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THE PROTEOLYTIC NATURE OF COMMERCIAL SAMPLES OF GALACTOSE OXIDASE

PURIFICATION OF THE ENZYME BY A SIMPLE AFFINITY METHOD

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Summary

Several commercially available samples of galactose oxidase (D-galactose: oxygen 6-oxidoreductase, EC 1.1.3.9) were found to contain high proteolytic activity on proteins such as fibrinogen, transferrin, albumin and casein. A simple, efficient method was devised for the purification of galactose oxidase which relies on the affinity of the enzyme for agarose (Sepharose 6B). The purified galactose oxidase was recovered in high yield free from proteolytic activity. The enzymic affinity for Sepharose and Sephadex was investigated to clarify the absorption mechanism.

Introduction

An earlier report from this laboratory [1] described the proteolytic nature of commercially available neuraminidase preparations obtained from the culture filtrate of *Clostridium perfringens*. A method was presented, to purify the enzyme of proteases, which has since been used for the desialylation of proteins [2,3] and cells [4,5] for in vivo study. Recently, in our studies on the oligosaccharide components of asialoproteins we have used some commercially available samples of galactose oxidase (D-galactose:oxygen 6-oxidoreductase, EC 1.1.3.9) derived from *Dactylium dendroides* or *Polyporus circinatus*. The present report describes the highly proteolytic nature of these products and provides a simple and efficient affinity procedure for isolating galactose oxidase from the contaminant proteases. A brief description of some properties of galactose oxidase is also included.

Materials and Methods

Sources of galactose oxidase. Samples were obtained from Sigma Chemical Co., St. Louis, Mo., from *P. circinatus* lot numbers 34C-0800, 102 units/mg solid and 114C-0108, 38 units/mg; P-L Biochemicals Inc., Milwaukee, Wis., from *D. dendroides* lot number 542-4, 125 units supplied, and Worthington Biochemical Corporation, Freehold, N.J., from *D. dendroides* no lot number given, contents 850 units of minimum activity 30 units/mg. All the above enzymic activities were quoted by the suppliers.

Purification of commercial galactose oxidase samples. A column of Sepharose 6B (10 × 1 cm) was equilibrated with 0.1 M ammonium acetate pH 7.2 at room temperature. Galactose oxidase (150—500 units as given by the distributor) was dissolved in 2—4 ml of 0.1 M ammonium acetate and loaded on the column. The major protein constituent was washed through the column and was devoid of galactose oxidase activity. After further washing with 0.1 M ammonium acetate the enzymic activity was detected in the effluent at approx. 5—6 times the void volume. The eluted enzyme was concentrated by pressure dialysis (8/32 inch visking tubing, obtained from Scientific Instruments Centre, London, U.K.) and frozen at −70°C.

For some preparations chromatography was undertaken on a column of Sepharose/D-galactosamine. D(+)-Galactosamine · HCl (Sigma) was covalently linked through the 2-amino group to Sepharose 6B by the CNBr method [6]. The conditions for chromatography were the same as those for Sepharose 6B.

Estimation of galactose oxidase activity. D(+)-galactose was purchased from B.D.H. Ltd. (Toronto) and horseradish peroxidase (type II) and *o*-dianisidine dihydrochloride from Sigma. Galactose was prepared as a 0.15 M aqueous solution. Peroxidase and *o*-dianisidine · diHCl were added to 0.05 M sodium phosphate buffer, pH 6.1 to give each a final concentration of 0.01%. One ml galactose solution was added to 1.8 ml of the freshly prepared peroxidase/*o*-dianisidine · diHCl reagent. After incubation at 37°C for 2 min to warm the reagents, 0.2 ml galactose oxidase was added and the reaction allowed to proceed for 5—15 min. Activity was stopped by adding 1 ml 4.5 M H_2SO_4 to the mixture and the absorbance measured at 550 nm. The unit of galactose oxidase activity was arbitrarily taken as the quantity of enzyme needed to produce an absorbance change of 1.00 per min at 37°C. A change of absorbance unit per min at 425 nm and 25°C for the peroxidase/*o*-toluidine assay system used by the distributing companies is equivalent to a change of 1.19 absorbance unit at 550 nm at 37°C for the peroxidase/*o*-dianisidine system. Unless otherwise stated the unit used in this report has been determined by the *o*-dianisidine method at 37°C.

