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SEPARATION AND PROPERTIES OF α-GALACTOSIDASE AND β-GALACTOSIDASE FROM *CAJANUS INDICUS*

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SUMMARY

Cajanus indicus α- and β-galactosidase which are inseparable by Sephadex gel-chromatography (Dey, P. M. (1973) Biochim. Biophys. Acta 302, 393–398), have now been separated by CM-cellulose chromatography. α-Galactosidase (EC 3.2.1.22) has been resolved into two isoenzymes and β-galactosidase (EC 3.2.1.23) into three. Various properties of the enzymes have been studied which have shown that they possess their own characteristic features.

INTRODUCTION

In a previous communication [1] we have reported the isolation of a specific β-L-arabinosidase from *Cajanus indicus*. This enzyme was separated on a Sephadex G-100 column from an α-galactosidase peak which also displayed α-D-fucosidase, β-L-arabinosidase and β-D-galactosidase activities. Hydrolysis of α-D-galactoside, α-D-fucoside and β-L-arabinoside by a homogeneous preparation of α-galactosidase is not uncommon because of their structural similarity [2]. α-Galactosidases have, however, been shown to have a rigid requirement of an α-galactosidic linkage. This would, therefore, indicate that the β-D-galactosidase activity shown by the α-galactosidase peak is due to a separate protein.

In this paper, we have reported the separation of β-galactosidase activity from that of α-galactosidase; the former has been resolved into three isoenzymes and the latter into two.

MATERIALS AND METHODS

Chemicals

p-Nitrophenyl-α-D-galactoside was prepared as described earlier [3]. *p*-Nitrophenyl-β-D-galactoside, 4-methylumbelliferyl-α-D-galactoside and -β-D-galactoside were purchased from Koch–Light Laboratories, Colnbrook, England. *p*-Nitrophenyl β-L-arabinoside was synthesized by the method of Feier and Westphal [4] and *p*-nitrophenyl-α-D-fucoside was a gift from Professor G. A. Levvy, Rowett Research Institute, Aberdeen.

Enzyme assay

p-Nitrophenyl-D-glycosides were used as substrates. Enzyme activity was assayed by adding appropriately diluted enzyme to a mixture of McIlvaine buffer [5], pH 5.5 and the substrate solution, held at 30 °C. The final assay volume was 1 ml; the substrate concentration was 0.5 mM. The enzyme reaction was terminated after incubation for 15 min by the addition of 5 ml of 0.1 M Na_2CO_3 solution. The amount of *p*-nitrophenol liberated was measured at 405 nm with a Unicam SP500 spectrophotometer. The reaction followed a linear course up to an absorbance of 0.7.

A unit of enzyme activity was defined as the amount which would liberate 1 μmole of *p*-nitrophenol per min at 30 °C. The specific activity was expressed as munits of enzyme per mg protein. Protein was measured by the Lowry method [6] using crystalline bovine serum albumin as a standard.

Sephadex gel chromatography

Sephadex G-100 column (2.5 cm × 98 cm) was prepared by the method recommended by Andrews [7] and equilibrated with McIlvaine buffer, pH 5.5, containing 0.1 M KCl. The column was maintained at 10 °C and eluted with a downward flow of 30 ml/h and 3-ml fractions were collected. Enzyme samples were dialysed against the column buffer before they were passed through the column.

CM-cellulose chromatography

CM-cellulose ion-exchange column (Whatman Column Chromedia, CM-52, 2.5 cm × 6 cm) was prepared according to the manufacturer's directions and equilibrated with McIlvaine buffer, pH 4.0. The sample was eluted using 60 ml buffer each time of varying pH values (pH 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5). The flow rate was maintained at 10 ml/h and 3-ml fractions were collected.

