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EFFECT OF IMMOBILIZATION ON STABILITY AND PROPERTIES OF *N*-ACETYL-β-D-HEXOSAMINIDASE FROM *Turbo cornutus**

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ABSTRACT

A mixture of glycosidases from the liver of the gastropod *Turbo cornutus* was co-immobilized with bovine serum albumin and glutaraldehyde, and then cast as membranes. The properties of immobilized *N*-acetyl-β-D-hexosaminidase were studied. The recovery of *N*-acetyl-β-D-hexosaminidase after immobilization was unaffected by increasing the concentration of glutaraldehyde, but was decreased by increasing the bovine serum albumin concentration. The immobilized enzyme showed enhanced resistance towards proteolytic and thermal inactivation. While the pH optimum for the soluble enzyme was 4.0, a bimodal pH curve with optima at 3.4 and 5.0 was observed after insolubilization. This bimodality was abolished when the immobilized enzyme was assayed in the presence of M NaCl. The K_m values, for *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, of the immobilized isoenzymes of *N*-acetyl-β-D-hexosaminidase were larger than those of their soluble counterparts. No loss of activity could be detected in the membrane after using it for 24 consecutive assays or after storage for at least 50 days at 4°.

INTRODUCTION

Co-crosslinking of an enzyme with a bulk protein, such as bovine serum albumin, by treatment with glutaraldehyde provides a technically simple and versatile method of immobilizing enzymes. Among the advantages of this method is the wide range of conditions of pH and temperature that can be used for the immobilization reaction. Catalase, uricase, and xanthine oxidase have been successfully immobilized by this method with retention of activity[1,2].

An extract of the liver of the gastropod *Turbo cornutus* containing twelve active, soluble glycohydrolases provides a convenient source of these enzymes for a study

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of the effect of immobilization on their kinetic properties. In a preliminary report, we demonstrated the general applicability of this method for the immobilization of glycosidases, with the finding that eight out of nine glycosidases investigated retained activities after co-immobilization with bovine serum albumin and glutaraldehyde[3]. In this paper, we report on the activity and stability of the immobilized *N*-acetyl-β-D-hexosaminidase (2-acetamido-2-deoxy-β-D-hexoside 2-acetamido-2-deoxy-β-D-hexohydrolase, EC 3.2.1.52) from *T. cornutus*.

EXPERIMENTAL

Materials. — A partially purified preparation containing glycosidic enzymes from the liver of *Turbo cornutus* was purchased from Miles Laboratories Inc. (Elkart, IN 46514), *p*-nitrophenyl glycosides from Research Products International Corp. (Elk Grove Village, IL 60007), glutaraldehyde (50%) from Fisher Scientific Co (Pittsburgh, PA 15219), trypsin (EC 3.4.4.4) (209.5 U/mg) from Worthington Biochemical Corp. (Freehold, NJ 07728), bovine serum albumin and soybean trypsin inhibitor from Sigma Chemical Co. (St. Louis, MO 63178) and pronase (45 000 proteolytic units/g) extracted from *Streptomyces griseus* from Calbiochem–Behring Corp. (San Diego, CA 92112).

Immobilization of the N-*acetyl-β-*D-*hexosaminidase.* — The *Turbo cornutus* glycosidase preparation was co-immobilized with bovine serum albumin and glutaraldehyde according to a modified procedure of Thomas and Broun[1]. In a typical experiment, the enzyme protein (100 mg) containing 8.6 U of *N*-acetyl-β-D-hexosaminidase and albumin (100 mg) were dissolved in 50 mM sodium citrate buffer (2 mL, pH 4.4), and 2.5% glutaraldehyde was added (0.65 mL). Each 100-μL aliquot of the mixture was spread on a glass plate in an area of ~1.2 cm × 1.2 cm. Membranes were formed in about 15 to 20 h at 4° in a humid environment. The membranes were wetted, removed from the glass plate with a razor, and suspended in the citrate buffer containing 0.3M glycine (5 mL) at 23° for at least 1 h to remove any remaining glutaraldehyde. The membranes were stored at 4° in 50mM citrate buffer (pH 4.4). The average thickness of each membrane, as determined by measuring the volume and area, was 80 μm. The volume of each membrane was estimated by the volume of sodium citrate buffer displaced by submerging the membranes in a conical, graduated centrifuge tube.

