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Purification of α-Galactosidase from Coconut Endosperm by Affinity Chromatography

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Abstract: Four specific adsorbants for the purification of α-galactosidase (α-D galactoside galactohydrolase EC 3.2 1.22) from coconut endosperm were prepared. The affinity gels prepared were Sepharose-4B-lysine-galacturonate, Sepharose-4B-lysine-galactosamine, Sepharose-4B-lysine-galactose-p-carboxyanilide and CH-Sepharose-4B-galactosamine. α-Galactosidase from coconut endosperm extract was partially purified by ammonium sulphate fractionation, DEAE-Sephadex chromatography and this partially purified preparation was further purified by affinity chromatography using these specific adsorbants. The adsorbed α-galactosidase was eluted either by using a linear gradient of increasing buffer concentration or by using the specific desorbant p-nitrophenyl-α-D-galactopyranoside. The specific activity of the purified enzyme tested with p-nitrophenyl-α-D-galactopyranoside as substrate was 20 units/mg protein. This represents a 900 fold increase in purification of the original crude extract and the yield was 67%. The purified enzyme was homogeneous by polyacrylamide gel electrophoresis.

1. Introduction

α-Galactosidases (α-D-galactosyl galactohydrolase E.C. 3.2.1.22) are widely distributed in nature and are commonly found in plant seeds.[21] Polysaccharides and oligosacchadrides containing α-D-galactosyl units accumulate in seeds during maturation and serve as storage products in resting seeds.[7] During germination oligosaccharides are utilized first followed by the polysaccharides. D-galactose, the initial product of hydrolysis is rapidly transformed and utilized through the glycolytic pathway and is an important source of energy for the growing seedling.[8]

α-Galactosidase is an important enzyme in the coconut endosperm as raffinose is the predominant oligosaccharide[4] and galactomannans, the major (61%) polysaccharide.[1] Balasubramaniam *et al*[2] showed the presence of two interconvertible α-galactosidase isoenzymes in the coconut kernel. The authors have purified the low molecular weight isoenzyme from coconut kernel to homogeneity and characterized it.[3] However the yield was only 12% and the amount of pure enzyme obtained was insufficient for studies to elucidate the mechanism of its

action. α-Galactosidase from coffee beans[11] and from human serum[13] have been purified by affinity chromatography using α-D-galactosylamine and Melibiose as affinity ligands.

In this paper the purification of α-galactosidase by affinity chromatography using galactosamine, galacturonic acid and galactose-p-carboxyanilide as affinity ligands is described.

2. Materials and Methods

Analytical grade BDH and Sigma Chemicals were used. Mature fresh coconuts were purchased locally. Sepharose 4B, CH-Sepharose-4B and DEAE-Sephadex A-25 were purchased from Pharmacia fine Chemicals. Cyanagen bromide was synthesized in the laboratory.[14]

Absorbance was measured using a Pye Unicam SP 800 UV Spectrophotometer. Centrifugation was carried out in an IEC B 20A refrigerated centrifuge.

2.1 Enzyme assay

α-Galactosidase activity was assayed as described by Dey and Pridham.[10] Suitably diluted enzyme preparation (0.1 ml) were incubated at 30°C with 0.5 ml of p-nitrophenyl-α-D-galactopyranoside (1mM) and 0.4 ml of 0.08 M McIlvaine buffer[18] (pH 5.5) for 15 min. The reaction was terminated by the addition of 0.1M Na_2CO_3 (5 ml) and absorbance measured at 405 nm. A unit of enzyme activity is defined as the amount of enzyme that hydrolyses 1 μ mole of substrate/min under these given conditions.

2.2 Protein estimation

Protein content was determined by the method of Lowry *et al*[16]. using crystalline bovine serum albumin as standard or by measuring the absorbance at 280 nm.

2.3 Estimation of sugars

Galacturonic acid, galactosamine and galactose-p-carboxyanilide in the washings were determined by the Dubois[11] method using authentic samples of the same sugars as standards.

