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EFFECT OF IMMOBILIZATION ON THE STABILITY AND SUBSTRATE SPECIFICITY OF α-D-GALACTOSIDASE ISOLATED FROM THE INVERTEBRATE *Turbo cornutus**

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ABSTRACT

A mixture of glycosidases from the liver of the invertebrate *Turbo cornutus* was co-immobilized with bovine serum albumin in the form of membranes by use of glutaraldehyde as a crosslinking reagent. The properties of the native and immobilized α-D-galactosidase were compared. Immobilization increased the stability of the α-D-galactosidase towards inactivation by heat, urea, or trypsin, and permitted repeated use of the membrane preparation without significant loss of activity. The untreated enzyme hydrolyzed the terminal α-D-galactopyranosyl group from melibiose, raffinose, stachyose, *O*-α-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-glucopyranosylceramide, and 2,3-di-*O*-acyl-1-*O*-[*O*-β-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl]-D-glycerol. The immobilized α-D-galactosidase also hydrolyzed the free oligosaccharides, but had no detectable activity towards the glycolipids.

INTRODUCTION

The gentle method of immobilizing enzymes by use of glutaraldehyde as a crosslinking reagent and bovine serum albumin as a carrier protein is useful in the study of the effects of immobilization on the properties of an enzyme. In a preliminary study on immobilization of glycosidases of *Turbo cornutus*, the feasibility of

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this technique was demonstrated[1]. Although reports have appeared on the properties of immobilized α-D-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) isolated from plant[2] and microorganisms[3], there has been no report on an immobilized α-D-galactosidase isolated from an invertebrate source. In this communication, the characteristics (activity, stability, and substrate specificity) of native α-D-galactosidase isolated from the marine invertebrate *Turbo cornutus* are compared with the characteristics of this enzyme in its immobilized form.

EXPERIMENTAL

Materials. — The following materials were obtained: *Turbo cornutus* liver extract containing α-D-galactosidase and other glycosidic enzymes[4] from Miles Laboratories Inc. (Elkhart, IN 46515); 4-nitrophenyl α-D-galactopyranoside from Research Products International Corp. (Mt. Prospect, IL 60056); stachyose, soybean trypsin inhibitor (STI), NAD, NADH, α-D-galactose dehydrogenase from *Pseudomonens fluorescens*, and bovine serum albumin (BSA) from Sigma Chemical Co. (St. Louis, MO 63778); 50% glutaraldehyde from Fisher Scientific Co., (Pittsburgh, PA 15219); raffinose and melibiose from Baltimore Biological Supply (Baltimore, MD 20014); plant 2,3-di-*O*-acyl-1-*O*-[*O*-β-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl]-D-glycerol (digalactosyl diglyceride) from P-L Biochemicals Inc. (Milwaukee, WI 53205); trypsin from Worthington Biochemical Corp. (Freehold, NJ 07728); sodium taurocholate from Calbiochem–Behring Corp. (San Diego, CA 092112); Triton X-100 from Rohm and Haas Co. (Philadelphia, PA 19105); and *O*-α-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-glucopyranosylceramide (CTH) from Supelco Inc. (Bellefonte, PA 16823). All other materials were of analytical reagent grade.

Enzyme assays. — The activity of α-D-galactosidase was assayed according to the method of Li and Li[5]. Typically, the reaction mixture (1 mL) contained 2mM 4-nitrophenyl α-D-galactopyranoside and the enzyme (3–6 milliunits) in 50mM sodium citrate buffer, pH 4.0. After incubation for 30 min at 25°, the reaction was terminated by the addition of 0.1M sodium borate buffer or 0.5M glycine, pH 9.8 (2 mL). When the immobilized enzyme was assayed, the membrane was removed prior to the addition of the borate or glycine buffer. The absorbance of the solution at 400 nm was measured. A unit of activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of 4-nitrophenol (extinction coeff., 17 700 mol^{-1}, ref. 5) per min at 25°. Hydrolysis of the complex carbohydrates was monitored by determining, with the method of Yeung *et al* [6], the amount of D-galactose released. All experiments were performed in duplicate.