Assay for soluble N-*acetyl-β-*D-*hexosaminidase.* — The activity of the enzyme was measured by the method of Li and Li[4]. Assays were conducted at 37° in 50mM sodium citrate buffer (1 mL, pH 4.0) containing 2mM *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside. For determinations of the K_m values, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside was employed. The reaction mixtures were incubated from 5 to 15 min, depending on the activity of the enzyme present. The reaction was stopped by adding 0.1M sodium borate buffer (2 mL, pH 9.8) and the absorbance was measured at 400 nm. One unit of enzyme is defined as the amount

of enzyme that releases 1 μmol of *p*-nitrophenol from *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside under the just described assay conditions.

Assay for immobilized N-*acetyl-β-D-hexosaminidase.* — Each membrane was placed in a 1.5 × 10-cm test tube and 2mM *p*-nitrophenyl glycoside in 50mM sodium citrate buffer (1 mL, pH 4.0) added. The tube was shaken in a water bath (37°). Control studies indicated that the reaction rate was not sensitive to the rate of shaking at the settings used. After an incubation of 5–10 min, an aliquot was removed from the suspension and added to 0.1M sodium borate buffer (pH 9.8) to make the final volume 3 mL. The absorbance of the solution at 400 nm was measured spectrophotometrically. The extinction coefficient of 17.7 · mM^{-1} · cm^{-1} for *p*-nitrophenol was used to calculate the specific activities of the enzymes[4]. Each membrane was washed 5 times with 50mM sodium citrate buffer (3 mL, pH 4.0) between assays.

The enzymes in the assays for soluble and immobilized *N*-acetyl-β-D-hexosaminidase contain both the A and B forms, unless other conditions are indicated.

Separation of A and B forms of N-*acetyl-β-D-hexosaminidase from* Turbo cornutus. — The two forms of the enzyme from the liver extract of the gastropod were separated by DEAE-Sephadex column chromatography, at pH 6.0, by a modification of the method of Tallman *et al.*[5].

Treatment of soluble and immobilized N-*acetyl-β-D-hexosaminidase with proteolytic enzymes.* — (*a*) *Trypsin.* Digestion of the soluble enzyme with trypsin was carried out by a modified method of Jorgensen[6]. The commercial glycosidase preparation (20 mg) and an equal amount of bovine serum albumin were dissolved in 25mM potassium phosphate buffer (1 mL, pH 7.5). Trypsin (2 mg) was added, and the mixture incubated at 37°. Digestion was stopped by adding an aliquot of the solution (usually 10 μL) to an equal volume of soybean trypsin inhibitor (2 mg/mL) in the phosphate buffer. One mg of trypsin inhibitor inactivated 1.4 mg of trypsin. The activity at zero time was determined before the addition of trypsin.

Each immobilized-enzyme membrane was digested with trypsin (0.30 mg) in 25mM phosphate buffer (1 mL, pH 7.5). At various intervals, the digestion was terminated by adding the soybean trypsin inhibitor (0.72 mg) in phosphate buffer (1 mL). The membrane was washed with the citrate buffer and then assayed for *N*-acetyl-β-D-hexosaminidase activity. The activity obtained from the membrane that had been incubated for 1 h in the phosphate buffer containing no trypsin was taken as the zero time value.

(*b*) *Pronase.* Digestion of the soluble enzyme with Pronase was conducted according to the method of Hashimoto *et al.*[7]. The glycosidase preparation (10 mg) and an equal amount of albumin were dissolved in 50mM potassium phosphate buffer (0.5 mL, pH 7.5), and a solution of pronase (0.5 mL, 1 mg/mL) in phosphate buffer was added. The mixture was then incubated at 37° and, at various intervals, 10-μL aliquots of the mixtures were removed and assayed for *N*-acetyl-β-D-hexosaminidase activity in 50mM citrate buffer (pH 4.0). Pronase had no activity in the sodium citrate buffer (pH 4.0).