2.4 Synthesis of galactose-p-carboxyanilide

Galactose-p-carboxyanilide was synthesized as described by Honeyman.[15] Thin layer chromatography was done on the synthesized material as described by Stahl *et al*.[20] The synthesized material gave a single spot on thin layer chromatography.

2.5 Inhibition of α-galactosidase by galactose-p-carboxyanilide, galactosamine and galacturonic acid

α-Galactosidase was incubated with constant concentrations of galactose-p-carboxyanilide (1 mM), galactosamine (10 mM) and galacturonic acid (6 mM) in 0.08 M McIlvaine buffer (pH 5.5) and with varying concentrations of substrate (2×10^{-4} to 5×10^{-4}M). The p-nitrophenol released was determined as described in section 2.2.

2.6 Preparation of Sepharose-4B-lysine

Activation of Sepharose-4B and binding of lysine was done as described by Cuatrecasas.[6] Sepharose-4B (7 ml packed volume) was washed and suspended in 3 m of distilled water. The suspension was held in an ice bath and stirred (continuously) with a glass rod. CNBr (in warm distilled water) was added to the suspension and the pH maintained at 11 for 15 min by the addition of 10 N NaOH. The activated gel washed with cold 0.2M $NaHCO_3$ (pH 8.5) was suspended in the same buffer. Lysine (0.2 m moles) dissolved in 6 ml of 0.2 of M $NaHCO_3$ (pH 8.5) was added to the suspension and mixed 'end over end' overnight at 6°C. The gel was washed with the same buffer. The washings were collected and the lysine content was determined using ninhydrin.[19] Sepharose-4B-lysine was prepared in batches and the lysine content was varied by changing the amount of CNBr used.

2.7 Preparation of Sepharose-4B-lysine-galactouronate gel

Lysine bound Sepharose-4B (Section 2.6) was washed with distilled water and stirred in 3 ml distilled water at room temperature (29°C). Galacturonic acid (0.5 mmoles) was added in 3 ml of distilled water to 1 m mole of 1-ethyl-3-(3-dimethyl amino-propyl) carbodiimide. The pH was maintained at 4.5 for 6 h by addition of 0.1N HCl. The gel was washed with distilled water and the galacturonic acid content in the washings was determined by the Dubois[11] method.

2.8 Preparation of Sepharose-4B-lysine-galactosamine gel

Sepharose-4B-lysine (Section 2.6) and galactosamine (0.15 m moles) were coupled using 0.3 mmoles of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide as described in Section 2.7.

2.9 Preparation of Sepharose-4B-lysine-galactose-p-carboxyanilide gel

Sepharose-4B-lysine (Section 2.6) and 1 m mole of galactose-p-carboxyanilide in 25 ml of 40% dimethyl formamide were coupled using 2 m moles of 1-ethyl-3 (3-dimethyl aminopropyl) carbodiimide as described in Section 2.7 but washing was done with 40% dimethyl formamide.

2.10 Preparation of CH-Sepharose-galactosamine gel

CH-Sepharose-4B (1.5 g) was swelled in 0.5 m NaCl and washed with 0.5 M NaCl followed by distilled water. Coupling of galactosamine was carried out as in Section 2.7 using 0.15 m moles of galactosamine and 0.3 m moles of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide.

2.11 Purification of α-galactosidase

All operations were carried out at room temperature (29°C) unless specified otherwise. Centrifugation was carried out at 4°C. Fractions collected after column chromatography were assayed for enzyme activity (Section 2.2) and the protein concentrations of each fraction was determined by measuring the absorbance at 280 nm.

2.11.1 Extraction

Coconut kernel scrapings were homogenized in McIlvaine buffer (pH 5.5) using a Waring blender. The extract (2ml/g) was passed through a cheese cloth and centrifuged at 25 000 g for 20 min. The supernatant obtained was adjusted to pH 3.8 using 0.5M citric acid. On standing for 30 min the acidified solution was centrifuged at 25 000g for 20 min. The pH of the acid supernatant was readjusted to pH 5.5 using saturated solution of Na_2HPO_4.