Immobilization of α-D-galactosidase. — The *Turbo cornutus* mixed-glycosidase preparation was co-immobilized with bovine serum albumin (BSA) and glutaraldehyde as a membrane according to the procedure of Thomas and Broun[7] in the following manner: BSA (200 mg) was dissolved in 0.1M sodium citrate buffer, pH 4.0, containing 0.02% sodium azide (3.0 mL). Enzyme protein (100 mg)

was dissolved in this buffer–BSA solution, and distilled water (0.88 mL) and glutaraldehyde (25%, 0.12 mL) were added. The solution (0.1-mL aliquots) was deposited onto a glass plate that had been previously marked off in 1.2 × 1.2 cm squares with a wax pencil, and the membranes were allowed to form at 4°. After 24 h, they were wetted with buffer and removed from the glass plate with a razor and washed with 0.3M glycine for at least 1 h in order to remove any excess of glutaraldehyde. The membranes were washed at 4° with 0.1M sodium citrate buffer, pH 4.0, in order to remove any unreacted enzyme, and stored in buffer (0.1M sodium citrate, pH 4.0, 0.02% sodium azide) at 4°. The immobilization procedure was carried out at pH 4.0 because the α-D-galactosidase was unstable to prolonged exposure to pH values near neutrality.

Enzyme assays using the naturally occurring glycolipid substrates CTH and di-D-galactosyldiglyceride were conducted as follows. The reaction mixture (0.5 mL) contained glycolipid (100 nmol), enzyme (0.5 mg, 4.3 milliunits) or 1 membrane (2.15 milliunits), 6mM sodium taurocholate, 0.04% Triton X-100, 50mM sodium citrate buffer, pH 4.0, and 0.01% sodium azide. The mixture was incubated at 25° for various time-intervals (0–72 h). D-Galactose release was determined by the method of Yeung *et al.*[6]. Controls were included both to insure that D-galactose dehydrogenase did not act on the intact substrates and to detect any D-galactose released from glycoproteins that may be present in the *Turbo cornutus*, mixed-glycosidase preparation.

Treatment of native α-D-galactosidase with trypsin. — Digestion of the native enzyme with trypsin was carried out with the modified method of Jorgensen[8]. A solution containing BSA (100 mg/mL), *Turbo cornutus*, mixed-glycosidase preparation (50 mg/mL), 25mM sodium citrate, and 50mM potassium phosphate (pH 7.6) was prepared. Trypsin (209.5 units/mg, 10.6 mg/mL) was dissolved in 0.1M potassium phosphate buffer, pH 7.6. To perform the experiment, the BSA–enzyme mixture (0.062 mL) was added to the trypsin solution (0.187 mL) to give a final volume of 0.25 mL, and incubated at 25°. The incubation times varied from 0 to 4 h. Trypsin and enzyme solutions were prepared just prior to the experiment. The reaction was stopped by the addition of 8% soybean trypsin inhibitor (STI) dissolved in the phosphate buffer (1 mg of STI neutralized 1.25 mg trypsin). Aliquots of this solution were assayed for α-D-galactosidase activity as previously described in the Enzyme assays section.

Treatment of the immobilized enzyme with trypsin. — A membrane containing BSA (5.0 mg) and *Turbo cornutus*, mixed glycosidase enzyme (2.5 mg) was placed in phosphate buffer (0.5 mL) and trypsin solution (0.145 mL) as described in the preceding paragraph. This solution (0.645 mL) was incubated at 25° for various time-intervals (0–4 h). At the end of the incubation period, STI (0.1 mL, 30 mg/mL) was added to stop the reaction. The membrane was washed with distilled water and assayed for α-D-galactosidase activity as described in the Enzyme assays section. The carbohydrates used in this study have the following structures: CTH, *O*-α-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-glucopy-

ranosylceramide; digalactosyl diglyceride, 2,3-di-*O*-acyl-1-*O*-[*O*-β-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl]-D-glycerol; melibiose, *O*-α-D-galactopyranosyl-(1→6)-D-glucose; raffinose, *O*-α-D-galactopyranosyl-(1→6)-*O*-β-D-glucopyranosyl-(1→2)-D-fructose; and stachyose, *O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→6)-*O*-β-D-glucopyranosyl-(1→2)-D-fructose.