Each immobilized-enzyme membrane was digested at 37° with pronase (0.18 mg)

in 50mM phosphate buffer (1 mL, pH 7.5). At different time-intervals, the digestion was stopped by washing the membranes with 50mM citrate buffer (pH 4.0), and the membranes were then assayed for *N*-acetyl-β-D-hexosaminidase activity. The enzymic activity of the membranes that had been incubated for 2 h at 37° in the phosphate buffer without pronase was taken as the control value.

RESULTS

Immobilization of N-*acetyl-β-*D-*hexosaminidase with bovine serum albumin and glutaraldehyde.* — The effect of varying glutaraldehyde concentrations on membrane formation and recovery of *N*-acetyl-β-D-hexosaminidase was studied. Glutaraldehyde was added to the protein solution described in the Methods section to make final concentrations of 0.1, 0.4, 0.6, 0.8, 1.2, 1.6, and 2.0%. The membranes formed at the two lowest concentrations of glutaraldehyde were too fragile to remove from the glass plate. This suggests that there is a critical glutaraldehyde concentration below which the membrane will not form properly. The enzymic activities of membranes made at glutaraldehyde concentrations ranging from 0.6 to 2.0% were compared and no difference was observed (data not shown). No enzymic activity was detected in the supernatant from these incubation mixtures. Membranes used in this work were prepared with glutaraldehyde concentrations of 0.6 to 0.7%.

The effects of varying bovine serum albumin concentrations, as a co-cross-linking agent, on the recovery of immobilized *N*-acetyl-β-D-hexosaminidase was examined. Membranes were formed with different amounts of bovine serum albumin but the same concentration of *N*-acetyl-β-D-hexosaminidase as described in the Methods section. Table I shows that the recovery of *N*-acetyl-β-D-hexosaminidase activity decreased when the albumin amount was increased from 20 to 200 mg. When less than 20 mg of albumin was present, the membranes formed did not remain intact for enzymic assays. In this work, 100 mg of albumin were used routinely, in our

TABLE I

EFFECT OF VARYING CONCENTRATIONS OF BOVINE SERUM ALBUMIN ON RECOVERY OF IMMOBILIZED *N*-ACETYL-β-D-HEXOSAMINIDASE

Bovine serum albumin[a] *(mg)*	*Relative activity*[b]
20	100
60	93
100	87
150	59
200	58

[a]The indicated amounts of albumin were co-immobilized with 100 mg of β-N-acetylhexosaminidase preparation from *T. cornutus*, as described in the text. [b]The activity was measured and expressed as % relative to that with the lowest albumin concentration. Each measurement represents the average of duplicate determinations.

TABLE II

EFFECT OF PROTEOLYTIC ENZYMES ON SOLUBLE AND IMMOBILIZED *N*-ACETYL-β-D-HEXOSAMINIDASES

Proteolytic enzymes	*Time of proteolysis (h)*	*Recovery of activities (%)*[a]	
		Soluble	*Immobilized*
Trypsin	0	100	100
	0.5	78	97
	0.75	71	108
	1.0	68	102
	24	[b]	93
Pronase	0	100	100
	0.33	69	96
	0.67	50	96
	1.0	39	102
	2.0	27	102

[a]Values are expressed as % of the activity of the enzyme not treated with the proteases. [b]Not determined.

immobilization procedure, in order to obtain a compromise between mechanical stability and maximum glycosidase activity of the membranes. Albumin did not affect the activity of the soluble enzyme (data not shown).

Although it has been reported that enzyme immobilization in the presence of substrate or competitive inhibitor enhances the recovery of enzyme activity[8], in our studies immobilization of *N*-acetyl-β-D-hexosaminidase in the presence of its product (2mM 2-acetamido-2-deoxy-D-galactose) did not affect the recovery of the enzyme activity. Similar results were obtained when the enzyme was co-immobilized in the presence of 2mM 2-acetamido-2-deoxy-D-glucose.

Effects of proteolytic enzymes on the soluble and immobilized N-*acetyl-β-D-hexosaminidase.* — The activity of the soluble enzyme decreased with increasing pre-incubation time in the presence of trypsin (Table II). In these experiments, bovine serum albumin was added to the incubation mixture containing the soluble enzyme to give the same ratio of enzyme protein to albumin protein as in the immobilized-enzyme membrane preparation. After preincubation for 1 h at a ratio of trypsin to enzyme and albumin protein of 1:20 (w/w), the activity of the soluble enzyme decreased by 32%. Doubling the amount of trypsin in the reaction mixture did not change the slope of the inactivation curve (data not shown). Under similar digestion conditions, the immobilized enzyme activity was not affected after pre-incubation with trypsin for 24 h.