Protein concentration was measured by the Lowry et al. method [7] using human serum albumin (Behringwerke A.G., Marburg, G.F.R.) as a reference protein.

Proteolytic activity of galactose oxidase preparations. Proteolysis was detected by measuring the release of radioactive peptides from ^{125}I-labelled proteins after incubation with galactose oxidase by a method described earlier [1]. The following proteins were used as substrates: human fibrinogen (Kabi Ltd., grade L); human transferrin and albumin (Behringwerke A.G.) and casein

(B.D.H. Ltd., Hammarsten grade). After trace labelling with ^{125}I using the iodine monochloride method [8] the concentration of each substrate was adjusted to 5 mg/ml in 0.05 M sodium phosphate buffer pH 6.1. The enzyme preparation (0.1 ml containing 0.2—25 units) was added to 0.4 ml of substrate and incubated at 37°C for 17 h. After measuring the total radioactivity of the sample in a Packard model 5986 multichannel analyser, 1 ml 20% (w/v) trichloroacetic acid was added and the protein precipitate centrifuged at 3000 rev./min for 10 min at room temperature. The supernatant was filtered through glass wool to remove any trace of floating precipitate and 0.5 ml aliquots were counted. The extent of proteolysis was calculated as the percentage of protein-bound radioactivity originally present in the incubate.

The affinity of galactose oxidase for Sepharose 6B. A sample of purified galactose oxidase was labelled with ^{125}I using the ICl technique [8]. Labelling efficiency was low (approx. 1%) and the extent of iodination was calculated to be $>10^{-3}$ atoms per molecule. As a result of iodination 17% of the enzymic activity was lost. The labelled sample (3 ml) was dialysed for 24 h at 2°C against 1 l 0.1 M ammonium acetate. 18 mg of galactose was dissolved in 1 ml of the enzyme and the sample was chromatographed on a Sepharose 6B column (10 × 1 cm) which was pre-equilibrated with 0.1 M ammonium acetate pH 7.2 containing 0.1 M galactose. Simultaneously, another 1 ml sample of ^{125}I-labelled galactose oxidase was chromatographed on a matched Sepharose 6B column in 0.1 M ammonium acetate but without galactose present. After chromatography the effluent fractions from both columns were counted. Similar experiments were also undertaken on columns of Sephadex G-200 in place of Sepharose.

Gel filtration. Chromatography of the purified enzyme was carried out on a Sephadex G-200 column (50 × 2.2 cm) equilibrated at room temperature with 0.01 M Tris · HCl containing 0.25 M NaCl. The loaded sample contained 0.1 mg galactose oxidase traced with ^{125}I-labelled galactose oxidase and human serum albumin (Behringwerke; 10 mg). The flow rate was controlled at 15 ml/h. Fractions were measured for radioactivity, absorbance at 280 nm and enzymic activity.

Results

Chromatography of galactose oxidase on Sepharose 6B

In Fig. 1, chromatography of a commercial sample of galactose oxidase on Sepharose 6B is depicted. The sharp protein peak (280 nm) which emerged first from the column was yellowish in colour and devoid of galactose oxidase activity. On further washing with 0.1 M ammonium acetate, galactose oxidase activity associated with a small protein peak was recovered well separated from the major peak. Subsequent elution with ammonium acetate or 0.15 M NaCl did not displace more protein or enzymic activity and it was estimated that >90% of the loaded enzyme was yielded by the column. After concentrating the pooled enzyme peak by pressure dialysis the sample was stored at −70°C. At this temperature enzyme samples have suffered no measurable loss of activity when kept for up to 4 months. One sample was purposely frozen and thawed daily over a 14-day period and measurements showed the activity to increase marginally. However, freeze drying the purified enzyme in 0.1 M ammonium