RESULTS AND DISCUSSION

Broun *et al.*[9] had great success in the application of glutaraldehyde in enzyme immobilization where the enzymes were crosslinked with bovine serum albumin. Covalent attachment of protein to polymeric supports is a known method of enzyme immobilization[2,9,10,11]. Glutaraldehyde immobilization is a gentle method because the reaction occurs under relatively mild conditions (low temperature and near neutral pH). This method has been successfully used for the immobilization of catalase, uricase, xanthine oxidase, and *N*-acetyl-β-D-hexosaminidase[7,12,13].

The α-D-galactosidase contained in the *Turbo cornutus*, mixed-glycosidase preparation could be immobilized with ~50% retention of its original hydrolytic activity towards a synthetic substrate (4-nitrophenyl α-D-galactopyranoside), *i.e.*, when 100 units of native α-D-galactosidase activity were used in the immobilization procedure, 50 units of enzyme activity were recovered in the membranes assayed under conditions optimum for the hydrolysis of the synthetic substrate by the membranes. Several reasons for the loss of activity upon immobilization are plausible. Possibly, all the enzyme is not bound in the membrane and subsequently is lost in the washings, or an amino acid essential for catalytic activity may be altered or obstructed (*e.g.*, lysine) during the polymerization reaction. Alternatively, many of the active sites may simply be rendered inaccessible.

Upon immobilization, the optimum pH of the enzyme shifted to a more acidic value lower than that of the native enzyme (data not shown). Both native and immobilized α-D-galactosidase are stable at 25°, and heat labile at 45° and 55° (Table I). However, after 5 min of incubation at 37°, the native enzyme lost half of its initial activity whereas the immobilized enzyme was unaffected. The immobilized enzyme was also more stable at 45°. When preincubated with 2M urea for 5 h at 25°, the immobilized enzyme retained all of its initial activity. Under identical conditions, the native enzyme lost 60% of its initial activity (Table II).

The observed increase in heat stability of immobilized α-D-galactosidase may be due to the bonding to the support (BSA), presumably at several sites, which thereby increases the activation energy for "uncoiling" the enzyme. Another possibility may be that the diffusional resistance of the unheated membrane does not allow the substrate to reach the masked active sites of the enzyme that are located deep within the membrane matrix. Upon heating, the membrane may become more permeable to the substrate, exposing once obstructed catalytic sites. Stability of the immobilized enzyme towards urea is probably due to rigidity resulting from the crosslinking to BSA.

TABLE I

EFFECT OF HEAT ON NATIVE AND IMMOBILIZED α-D-GALACTOSIDASE[a]

Temp. (degrees)	*Relative activity remaining (%)*	
	Native	*Immobilized*
4	100	100
25	98	105
37	48	103
45	22	65
55	10	12

[a]Native and immobilized α-D-galactosidase were preincubated at the indicated temperature for 5 min. After preincubation, they were placed in an ice bath and subsequently assayed for α-D-galactosidase activity as described.

TABLE II

COMPARISON OF STABILITY OF NATIVE AND IMMOBILIZED α-D-GALACTOSIDASE TOWARDS UREA TREATMENT[a]

Preincubation time (h)	*Activity remaining (%)*	
	Native	*Immobilized*
0	100	100
2	70	97
5	40	100

[a]The native and immobilized enzyme preparations were preincubated at 25° in 50mM sodium citrate, pH 4.0, containing 2M urea. After the preincubation period, the samples were dialyzed at 4° against 20mM sodium citrate (250 mL), pH 4.0, for 1 h with 2 changes. Controls were preincubated identically, except that urea was not included in the preincubation solution. Following dialysis, α-D-galactosidase was assayed as described in the Enzyme assays section.

