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PURIFICATION OF α-GALACTOSIDASE FROM COCONUT

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Key Word Index—*Cocos nucifera*; Palmaceae; coconut; α-galactosidase; purification; hydrophobic chromatography.

Abstract—α-Galactosidase from coconut endosperm was purified to homogeneity with a 490-fold increase in specific activity. The yield was 70% and the specific activity was 24.5 units/mg protein. The purification procedure included extraction, acidification, ammonium sulphate fractionation and hydrophobic chromatography. The hydrophobic gel (Sepharose-4B-capranilide) had a capacity of 0.63 mg of α-galactosidase per ml of gel. Purified α-galactosidase was a glycoprotein with a carbohydrate content of 12%. The molar extinction coefficient was 8.7×10^4/M/cm.

INTRODUCTION

α-Galactosidases (α-D-galactosyl galactohydrolase, EC 3.2.1.22) are widely distributed in nature and commonly found in plant seeds [1]. Polysaccharides and oligosaccharides containing α-D-galactosyl units accumulate in seeds during maturation and serve as storage products in resting seeds [2]. α-Galactosidase is an important enzyme in the coconut endosperm, as here galactomannans are the major polysaccharide (61%) [3]. α-Galactosidase also plays an important role in development of the cell walls of coconut and other palm seeds [4]. Balasubramaniam *et al.* [5] showed the presence of two interconvertible α-galactosidase isoenzymes in coconut endosperm of M_rs 121 000 and 21 000. Majer *et al.* [6, 7] have partially purified α-galactosidases from coconut and indicated its importance in germination. In this paper we describe purification of α-galactosidase from coconut to homogeneity.

RESULTS AND DISCUSSION

The purification of α-galactosidase from coconut kernel is summarized in Table 1. The overall yield was 70% and the enzyme was purified 490-fold. The hydrophobic interaction chromatography step (Fig. 1) gave a 6-fold increase in purity. The amount of ε-amino-*n*-caproic acid bound to Sepharose-4B was 38 μmol/ml gel while the aniline bound to caproic acid was 30 μmol/ml gel. This shows that *ca* 79% of the carboxylic acid groups of caproic acid had been blocked by aniline and the synthesized ligand exhibited mainly hydrophobic properties. In our laboratory attempts were made to purify α-galactosidase from coconut kernel by binding 2-amino-2-deoxy-D-galactopyranose to CH-Sepharose-4B [8]. Bound ligand was calculated to be 20 μmol/ml gel. The capacity of this gel for α-galactosidase was only 4 μg/ml of gel and the results indicated that purification may be due to hydrophobic interactions rather than affinity chromatography. The use of the hydrophobic gel Sepharose 4B-capranilide increased the capacity for α-galactosidase to 630 μg/ml gel. Isoelectric focusing of the purified α-galactosidase gave a single band indicating a homogeneous protein.

Purified α-galactosidase was shown to be a glycoprotein containing 12% carbohydrate by the Dubois method [9]. The amino acid composition of the purified enzyme is given in Table 2. The protein has a high acidic amino acid content. Unlike in α-galactosidase from *Lens culinaris* [10] aspartic and glutamic acid must be present in the acidic form as the isoelectric point of the low M_r α-galactosidase from coconut is 3.8 [11]. The amino acid composition of coconut α-galactosidase is similar to that of α-galactosidase from soybean [12]. Amino acid analysis also showed that α-galactosidase is a glycoprotein and its glucosamine content is 7.5% of the total carbohydrates. The M_r calculated from the amino acid composition and the carbohydrate content for α-galactosidase is 19 875. The M_r of the low M_r α-galactosidase isoenzyme from coconut determined by gel filtration was 21 000 [5].

The molar extinction coefficient of α-galactosidase at 280 nm determined from UV spectra in conjunction with amino acid analysis was 8.7×10^4/M/cm.

We conclude that the hydrophobic interaction chromatography is a good method for large scale purification of α-galactosidase from coconut due to the high capacity of the synthesized gel for α-galactosidase.

EXPERIMENTAL

Mature coconuts were purchased from the market. CNBr was synthesized in the laboratory [13]. All other chemicals were of analytical grade.

Preparation of hydrophobic gel. Sepharose 4B (80 ml) was activated with CNBr (15 g) as described in ref. [14]. Activated gel was reacted with ε-amino-*n*-caproic acid (6 mmol) in 80 ml of 0.1 M $NaHCO_3$ (pH 8.5) for 24 hr at 6°. The gel was washed with H_2O and the caproic acid content of the washings were

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Table 1. Purification of α-galactosidase from coconut kernel

Procedure	Volume (ml)	Total enzyme activity (units)*	Total protein (mg)†	Specific activity (units/mg protein)	Recovery (%)	Purification (fold)
Extract	4950	2620	51000	0.05	100	1
14000 *g* supernatant	3650	2590	15300	0.17	99	3
Supernatant from acid (pH 3.8) precipitation	4220	2530	1270	2.0	96	40
45–60% $(NH_4)_2SO_4$ fraction after dialysis	334	1890	468	4.03	72	81
Hydrophobic chromatography	375	1840	75	24.5	70	490

*1 Unit = 1 μ mol/min *p*-nitrophenol liberated in standard assay (see text).
†Protein measured by the method of Lowry *et al.* [18] using bovine serum albumin as standard.