Isoelectric focusing

Ampholine, carrier ampholyte, from LKB, Sweden, with a pH range 5–8 was used. The focusing was carried out on a Shandon electrophoresis apparatus (Kohn model U77); Whatman No. 3 paper (16 cm × 3 cm) was used as a solid support. The paper strips were soaked in Ampholine solution prepared by diluting 2.5 ml of the 40% solution to 100 ml with distilled water; arginine and lysine were added to a final concentration of 0.05% each in order to stabilize the pH gradient at the alkaline end. Enzyme sample (10–20 μg) was applied in the middle of each strip in the form of a band. The electrode vessels contained 0.2 M H_2SO_4 at the anode and 0.4 M ethylenediamine at the cathode. The contacts between the electrophoretogram and the electrode vessels were made with similar filter paper wicks. Electrophoresis was carried out for 15 h using 0.8 mA per strip. At the end of the run, the paper surface was sprayed with the appropriate 4-methylumbelliferylglycoside solution (5 mg/10 ml McIlvaine buffer, pH 5.0) and incubated at 37 °C. The enzyme activities were detected under a ultraviolet lamp as distinct fluorescent bands. The active bands were cut out in duplicate experiments before spraying the substrate and eluted with water for the determination isoelectric points.

Ultrafiltration

Enzyme solutions obtained at various stages of purification were concentrated

by using an Amicon ultrafiltration cell (Amicon Corporation, Lexington, Massachusetts). Diaflo PM10 membranes, which exclude materials under 10 000 molecular weight, were used under a nitrogen pressure of 30 lb/inch^2.

RESULTS AND DISCUSSION

Isolation of the enzymes

Cajanus indicus seeds were powdered (640 g) and washed twice with 500-ml portions of acetone (−15 °C). This treatment removed acetone-soluble lipids and colouring materials. The air-dried powder was suspended in McIlvaine buffer, pH 5.5 (1200 ml) for 24 h at 4 °C and then centrifuged to obtain a clear enzymically active supernatant. The specific activity of the α- and β-galactosidase was 3.2 and 0.76, respectively.

The pH of the enzyme solution, which was 6.2, was adjusted to 4.8 by dropwise addition of citric acid (1 M) at 4 °C with continuous stirring. A heavy precipitate of inactive proteins was formed; this was completed on leaving the suspension for 24 h at 4 °C. Precipitated protein was discarded by centrifugation; the clear supernatant was found to contain most of the galactosidase activities. α-Galactosidase was enriched by 11-fold with a 70% recovery and the values for β-galactosidase were 8-fold and 95%, respectively.

The active supernatant solution from the above step was subjected to $(NH_4)_2SO_4$ fractionation. The solution was stirred for 5 h at 0–30% saturation and the precipitate discarded. At 30–50% saturation, the solution was stirred for 24 h and the precipitate thus obtained was suspended in McIlvaine buffer, pH 5.5, and dialysed against 3 l of the same buffer at 4 °C. A small amount of precipitate formed during dialysis was discarded. The same process of $(NH_4)_2SO_4$ fractionation was carried out with this dialysed solution. Both fractionation steps provided an overall increase of 5.6-fold in the specific activity of α-galactosidase with a 70% recovery. β-Galactosidase was enriched by 4.7-fold with 85% recovery.

This enzyme preparation was passed through a Sephadex G-100 column. As shown earlier [1], the α- and β-galactosidase activities were eluted as a single peak. There was a considerable increase in the specific activities of these enzymes as a result of separation of a large inactive protein peak. The enhancement of the specific activities of the two enzymes was 2.5-fold and 2.1-fold with recoveries of 79% and 85%, respectively.

The purified enzyme sample was then further chromatographed on a CM-cellulose column. The β-galactosidase activity was resolved into three peaks (I, II and III) and were eluted in the pH range 4.5–5.0, whereas two isoenzymes of α-galactosidase (I and II) were separated in the pH range 6–6.5 (Fig. 1). Each of the enzyme peaks was concentrated separately. The specific activities are expressed in Table I. The enzyme recovery in this step was 65% for the combined β-galactosidase activity and 70% for α-galactosidase.

Electrophoretic properties

The two forms of α-galactosidase were well separated on isoelectric focusing; the pI values were 6.2 and 7.1 for I and II, respectively. The pI values for β-galactosidase I, II and III were 7.1, 6.5 and 6.1, respectively.