2.11.2 Ammonium sulphate fractionation

The acid supernatant (pH 5.5) was fractionated using solid ammonium sulphate. The precipitate collected between 45 - 60% saturation was dissolved in McIlvaine buffer (pH 5.5) and dialysed against 0.008M McIlvaine buffer (pH 5.5).

2.11.3 DEAE-Sephadex A-25 gel chromatography

α-Galactosidase from ammonium sulphate fractionation step (40 mg in 60 ml) was applied to a column of DEAE-Sephadex A-25 (2.4×28 cm) previously equilibrated with 0.008 M McIlvaine buffer (pH 5.5). The column was washed with 60 ml of the same buffer. The column was eluted with a 2:1 gradient consisting of 0.008 M (200 ml) and 0.16M (100 ml) McIlvaine buffer (pH 5.5). The fractions containing enzyme activity were pooled and dialysed against 0.008M McIlvaine buffer (pH 5.0).

2.11.4 Sepharose-4B--lysine-galacturonic acid gel chromatography

To Sepharose-4B-lysine-galactouronate gel (0.5×3 cm) was applied 2 ml (0.1 mg) of DEAE-Sephadex A-25 eluted enzyme (Section 2.11.3). The gel had been equilibrated with 0.008M McIlvaine buffer (pH 5.0). After application allowed a period of 15 min for the adsorption of the enzyme. The column was eluted with 8 ml of the same buffer and later with the same volume of 0.08 M McIlvaine buffer (pH 5.0).

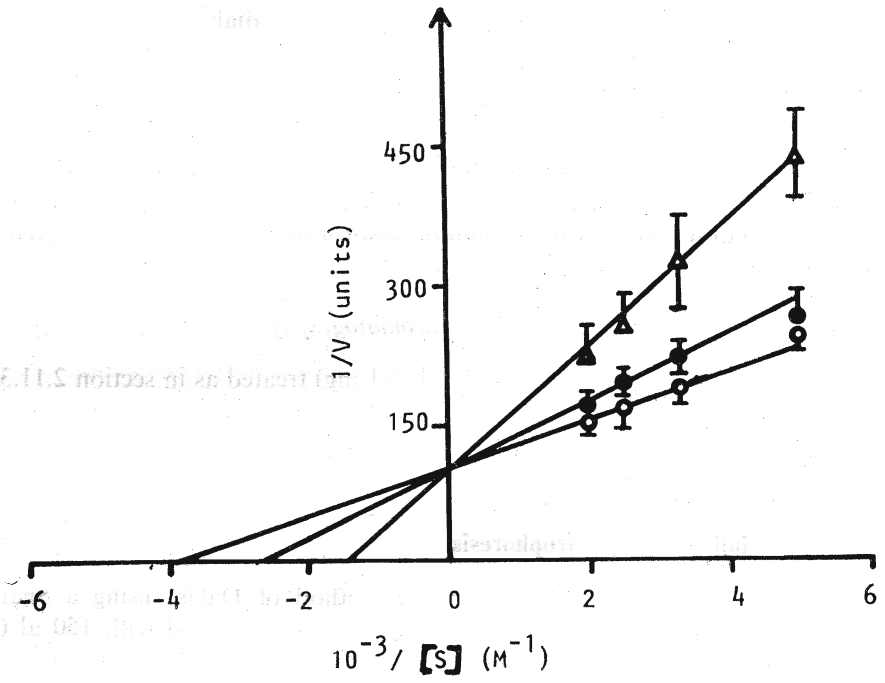

Figure 1: Lineweaver-Burk plot for the inhibition of α -galactosidase by galacturonic acid and galactose-p-carboxyanilide. O, none; ●' galacturonic acid (10 mM); △, galactose-p-carboxyanilide (1mM). Bars indicate standard deviations obtained from four experiments. No inhibition was observed when galactosamine (10 mM) was used.