Immobilization also enhanced the stability of α-D-galactosidase towards trypsin, as demonstrated in Fig. 1. This greater stability could be due to the crosslinking of free lysine residues with glutaraldehyde, which would reduce the amount of sites available for attack by trypsin. This stability could also result from the exclusion of the trypsin molecules by the glutaraldehyde-crosslinked protein matrix.

Immobilized α-D-galactosidase showed a substrate specificity similar to that of the native enzyme (Table III). Both the immobilized and the native forms of the enzyme were able to cleave the terminal α-D-galactopyranosyl group from raffinose, melibiose, and stachyose. The native enzyme had specific activities towards these substrates of 75.0, 37.2, and 18.6 nmol · h^{-1} · mg^{-1} of protein, respectively. The immobilized form of the enzyme had specific activities towards these substrates of 32.4, 27.0, and 3.0 nmol · h^{-1} · mg^{-1} of protein, respectively. D-Galactose could be cleaved from CTH (2.4 nmol · h^{-1} · mg^{-1}) and digalactosyl diglyceride (6.2 nmol · h^{-1} · mg^{-1}) by the native enzyme. Under the assay condi-

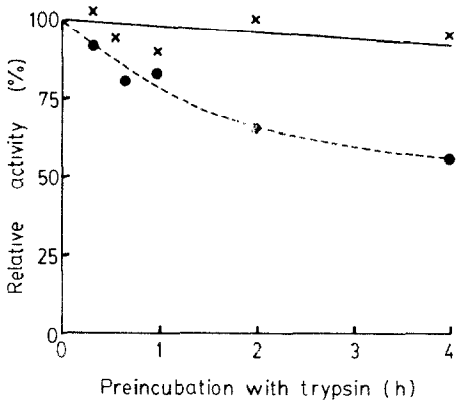

Fig 1. The effect of trypsin on native and immobilized α-D-galactosidase The native (●——●) and immobilized (×——×) enzymes were treated with trypsin and assayed for α-D-galactosidase activity as previously described The ratios of trypsin to BSA to mixed-glycosidase preparation was 1 0 mg 3 13 mg 1.57 mg, respectively

TABLE III

SUBSTRATE SPECIFICITY OF NATIVE AND IMMOBILIZED α-D-GALACTOSIDASE FROM *Turbo cornutus*[a]

Substrate	*Substrate conc.* (mM)	*Specific activity (nmol h^{-1} mg^{-1})*	
		Native	*Immobilized*
Raffinose	10	75	27 0
Melibiose	10	37.2	32.4
Stachyose	10	18.6	3 0
Digalactosyl diglyceride[b]	2	6.2	0
CTH[b]	2	6.2	0

[a]The assays were conducted at 25° in 50mM sodium citrate buffer, pH 4 0, and the amount of D-galactose released was determined by the method of Yeung *et al* [8] [b]These assays also contained 6mM sodium taurocholate, 0 04% Triton X-100, and 0.01% sodium azide These compounds have a negligible effect on the native enzyme

tions employed, the hydrolytic activity towards these glycolipids could not be demonstrated with the immobilized enzyme. The inability of the immobilized enzyme to cleave the terminal α-D-galactopyranosyl group from CTH and digalactosyl diglyceride is perhaps due to the formation, in aqueous solution, of micelles that become too large to penetrate the membrane matrix. The specific activity of the immobilized enzyme towards di-, tri-, and tetra-saccharides decreased with the increase in molecular weight of the substrate (Table III). Presumably, this is due to the lower diffusion rates of the larger substrates and steric hindrances within the membrane matrix.

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