient.

coefficient for adsorption. The abscissa in Figure 4 shows the molar concentrations of NaCl in the solution. As can be seen, the partition coefficient decreases sharply with an increase in ionic strength. Above a NaCl concentration value of 0.2 M, very little adsorption takes place, and at NaCl concentrations above 2.0 M the partition coefficient reaches its minimum (i.e., $f = 5.0$). Nonetheless, as we shall see later, even at these ionic strength values the enzyme could not be completely desorbed from the affinity support.

Dalmia and Nikolov (12) found an increase in adsorption with increasing ionic strength in their studies of the glucoamylase/raw starch system. They suggest that electrostatic repulsions exist among enzyme molecules on the starch surface, and that a high ionic strength therefore weakens nonspecific interactions.

Adsorption Isotherms

Figure 5a shows the equilibrium isotherm for AMG at pH 4.5. This pH was selected for the isotherm experiments because it was found to be the optimum for AMG enzyme activity and stability, as described earlier.

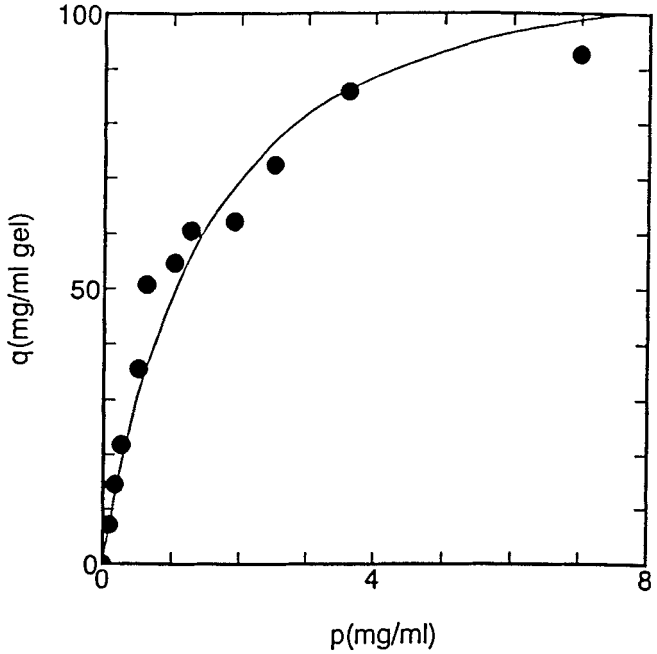

FIG. 5a Equilibrium isotherm for AMG enzyme. Line corresponds to Langmuir isotherm using values reported in Table 1.

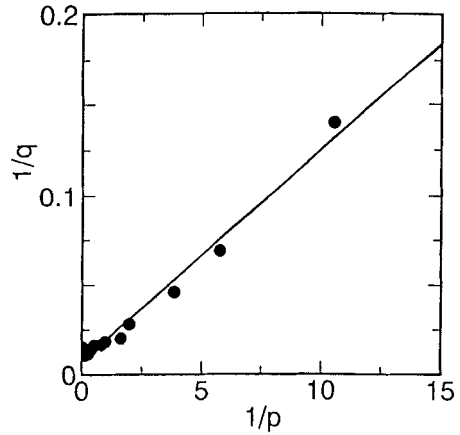

FIG. 5b Linearized adsorption isotherm of AMG on β-CD–chitosan at pH 4.5.

TABLE 1
Adsorption Isotherm for AMG/β-CD–Chitosan System

q_m (mg adsorbed enzyme/mL hydrated gel)	K_p (mg free enzyme/mL solvent)
120.5	1.4

The solid line shows the best fit Langmuir isotherm using the parameters reported in Table 1. The adsorption isotherm was described by the Langmuir expression which shows the conventional description of phase equilibria in adsorption processes:

$$q = \frac{q_m p}{K_p + p} \tag{2}$$

The linearized form of this equation is

$$\frac{1}{q} = \frac{K_p}{q_m}\frac{1}{p} + \frac{1}{q_m} \tag{3}$$

where q_m is the maximum binding capacity of the gel and K_p is an appropriate constant.

The parameters were estimated from the slope and intercept of a linearized form of the Langmuir expression shown in Eq. (3) and Figure 5b. As seen in Table 1, the maximum adsorption capacity is 120.5 mg/mL, and the dissociation coefficient was found to be 1.4 mg/mL for AMG. A linear approximation can be obtained for free enzyme concentrations below 1 mg/mL, which is the range used for partition coefficient experiments.