2.11.5 Sepharose-4B-lysine-galactose-p-carboxyanilide gel chromatography

To Sepharose-4B-lysine-galactose-p-carboxyanilide column (0.5 × 9.0 vm) equillibrated with 0.008M McIlvaine buffer (pH 5.0) was applied 2 ml of DEAE-Sephadex A-25 eluted enzyme (0.1 mg) prepared as in section 2.11.3. After 15 min of adsorption the enzyme was eluted with 30 ml of 0.008 M McIlvaine buffer (pH 5.0) and 15 ml of 0.1 M McIlvaine buffer (pH 5.0). At this point 1 ml of 20 mM p-nitrophenyl-α-D-galactopyranoside was applied to the column and eluted with 15 ml of 0.1M McIlvaine buffer (pH 5.0). The eluate was dialysed against the same buffer and assayed for enzyme activity.

2.11.6 Sepharose-4B--lysine-galactosamine gel chromatography

To the Sepharose-4B-lysine-galactosamine gel equillibrated with 0.008M McIlvaine buffer (pH 5.0) was applied 2 ml of DEAE-Sephadex A-25 eluted enzyme (0.1mg) treated as in section 2.11.3. After 15 min of adsorption the enzyme was eluted with the same buffer.

2.11.7 CH-Sepharose-galactosamine gel chromatography

DEAE-Sephadex A-25 eluted enzyme 2 ml (0.1 mg) treated as in section 2.11.3 was applied to the CH-Sepharose-galactosamine gel equilibrated with 0.008M McIlvaine buffer (pH 5.0). Allowed for 15 min and eluted with 25 ml of the same buffer and 30 ml of 0.1M McIlvaine buffer (pH 5.0).

2.12 Polyacrylamide gel electrophoresis

The electrophoresis was carried out by the method of Davis[6] using a Shandon apparatus. Polyacrylamide gels (5%) were prepared and loaded with 150 μl (5 μg protein) of the purified enzyme (diluted 2:1 with glycerol). McIlvaine buffer pH 6.5 was used in the reservoir. Gels were stained for proteins using Coomassie Brilliant Blue. α-Galactosidase activity was determined by incubating with 4-methyl umbelleferyl-α-D-galactoside (2.5 mM) at room temperature for 30 min. The reaction was terminated with 0.5 M NaOH and the fluorescent bands were observed under UV-light.

3. Results

3.1 Inhibition of α-galactosidase

Galacturonic acid and galactose-p-carboxyanilide, are competitive inhibitors of α-galactosidase while galactosamine did not inhibit the enzyme at a concentration of 10 mM (Figure 1). The Ki values calculated from the graph were 2×10^{-2}M and 1.8×10^{-4} M for galacturonic acid and galactose-p-carboxyanilide respectively.