In similar experiments with Pronase, <30% of the activity was recovered after a 2-h proteolysis of the soluble enzyme. In contrast, no loss of the activity of the immobilized enzyme could be detected over the same incubation period (2 h). Prolonged incubation (16 h) of the immobilized enzyme with Pronase, however, resulted in extensive degradation of the membrane. The membranes did not remain intact

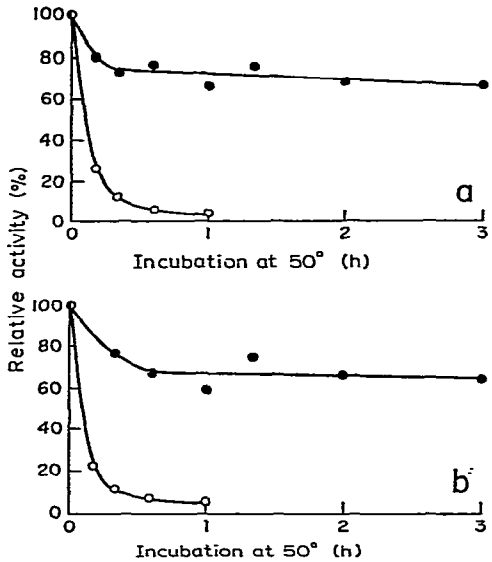

Fig. 1. Effect of heat on soluble and immobilized *N*-acetyl-β-D-hexosaminidases (A and B forms). Immobilized enzyme membranes and soluble enzymes were pre-incubated at 50° in 50mM sodium citrate buffer (pH 4.0). At the time-intervals indicated in the figure, the incubation mixtures were cooled and assayed for enzymic activities. The activity of the soluble (—○—) and immobilized enzyme (—●—), after the heat treatment, was expressed as % of the activity before the treatment: (a) A form, (b) B form.

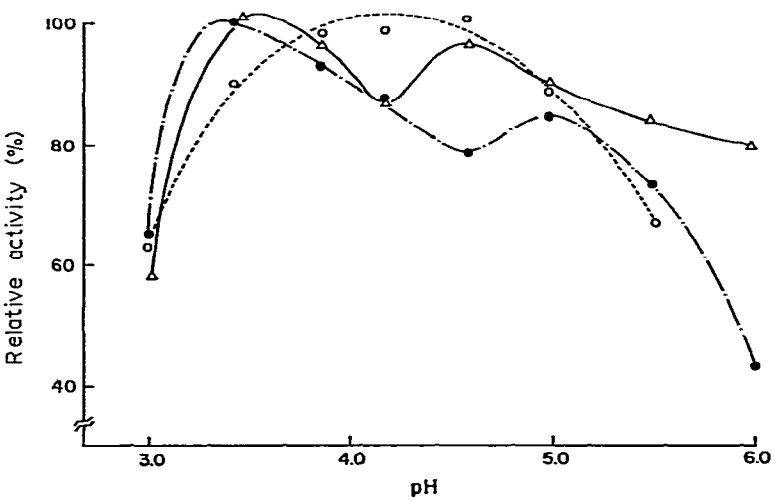

Fig. 2. pH profile of immobilized *N*-acetyl-β-D-hexosaminidase. Assays were conducted at 37° in mM substrate and 25mM sodium citrate buffer containing no (·—·), 0.5M (—△—), and M sodium chloride (—○—). All data points on each curve are expressed as % of the maximum activity obtained in each experiment. The maximal activities are 3.9, 4.2, and 4.1 units/membrane for no, 0.5M, and sodium chloride, respectively.

and could not be used for enzymic assays. At the end of 24 h, the membranes were completely solubilized.