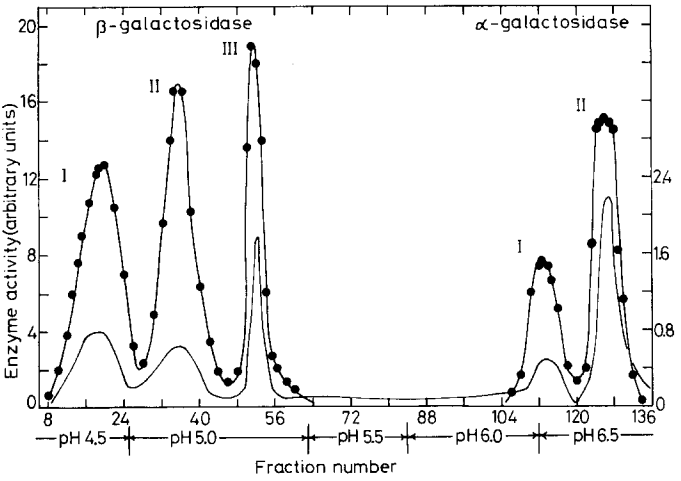

Fig. 1. Separation of the isoenzymes of α- and β-galactosidase by CM-cellulose chromatography. Details are described in the text. ——, A_{280nm}

TABLE I

SPECIFIC ACTIVITIES OF PURIFIED α- AND β-GALACTOSIDASES

Enzyme	Specific activity
α-Galactosidase I	1600
α-Galactosidase II	1000
β-Galactosidase I	180
β-Galactosidase II	230
β-Galactosidase III	150

Thermal stability

α-Galactosidase I was less stable at 50 °C than that of II (Fig. 2), however, both forms were highly thermolabile as compared to the almond enzyme [8] or the broad bean enzyme [9].

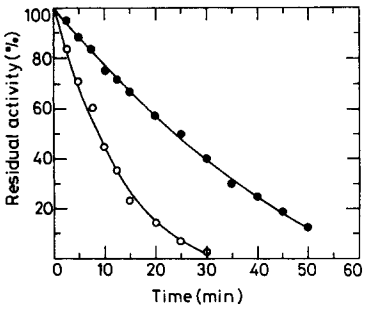

Fig. 2. Inactivation of α-galactosidase I (○—○) and II (●—●) at 50 °C in McIlvaine buffer, pH 5.5. Enzyme activity was measured at given time intervals by the usual assay method. Results are expressed as the percentage of the control (the $A_{405\ nm}$ for enzyme activity was in the range of 0.5–0.6 at the zero time).

The thermal stability of the three forms of β-galactosidase are shown in Fig. 3. At 55 °C the enzyme I seems to be less stable than that of II and III; the latter two are inactivated to a similar extent. However, the enzyme III is less stable than that of II at 60 °C. There seems to be no relation between the stability of the enzyme forms with their specific activities.

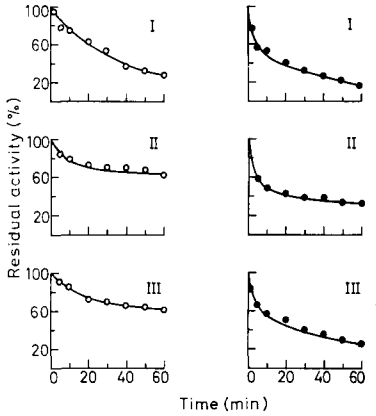

Fig. 3. Inactivation of β-galactosidase (I, II and III) at 55 °C (○—○) and 60 °C (●—●) in McIlvaine buffer, pH 4.0. Enzyme activity was measured at given time intervals by the usual assay method. Results are expressed as the percentage of the control (the $A_{405\ nm}$ for enzyme activity was in the range of 0.5–0.6 at the zero time).

pH optima and K_m values

The pH activity profiles of α- and β-galactosidases are shown in Fig. 4. The α-galactosidase I and II have distinct pH optima at 5.0 and 4.5, respectively. Both the enzymes showed single peaks as compared to double optima in several cases [2], with respect to *p*-nitrophenyl-α-D-galactoside as substrate.