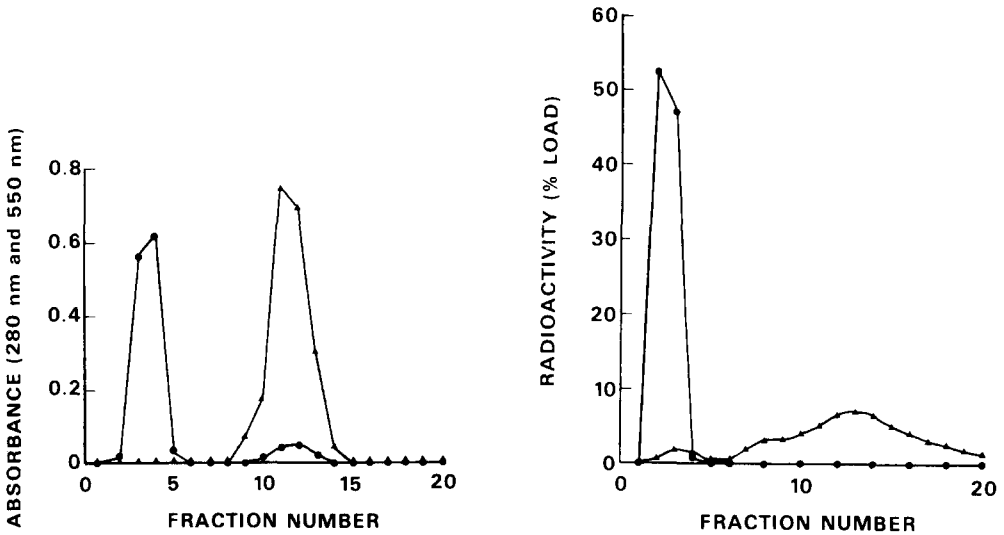

Fig. 1. Fractionation of a commercial sample of galactose oxidase (Sigma; Lot no. 114C-0108) on Sepharose 6B (10 × 1 cm). The enzyme (150 units dissolved in 2 ml 0.1 M ammonium acetate) chromatographed through the column during elution with 0.1 M ammonium acetate buffer, pH 7.2. The fraction volume was 5 ml. ●———●, absorbance at 280 nm; ▲———▲, galactose oxidase activity (550 nm) of 20-μl fraction measured as described in the text. All proteolytic activity was associated with the major 280 nm peak. The fractionation of Worthington and P.-L. Biochemicals galactose oxidase samples on Sepharose 6B gave similar elution profiles.

Fig. 2. The effect of galactose (0.1 M) on the chromatography of ^{125}I-labelled galactose oxidase on Sepharose 6B (10 × 1 cm). After loading the enzyme (1 ml volume) the column was eluted either with 0.1 M ammonium acetate pH 7.2 (▲———▲) or with 0.1 M ammonium acetate containing 0.1 M galactose (●———●). The fraction volumes were 5 ml.

acetate led to a loss of >90% of the activity when the reconstituted enzyme was compared to a control sample which had been stored at −70°C. Specific enzymic activities for the purified enzyme ranged from 303 to 313 units/mg, the weight of protein being obtained by the Lowry et al. method relative to a human serum albumin standard. Using 280 nm absorbance to determine protein content, the specific activity of one sample was calculated to be 96.7 units per $A^{1\,cm}_{280\,nm}$ 1.00.

Proteolytic activity of galactose oxidase preparations

The proteolytic activities of galactose oxidase samples obtained from three suppliers are shown in Table I. The enzyme samples showed great proteolytic activity on all substrates when present in quantities greater than 10 galactose oxidase units although differences in the extent of proteolysis were observed for certain substrates. When smaller amounts of enzyme (<1 unit) were taken protease activity on transferrin and albumin was less pronounced although still appreciable on fibrinogen and casein. The relative activities of different concentrations of the same enzyme preparation were not proportional to the amount of added enzyme which was possibly due to a depletion of the favoured peptide bond(s) for cleavage.