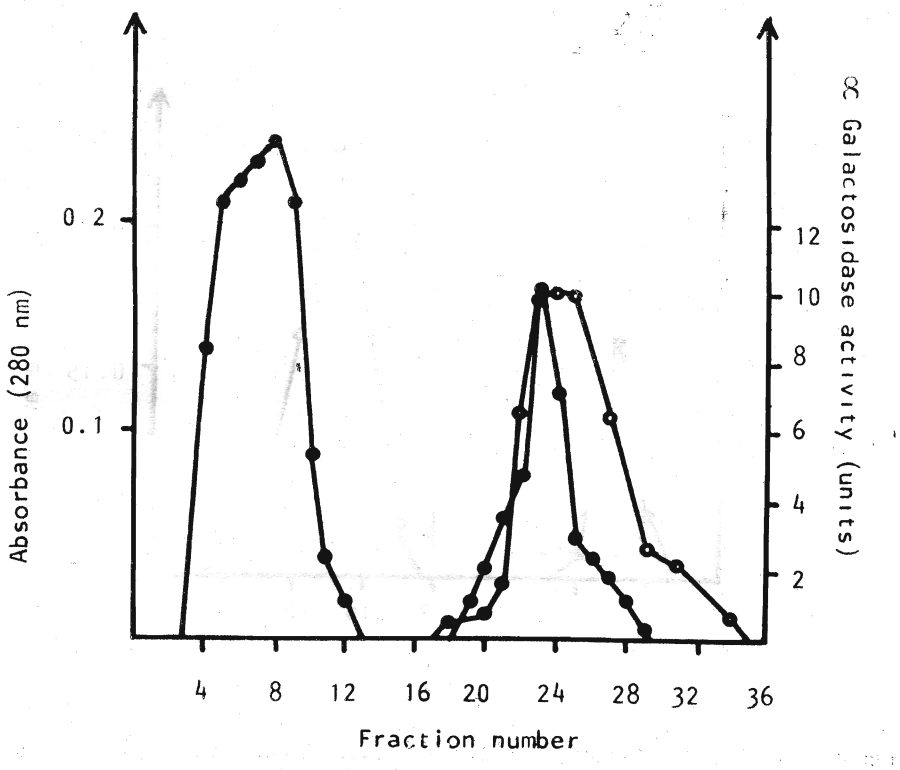

Figure 2: DEAE-Sephadox A-25 chromatography of α -galactosidase. The column 2.4 x 28 cm) was eluted with a 2:1 gradient of 0.008M (200 ml): 0.16M (100 ml) McIlvaine buffer (pH 5.5), flow rate was 30 ml/h. and 10 ml fractions were collected. ●—●, Absorbance at 280 nm; o—o , enzyme activity.

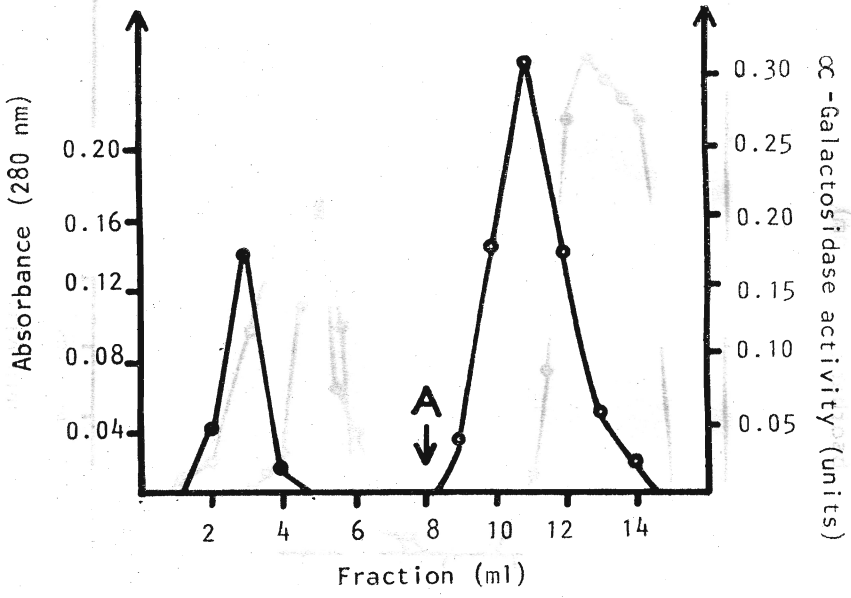

Figure 3: Affinity chromatography using Sepharose-4B-lysino-galacturonate column (0.5 x 3 cm). Applied 2 ml (0.1 mg) of DEAE-Saphadex A-25 eluted enzyme dialysed against 0.008M McIlvaine buffer (pH 5.0) and eluted with the same buffer. At A, eluted with 0.1M McIlvaine buffer (pH 5.0). Flowrate 10 ml/h, fraction volune 1 ml. ●—●, Absorbance at 280 nm; 0—0, Enzyme activity.

3.2 Preliminary purification of α-galactosidase

Preliminary purification of α-galactosidase (Table 1) by acidification, $(NH_4)_2SO$ fractionation and DEAE-Sephadex A-25 chromatography gave on overall yield of 78% and the α-galactosidase was purified 320 fold. DEAE-Sephadex A-25 chromatographic step (Figure 2) gave an 8 fold increase in purity (Table 1). When tested by polyacrylamide gel electrophoresis this enzyme solution had two protein bands and a single enzyme activity band.