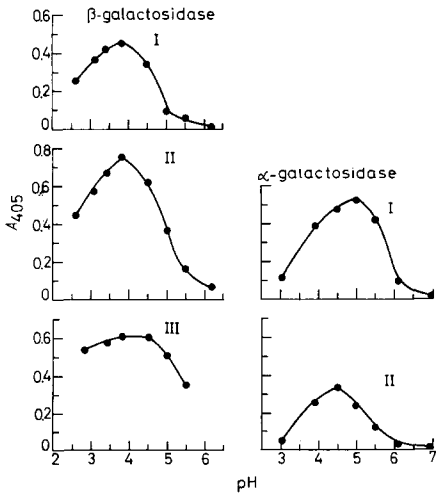

Fig. 4. Dependence on pH of α- and β-galactosidases. The enzyme samples were incubated with their respective *p*-nitrophenylgalactosides in McIlvaine buffer (pH 2.5–7.0) under the standard assay conditions. *p*-Nitrophenol liberated was estimated at 405 nm and plotted against pH.

TABLE II

K_m VALUES OF α- AND β-GALACTOSIDASES FOR THE HYDROLYSIS OF *p*-NITROPHENYLGALACTOSIDES

The concentration of the substrates ranged from 0.1 to 1.0 mM. The incubation with the enzyme was carried out according to the standard assay procedure at optimal pH values of the enzymes.

Enzyme	K_m ($M \times 10^{-4}$)
α-Galactosidase I	6.3
α-Galactosidase II	1.2
β-Galactosidase I	14.8
β-Galactosidase II	3.0
β-Galactosidase III	12.0

All the forms of β-galactosidase (I, II and III) displayed optimum activity at approx. pH 4.0 (Fig. 4). Enzyme III, however, showed a wider range of pH optimum (3.5–4.5).

Table II shows a lower affinity of α-galactosidase I for its substrate than that of II. The K_m values of the three forms of β-galactosidase are distinguishable from each other (Table II).

Substrate specificity

The α-galactosidase preparations failed to hydrolyse methyl-β-D-galactoside and lactose but hydrolysed the α-galactosidic compounds as shown in Table III. The enzyme II hydrolysed the substrates faster than enzyme I. Both isoenzymes hydrolysed *p*-nitrophenyl-β-L-arabinoside and *p*-nitrophenyl-α-D-fucoside in a similar way as

TABLE III

SUBSTRATE SPECIFICITY OF α- AND β-GALACTOSIDASES

The enzyme digest (100 μl) made in McIlvaine buffer (at optimal pH values of α- and β-galactosidases, respectively) contained 0.4 μM of each oligosaccharide (and methyl-β-D-galactoside) or 50 μg of galactomannan and 20 munits of the respective enzyme. This mixture was prepared on a piece of parafilm (Gallenkamp, England) layed over an ice-slab. The reaction mixture was taken up in a capillary tube which was then sealed and incubated at 30 °C. The reaction was terminated after the given time interval by immersing the capillary tube in boiling water. The amount of galactose released during incubation was determined enzymatically by the ultraviolet method using the Test Combination for galactose (The Boehringer Corporation, London, Ltd.).

Substrate	Incubation time (min)	% Galactose released				
		α-Galactosidase		β-Galactosidase		
		I	II	I	II	III
Melibiose	10	15.9	35.3	No hydrolysis		
Raffinose	10	19.5	38.5	No hydrolysis		
Stachyose	10	18.0	36.7	No hydrolysis		
Guar galactomannan	120	10.1	11.3	No hydrolysis		
Locust bean galactomannan	120	9.1	12.0	No hydrolysis		
Methyl β-D-galactoside	20	No hydrolysis		3.5	8.0	5.1
Lactose	20	No hydrolysis		16.0	10.8	11.5

shown previously [1]. The hydrolysis of melibiose was slower than raffinose; for several α-galactosidases such a behaviour has been attributed to the reducing glucose moiety of the disaccharide [10–12]. Guar gum (mannose/galactose = 1.5) and locust bean gum (mannose/galactose = 3.1) were hydrolysed to a similar extent by both the enzymes; the rate of hydrolysis was considerably slower than that of the oligosaccharides.

All the forms of β-galactosidase hydrolysed the β-galactosides as shown in Table III. Lactose (β-D-galactosyl-(1 → 4)-D-glucose) was a better substrate than methyl-β-D-galactoside.

ACKNOWLEDGEMENTS

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