After Sepharose 6B chromatography the yellow protein peak contained es-

TABLE I

PROTEOLYTIC ACTIVITY OF COMMERCIAL SAMPLES OF GALACTOSE OXIDASE

Each substrate was traced with its ^{125}I-labelled derivative and incubated in duplicate for 17 h at 37°C with 0.1 ml of a galactose oxidase sample. The reaction volume was 0.5 ml and the buffer was 0.05 M sodium phosphate pH 6.1. After counting, 1.0 ml 20% trichloroacetic acid was added to each incubate and following centrifugation and filtration 0.5 ml of the supernatant was counted to determine the trichloroacetic acid-soluble radioactivity. The enzyme units are described in the text. N.D., not determined.

Protein substrate	Concentration (mg/ml)	Proteolytic activity (%)					
		Sigma *		Sigma **	P.-L.	Worthington	
		134 U/ml	2.8 U/ml	3.6 U/ml	150 U/ml	254 U/ml	5.1 U/ml
Fibrinogen	5.0	81.7	57.5	3.7	71.8	71.5	10.3
Casein	5.0	74.4	47.2	1.4	N.D.	34.2	3.4
Transferrin	5.0	54.5	4.7	0.2	5.2	6.0	0.4
Albumin	5.0	86.0	5.0	0.2	4.0	3.4	0.3

* Lot No. 34C-0800.
** Lot No. 114C-0108.

sentially all the proteolytic activity. Elution of galactose oxidase activity did not coincide with any detectable proteolytic activity. Concentrated preparations of purified galactose oxidase were tested for proteolytic activity and the results from representative experiments are given in Table II. Proteolytic activity was either absent or detected in only trace amounts.

The affinity of galactose oxidase for Sepharose 6B and Sephadex G-200 gels

Two samples of ^{125}I-labelled galactose oxidase were simultaneously chromatographed on Sepharose 6B columns of the same size. One column had been equilibrated with 0.1 M ammonium acetate buffer containing 0.1 M galactose, the other column with ammonium acetate alone. In the presence of galactose, ^{125}I-labelled galactose oxidase passed through without retardation whereas the control sample (i.e. no galactose present) was eluted with the expected effluent volume (Fig. 2). Retardation of the enzyme was also observed when the column was equilibrated with 0.01 Tris · HCl pH 7.2 containing 0.25 M NaCl although not so pronounced as in the presence of 0.1 M ammonium acetate. The results suggested that galactose successfully competed for the enzyme

TABLE II

PROTEOLYTIC ACTIVITY OF GALACTOSE OXIDASE AFTER PURIFICATION ON SEPHAROSE 6B

The reaction conditions and method of calculation are the same as those described in Table I.

Protein substrate	Concentration (mg/ml)	Proteolytic activity (% trichloroacetic acid-soluble radioactivity)			
		Sigma		P.-L. 10 U/ml	Worthington 34 U/ml
		1.4 U/ml	7 U/ml		
Fibrinogen	5.0	0	0.1	0.1	0.1
Casein	5.0	0	<0.1	0	0
Transferrin	5.0	0	<0.1	0	<0.1
Albumin	5.0	0	0	0	0

and therefore prevented affinity for the Sepharose matrix. Sepharose/galactosamine was prepared for use as a column matrix further to retard the enzyme by providing available terminal galactose units for binding. However, the chromatography was not influenced by this conjugate and the enzyme eluted in an effluent volume similar to that obtained with Sepharose alone. Moreover, if the Sepharose column was replaced with a column of Sephadex G-200 (10 × 1 cm), galactose oxidase activity was retarded significantly (although much less markedly than with Sepharose) in the presence of 0.1 M ammonium acetate but not when 0.1 M galactose was used as eluant. Finally, a sample of Sepharose was freeze dried and 10—50-mg aliquots were added to 5 units of galactose oxidase in the presence of peroxidase and *o*-dianisidine/diHCl to determine whether the matrix was also a substrate for the enzyme. After 20 h incubation at pH 6.1 together with the appropriate enzyme, substrate and reagent blanks, no significant enzymic action on Sepharose was detected.