3.3 Purification by affinity chromatography

In all affinity chromatography purifications DEAE-Sephadex A-25 eluted α-galactosidase, dialysed against 0.008M McIlvaine buffer (pH 5.0) was applied to the columns equilibrated with the same buffer. The control experiment showed that the dialysed enzyme when applied to a Sepharose-4B column was eluted out in void volume. This indicates that α-galactosidase had no affinity to Sepharose-4B.

The synthesized Sepharose-4B-lysine-galactouronate gel had a bound lysine content of 124 μ moles/ml and a galacturonic acid content of 112 μmoles/ml. The addition of large amounts of CNBr (330 mg/ml) had probably led to a highly activated gel leading to increased coupling of the ligand. When DEAE-Sephadex A-25 eluted enzyme was applied to the column, contaminating proteins were eluted out with the same buffer while the enzyme was eluted out only with increasing molarity of the buffer (Figure 3). Specific activity of the purified enzyme was 20 units/mg protein. Recovery for the affinity chromatographic step was 86% and the capacity of the gel for α-galactosidase was 42 μg/ml.

The synthesized Sepharose-4B-lysine-galactosamine gel had a bound lysine content of 43 μmoles/ml and the bound galactosamine content was 21 μmoles/ml. The DEAE-Sephadex A-25 eluted enzyme was only partially separated from contaminating proteins by this affinity gel (Figure 4). The synthesized CH-Sepharose-4B-galactosamine gel had a galactosamine content of 20 μmoles/ml. The major part of the enzyme applied to this column was eluted out by increasing the molarity of the buffer (Figure 5). Selected fractions were concentrated and the specific activity of the pooled enzyme was found to be 20 units/mg protein. The recovery for the purification step was 60%. The capacity of the gel for α-galactosidase was 4 μg/ml.

Sepharose-4B-lysine-galactose-p-carboxyanilide gel had a lysine content of 29 μmoles/ml and the galactose-p-carboxyanilide content was also 29 μmoles/ml. When the DEAE-Sephadex A-25 eluted enzyme was applied to this column, 50% of the enzyme was eluted out by increasing the morality of the buffer while the rest was eluted out by the substrate. (Figure 6). Both fractions were pooled dialysed and concentrated. The specific activity was determined to be 20 units/mg protein and the capacity of the gel for α-galactosidase was 11 μg/ml. The recovery for the purification step was 67%.

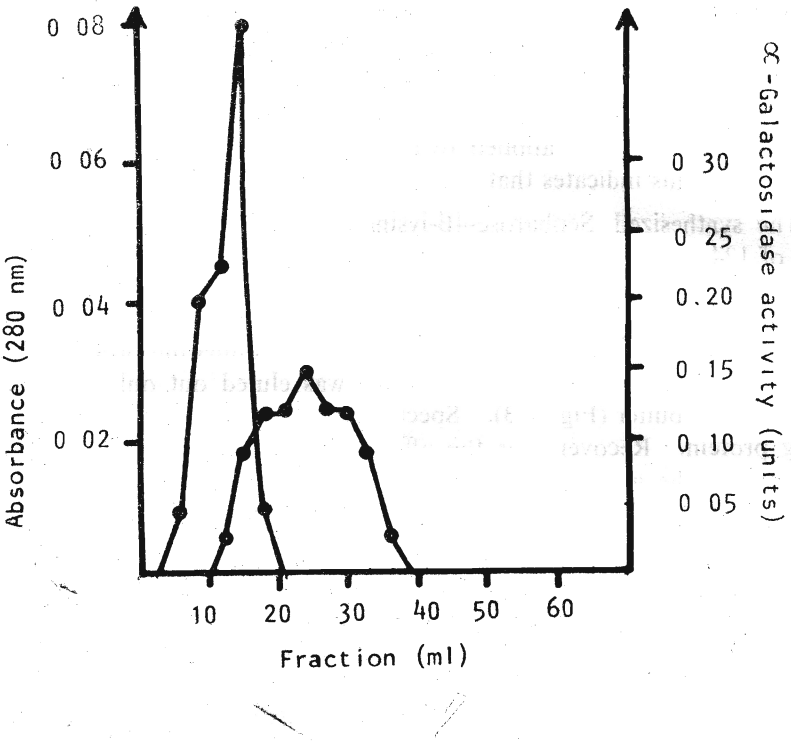

Figure 4: Affinity chromatography using Sepharose-4B-lysino-galactosamine column (0.5 x 8 cm). Applied 2 ml (0.1 mg) of DEAE-Sephadex A-25 eluted enzyme dialysed against 0.008M McIlvaine buffer (pH5.0). Eluted with the same buffer flowrate 10 ml/h and 3 ml fractions were collected. ●—●, Absorbance at 280 nm; O—O enzyme activity.