Effect of heat on soluble and immobilized N-*acetyl-β-D-hexosaminidase.* — Evidence from electrophoretic and column chromatography data indicates that two forms of *N*-acetyl-β-D-hexosaminidase exist in the *T. cornutus* preparation[9]. The two forms were separated by ion-exchange chromatography, and each was immobilized. Fig. 1 shows the remaining activity of the soluble and immobilized enzymes after pre-incubation in a 50° water bath for various time-intervals. The soluble enzymes were rapidly inactivated by the heat treatment. After 1 h, <5% of each activity remained. The immobilized isoenzymes were relatively more stable than the soluble, since over 65% of each activity was recovered after the heat treatment for 3 h.

Effect of pH on soluble and immobilized N-*acetyl-β-D-hexosaminidase.* — Pre-incubation of the enzyme at 37° in 0.25M glycine buffer (pH 2.8) resulted in a rapid loss of *N*-acetyl-β-D-hexosaminidase activity. In 40 min, the activities of soluble and immobilized enzymes each decreased by approximately 65% (data not shown).

The pH optimum for the soluble enzyme has been reported by Muramatsu[10] to be 4.0 when assayed with phenyl 2-acetamido-2-deoxy-β-D-hexopyranoside as the substrate in citrate–phosphate buffer. We have obtained the same value under our experimental conditions, when the pH was varied from 3.0 to 6.0. Assays of immobilized *N*-acetyl-β-D-hexosaminidase activity showed two pH optima, one at 3.4 and the other at 5.0 (Fig. 2). When the assays were conducted on 0.5M sodium chloride solutions, pH optima of 3.6 and 4.6 were observed. In the presence of M sodium chloride, a broad pH optimum from 4.0 to 4.5 was observed. When immobilized A form of the enzyme was assayed in the absence of sodium chloride, a similar bimodal pH curve (optima at 3.6 and 5.0) was observed (data not shown).

Effect of urea treatment on N-*acetyl-β-D-hexosaminidase.* — The enzyme was dissolved in an 8M urea solution and incubated for 15 min at 23°. When aliquots of the solution were assayed at pH 3.4 and 5.0, the residual activities were 18 and 25%, respectively. Each membrane was incubated with 8M urea (1 mL) under similar conditions, and then washed with the citrate buffer. The enzymic activities remaining in the membranes were 23 and 12% when assayed at pH 3.4 and 5.0, respectively.

Stability of immobilized N-*acetyl-β-D-hexosaminidase towards usage and storage.* — Two membranes, which were used for 24 consecutive assays, were found to retain all their enzymic activities. Storage of the membranes for 50 days at 4° in 50mM citrate buffer (pH 4.4) also did not result in any loss of the enzymic activity.

DISCUSSION

Previous studies have shown that glycosidic enzymes can be successfully immobilized by various means with retention of activity. β-Galactosidase (EC 3.2.1.23) has been immobilized on aminoethylcellulose[11], collagen[12], and inert protein[15], and by entrapment in cellulose triacetate fibers[13]. β-Glucosidase (EC 3.2.1.21) has been insolubilized on polyacrylic-type copolymers[14] and in a porous matrix[15]. These

studies, however, dealt with the immobilization of individual glycosidases and presented no evidence that these methods could be of general use for immobilizing other glycosidases.

The glutaraldehyde–albumin co-crosslinking technique provides a simple, general method for preparing immobilized glycosidases. The yield of immobilized-enzyme activity, while unaffected by increasing glutaraldehyde concentrations, was diminished by increasing the amount of bovine serum albumin contained in each membrane. Both observations may be explained by the influence of diffusional limitations on the substrate within the membrane matrix. At less than saturating substrate concentrations, a substantial portion of the substrate would be degraded before it reaches the enzyme sites in the membrane interior[16]. Thus, there is more catalytic potential within the membrane than is reflected in the observed reaction rates. Some of this occluded enzyme activity could be destroyed by increasing the amount of glutaraldehyde without that loss being reflected in the net reaction-rate. In contrast, increasing the amount of albumin yielded thicker membranes, so that a greater portion of the total catalytic capacity of the membranes was contained in the membrane interior and, thus, was less accessible to the substrate. The overall-observed reaction rate per membrane would therefore be expected to decline.