The gel filtration properties of the purified enzyme were further investigated using a Sephadex G-200 (50 × 2.2 cm). If the column was equilibrated with 0.01 M Tris · HCl, containing 0.25 M NaCl at pH 7.2, the enzyme behaved as macromolecule with an M_r of approx. 39 000 (Fig. 3) as determined by the V_e/V_o ratio technique of Determann [9] with human serum albumin as a reference protein (M_r 69 000). The profile obtained with ^{125}I-labelled galactose oxidase compared closely with the enzymic activity and the value of 39 000 compares well with the M_r of 42 400 determined by Kelly-Falcoz et al. [11] using an equilibrium centrifugation technique. However, when the same column was equilibrated with 0.1 M ammonium acetate containing 0.1 M galactose (pH 7.2) the enzyme chromatographed with an M_r of approx. 52 000, a value which agrees more closely with an earlier result (55 000) of Bauer et al. [10] from sedimentation velocity and equilibration measurements.

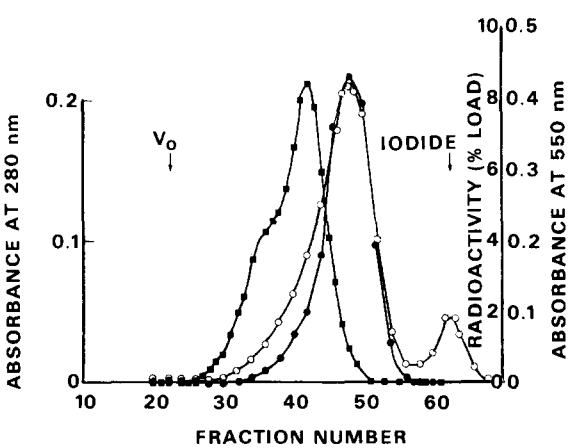

Fig. 3. Chromatography of purified galactose oxidase (traced with ^{125}I-labelled galactose oxidase) and human serum albumin (10 mg) on a column of Sephadex G-200 (50 × 2.2 cm) equilibrated with 0.01 M Tris · HCl containing 0.25 M NaCl. ■———■, absorbance at 280 nm; ○———○, radioactivity; ●———●, galactose oxidase activity. The fraction volumes were 3.4 ml.

Discussion

The study demonstrates the alarmingly proteolytic nature of certain commercial samples of galactose oxidase and researchers using such enzyme preparations should consider the possible effects on substrates of a proteinaceous or cellular nature. A simple, single-step purification method is given which effectively removes the proteolytic contaminants and allows good recovery of highly purified galactose oxidase. The purification procedure takes advantage of an affinity between galactose oxidase and Sepharose. The agarose gel retards the enzyme during chromatography in the presence of neutral pH buffers such as 0.1 M ammonium acetate or 0.01 M Tris · HCl containing 0.25 M NaCl.

Equilibrating the agarose column with 0.1 M galactose prevents enzyme binding to the matrix. Briefly, the primary structure of agarose can be described as a linear polysaccharide consisting of an alternating arrangement of D-galactose and 3,6-anhydro-L-galactose units with alternating β-(1—4) and α-(1—3) links [12]. At first, enzyme affinity was explained by the formation of an enzyme-substrate complex. However, no significant reaction was observed between galactose oxidase and Sepharose even after 20 h incubation at 37° C. The affinity of galactose oxidase could be more closely compared to a partial recognition of Sepharose as a galactose substrate, the short-lived "complex" being reversible and resulting in a delay of enzyme emergence from the column. Inhibition of enzyme binding, in the presence of 0.1 M galactose, is probably mediated by the reaction product galactohexodialdose.

Enzyme retardation by Sephadex is more difficult to explain. Sephadex is a dextran polymer largely composed of D-glucose units linked by β-(1—6) bonds but, as shown in several earlier studies [13,14], glucose is not a substrate for galactose oxidase. However, the presence of galactose or galactohexodialdose may possibly alter the shape of the galactose oxidase molecule which could account for the change in V_e/V_o ratio observed during Sephadex G-200 chromatography.

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