TABLE 1. – Purification of α – galactosidase from coconut endosperm

Purification step	*Volume (ml)*	*Total enzyme activity (units)*	*Total protein (mg)*	*Specific activity (units/mg protein)*	*Recovery (%)*	*Purification (fold)*
Extract	650	228	10400	0.022	100	1
Supernatant of extract centrifuged at 25 000 g	550	215	2035	0.106	94	5
Supernatant of Acidified solution centrifuged at 25 000 g	660	211	264	0.799	93	36
Ammonium-sulphate fractionation	140	182	196	0.929	80	42
DEAE-Sephadex A-25 chromatography	422	177	25	7.08	78	322
Affinity chromatography by Sepharose-4B-lysine-galacturonate column and concentration	211	152	7.6	20.0	67	909

3.4 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of purified α-galactosidase having a specific activity of 20 units/mg protein gave single and coincident protein and α-galactosidase activity bands.

4. Discussion

Insoluble derivatives of 1-amino sugars have been successfully employed in the affinity chromatography of lectins.[12] N-ε-amino caproyl-Nε-aminocaproyl-α-D-galactopyranosyl-Sepharose-4B conjugate was successfully used for purification of α-galactosidase from coffee beans.[13] This ligand is a competitive inhibitor of α-galactosidase from coffee beans and the Ki value is 3×10^{-4}M. As α-D-galactophyranosylamine is not available commercially, we have used galacturonic acid, galactose-p-carboxyanilide and galactosamine (2-amino-2-deoxy-D-galactopyranose) as affinity ligands.

Galactosamine (2-amino-2-deoxy-D-galactopyranose) does not inhibit α-galactosidase from coconut endosperm. Thus it is unsuitable for use as a affinity ligand. However CH-Sepharose-galactosamine gel has a weak affinity for α-galactosidase. This affinity is probably due to the hydrophobic properties of the spacerarm and the purification of α-galactosidase by hydrophobic interaction chromatography is worth looking into.

Sepharose-4B-lysine-galactose-p-carboxyanilde gel has the highest affinity for α-galactosidase though the capacity is lower than the Sepharose-4B-lysine-galacturonic acid gel. Decrease in capacity may be due to the lower ligand content of the immobilized galactose-p-carboxyanilide compared to the immobilized galacturonic acid. Both these gels could be used for purification of α-galactosidase but the use of galacturonic acid ligand is preferable due to the ease in eluting the enzyme out of the column and as capacity and percentage recovery are also higher.

The percentage recovery for purification by Sepharose-4B-lysine-galacturonic acid gel is 67% and the specific activity is 20 units/mg protein Both values are higher than the values obtained by purification using gel filtration.[2] Recovery for gel filtration method is 12% and specific activity is 14 units/mg protein. Increase in specific activity could be due to conversion of the low molecular weight isoenzyme to a higher molecular weight isoenzyme during purification by affinity chromatography.[9]

The authors are presently studying the use of hydrophobic ligands to purify α-galactosidase from coconut endosperm.