The altered pH profile that was observed may be attributed to fixed-charge effects in the immobilized-enzyme matrix. The possibility that this bimodal pH profile is due to the presence of the two isoenzymes, each having a different pH optimum after immobilization, is ruled out, because the immobilized A form also showed similar biphasic pH curves. This altered pH profile persists at moderate ionic-strength, although it is shifted somewhat. The bimodality of the profile was eliminated at high ionic-strength. These results provide strong evidence that the explanation for this bimodal pH profile lies in the nature of the fixed charges of the membrane matrix. If the matrix were positively-charged, protons and other cations in the bathing medium would be excluded from the matrix. Consequently, the pH inside the membrane would be higher than that of the bathing solution. Conversely, if the membrane contained negative-fixed charges, the pH inside the membrane matrix would be lower than in the bulk solution. However, in order to explain a bimodal pH curve, it must be assumed that the sign of the fixed charge changes as the pH of the bulk medium is varied between 3.0 and 6.0. There now could be two external pH values at which the internal pH would be optimal for enzymic activity, one above and one below the isoelectric pH of the membrane matrix. Evidence that this effect is a general one, not specific for *N*-acetyl-β-D-hexosaminidase, has been obtained in this laboratory in experiments using immobilized α-D-galactosidase[17]. Immobilized α-D-galactosidase also displayed a bimodal pH curve. This bimodal pH profile could also be abolished by assaying the enzyme at high ionic-strength.

Both trypsin and pronase inactivated the unmodified, soluble *N*-acetyl-β-D-hexosaminidase. The behavior of the immobilized enzyme towards proteolysis is of particular interest. The immobilized enzyme is resistant to proteolysis by trypsin for up to 24 h. This enhanced stability of the immobilized enzyme is probably caused

TABLE III

K_m OF *N*-ACETYL-β-D-HEXOSAMINIDASES A AND B FOR *p*-NITROPHENYL 2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSIDE

N-*Acetyl-β-D-hexosaminidase*[a]		*Apparent* K_m *(mM)*[b]
Soluble	A	2.9
	B	3.2
Immobilized	A	4.2
	B	6.0

[a]Assays were conducted, as described in the text, with various concentrations of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside as the substrate. [b]The apparent K_m values were obtained from least-square fits of Lineweaver–Burk plots.

by a lowered lysine content of the membranes[18] and, therefore, a decreased susceptibility to proteolysis by trypsin. Such stability could also be a result of the exclusion of trypsin molecules by the glutaraldehyde-cross-linked protein matrix. The latter explanation is supported by the observation that the immobilized enzyme is also resistant to digestion by Pronase, which hydrolyzes peptide bonds nonspecifically.

The apparent K_m values for the immobilized isoenzymes are about twice of those for the soluble forms (see Table III). The elevated K_m values of the immobilized enzymes may also be attributed to mass-transfer limitations. Theoretical calculations by Hamilton and coworkers show that, from Lineweaver–Burk plots, enzymic reactions which are influenced by diffusion may yield K_m values as much as three times larger than those obtained for nondiffusion-limited reactions, depending on the assay conditions[19].

N-Acetyl-β-D-hexosaminidase isolated from various sources, including the "hepatopancreas" of the marine bivalve, *Mytilus edulis*, has been shown to exist as isoenzymes[5,20–22]. We have obtained evidence, from ion-exchange column chromatography and electrophoretic experiments, that two forms of the enzyme are also present in the gastropod, *T. cornutus*[9]. Both forms of the soluble enzyme were rapidly inactivated by heat. The instability of both isoenzymes of *N*-acetyl-β-D-hexosaminidase towards heat treatment has also been reported by Tallman *et al.*[5], who isolated the isoenzymes from human placenta[5], and by Mozo *et al.* who studied the isoenzymes in the hepatopancreas of *M. edulis*[22]. Fig. 1 shows that the stability of both immobilized isoenzymes of *N*-acetyl-β-D-hexosaminidase towards heat treatment was greatly enhanced by immobilization. Enhanced stability of other immobilized enzymes towards heat treatment has also been demonstrated[23].

Immobilization, however, does not enhance the stability of enzymes under all conditions. When the stabilities of the soluble and immobilized enzymes were compared in the presence of 8M urea, both enzymes were similarly inactivated.

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