Desorption of the Enzyme from the Matrix

Desorption of AMG enzymes at different pH and ionic strength values was done at 25°C. As can be seen Table 2, which shows the percentage

TABLE 2
Elution of AMG Enzyme from the Affinity Chromatography Media

Elution buffer	Recovery (%)
1 M NaCl in 0.1 M acetate buffer, pH 4.5	26.6
2 M NaCl in 0.1 M acetate buffer, pH 4.5	31.4
0.5 M NaCl in 0.1 M acetate buffer, pH 5.0	38.8
2 M NaCl in 0.1 M borate buffer, pH 8.0	100
0.1 M Borate buffer, pH 8.0	100

recovery of the enzyme, desorption of the enzyme was achieved with 0.1 M borate buffer and pH 8.0, with/without 2 M NaCl. With salt present, pH 4.5 and 5.0 acetate buffers did not help elute the enzyme completely. The optimal pH for elution is 8.0. There is no need to remove any salt, and the enzyme is stable in this buffer solution.

As mentioned earlier, even though the partition coefficient was found to be very small according to the ionic strength experiments, salt had little effect on desorption. This may be due to the way we conducted our experiments. Salt was added with enzyme at the initial stage of adsorption, and there was salt ions and enzyme competition for the adsorption sites.

CONCLUSIONS

In this study, equilibrium studies were carried out for the AMG/β-CD–chitosan system. The effect of process parameters (pH, ionic strength, temperature) on the equilibrium partition coefficients were studied in order to optimize the adsorption conditions for AMG enzymes. It was shown that adsorption equilibrium partition coefficients were affected by pH, ionic strength, and temperature.

The findings showed that pH between 4.0–4.5, temperature of 20–25°C, and low ionic strength values were the optimal operating conditions. Those values also correspond to the pH and/or temperature stability of the enzyme and the maximum gel adsorption capacity.

The adsorption isotherm of AMG measured for pH 4.5 at 25°C is a good fit to the Langmuir model. The model parameters were estimated from the linearized form of the isotherm equation, and q_m and K_p were 120.5 mg/mL and 1.4 mg/mL solvent, respectively.

The enzyme was successfully eluted from the affinity matrix with a 0.1 M borate buffer, pH 8.0. This simplifies the operations because there will be no need to remove any salts from the medium.

The results of this study indicate the importance of process parameters for affinity chromatography. Selection of the optimum process parameters (e.g., pH, temperature) is important not only for the maximum adsorption conditions but also for removing other contaminating enzymes.

The experimental data presented here can be used to design an appropriate scheme to purify AMG using affinity chromatography since the experimental results show the feasibility of glucoamylase adsorption–desorption using cyclodextrin immobilized chromatographic supports. The drawback of this system is the low adsorption capacity of the matrix: 22 μmol/g dry resin (the CD concentration is 103 μmol CD/g dry resin).

Studies on adsorption–desorption kinetics and of the effects of particle size and impurities are underway.

NOMENCLATURE

f	partition coefficient (mg·mL^{-1} gel/mg·mL^{-1} solvent)
K_p	dissociation constant for adsorbent–adsorbate complex (mg/mL solvent)
p	equilibrium concentrations of free enzyme (mg free/mL solvent)
q	equilibrium concentration of adsorbed enzyme (mg adsorbed/mL gel)
q_m	maximum adsorption capacity (mg adsorbed/mL gel)

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REFERENCES

1. B. C. Saha and J. G. Zeikus, *Starch/Staerke*, *41*, 57 (1989).
2. P. J. Reilly, in Separation and Purification, 3rd ed. (E. S. Perry and A. Weissberger, Eds.), Wiley, New York, 1978, pp. 185–205.
3. B. V. McClearly, T. S. Gibson, H. Sheehan, A. Casey, L. Horgan, and J. O'Flaherty, *Carbohydr. Res.*, *185*, 147–162 (1989).
4. K. Aunstrup, in *Applied Biochemistry and Bioengineering*, Vol. 2 (B. Wingard, E. Katchalshi-Katzir, and L. Goldstein, Eds.), Academic Press, New York, 1979, p. 306.
5. K. Fukuda, Y. Teramoto, M. Goto, J. Sakamoto, S. Mitsuiki, and S. Hayashida, *Biosci. Biotech. Biochem.*, *56*(4), 556–559 (1992).
6. J. H. Pazur, in *Biotechnology of Amylodextrin Oligosaccharides* (R. B. Friedman, Ed.), American Chemical Society, Washington, D.C., 1991, pp. 51–72.
7. S. Hayashida, K. Nakahara, W. Kanlayakrit, T. Hara, and Y. Teramoto, *Agric. Biol. Chem.*, *53*, 143–149 (1989).
8. G. L. Peterson, *Anal. Biochem.*, *83*, 346 (1977).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, *193*, 265–275 (1951).
10. Novo Nordisk, *Product Sheet and Analytical Notes*, 1992.
11. S. Harsa, D. L. Pyle, and C. A. Zaror, in *Separations for Biotechnology*, 2 (D. L. Pyle, Ed.), Elsevier Applied Science, London, 1990, pp. 345–355.
12. B. K. Dalmia and Z. L. Nikolov, *Enzyme Microb. Technol.*, *13*, 982–990 (1991).

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