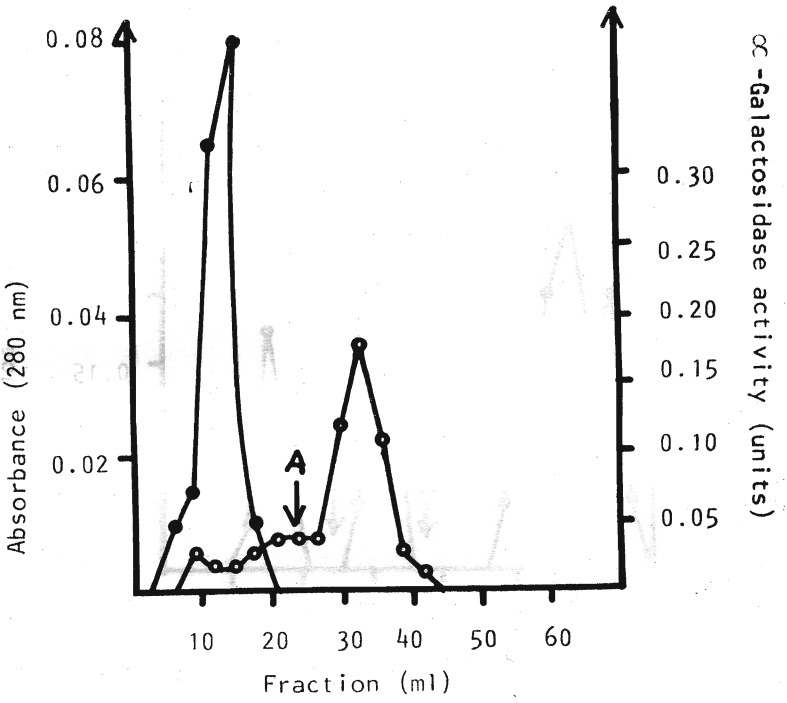

Figure 5: Affinity chromatography using CH-Sepharose-galactosamine column (0.5 x 8 cm). Applied 2 ml (0.1 mg) of DEAE-Sephadex A-25 eluted enzyme dialysed againts 0.008M McIlavaine buffer (pH 5.0) and eluted with the same buff At A, eloted with 0.1M McIlvaine buffer (pH 5.0). Flow rate 10 ml/h and collected 3 ml fractions ●—● , Absorbance at 280 nm; O—O, enzyme activity.

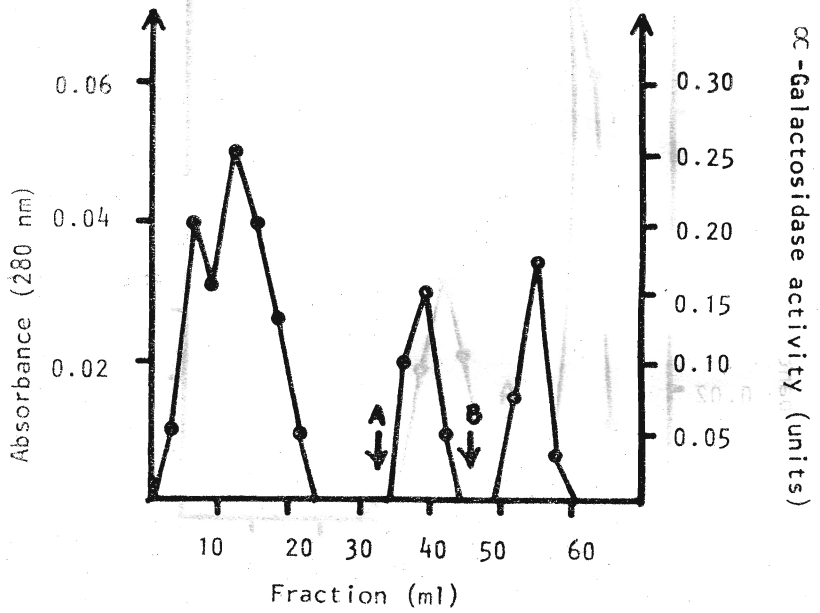

Figure 6: Affinity chromatography using Sepharose-4B-lysino galactose-p-carbozyanilie column (0.5 x 9.0 cm). Applied 2 ml of DEAE-Sephadex A-25 eluted enzyme dualysed against 0.008M McIlvaine buffer (pH 5.0). Eluted with the same buffer and at A, eluted with 0.1M McIlvaine buffer (pH 5.0), at B, eluted with p-nitrophenyl-α-D-galactopyranoside. ●—●, Absorbance at 280 nm; O—O, enzyme activity.

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