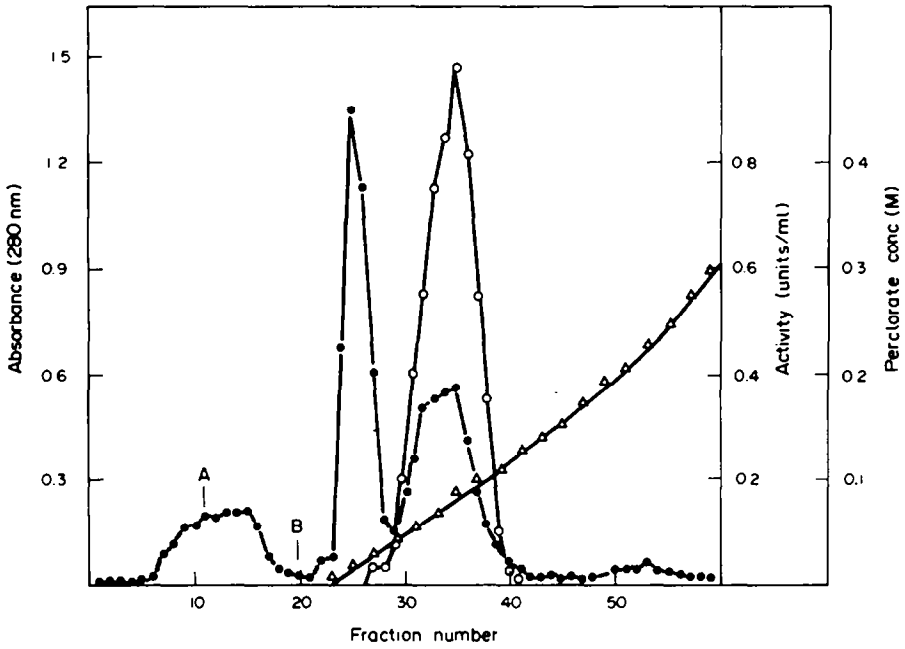

Fig. 1 Hydrophobic chromatography of α-galactosidase. Sample, 168 mg (120 ml); column dimensions 1.4 × 28 cm. Eluants: A, 40% $(NH_4)_2SO_4$ in 0.01 M McIlvaine buffer (pH 5.5); B, 2:1 gradient of 0.01 M McIlvaine buffer (pH 5.5) and the same buffer containing 0.5 M $NaClO_4$ (pH 5.5). Flow rate 30 ml/hr. Fraction volume 10 ml. ●——●, *A* at 280 nm; ○——○, activity (units/ml); Δ—Δ, $NaClO_4$ (M).

determined by the ninhydrin method [15]. Redistilled aniline (6 mmol in 80 ml of 40% DMF) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (7 mmol) were added to the caproic acid bound gel and the pH was maintained at 5 for 10 hr at room temp. (29°). The gel was washed with 40% dimethylformamide, and the aniline content of the washings was determined by measuring the *A* at 280 nm. The gel was further washed with H_2O and 0.01 M McIlvaine buffer (pH 5.5) [16] containing NaN_3 (0.02% w/v).

Enzyme assay. Enzyme assay was done according to ref. [17] using *p*-nitrophenyl-α-D-galactopyranoside as the substrate.

Protein determination was done either by *A* at 280 nm or by the method of ref. [18].

Purification of α-galactosidase. All operations were done at room temp. (29°) and all centrifugation at 4°.

Extraction. Coconut kernel scrapings in cold (6°) McIlvaine buffer (pH 5.5) were homogenized in a prechilled Waring blender. The extract (2 ml/g) was passed through cheesecloth and centrifuged at 14000 *g* for 20 min. The supernatant was retained. The supernatant was adjusted to pH 3.8 using 0.5 M citric acid and was allowed to stand for 30 min. The acidified soln was centrifuged at 14000 *g* for 20 min and the pH of the acid supernatant was adjusted to pH 4.5 using satd Na_2HPO_4.

Ammonium sulphate fractionation. The acid supernatant (pH 4.5) was fractionated using solid $(NH_4)_2SO_4$. The fraction between 45–60% satn showed the highest sp. act. The ppt was dissolved in 0.01 M McIlvaine buffer (pH 5.5) and dialysed against the same buffer. The ppt observed after dialysis was removed by centrifugation at 25000 *g* for 20 min and the supernatant was retained.

Hydrophobic interaction chromatography. A column of Sepharose-4B conjugated to capranilide (1.4 cm × 28 cm) was

Table 2. Amino acid composition of coconut α-galactosidase

Amino acid	nmol	Residues/molecule*
Asp	208.61	19.1 (19)
Thr	98.65	9.0 (9)
Ser	139.74	12.8 (13)
Glu	140.73	12.9 (13)
Pro	69.08	6.3 (6)
Gly	170.90	15.7 (16)
Ala	158.59	14.5 (15)
Half Cys†	38.90	3.6 (4)
Val	100.64	9.2 (9)
Met	37.91	3.5 (4)
Ile	76.42	7.0 (7)
Leu	127.63	11.7 (12)
Tyr	62.92	5.8 (6)
Phe	42.48	3.9 (4)
His	28.19	2.6 (3)
Lys	74.63	6.8 (7)
Try‡	69.47	6.4 (6)
Arg	65.11	6.0 (6)
Glucosamine	13.10	1.2 (1)

*Calculate for the M_r 19875 with a 12% carbohydrate content. The nearest integer is given in parentheses.

†Determined after performic acid oxidation.

‡Determined after hydrolysis with *p*-toluenesulphonic acid.

equilibrated with 0.01 M McIlvaine buffer (pH 5.5) containing 40% $(NH_4)_2SO_4$. Dialysed enzyme sample (120 ml) from the $(NH_4)_2SO_4$ fractionation step was adjusted to 40% satn with $(NH_4)_2SO_4$ and was applied to the column. The sample was washed with 80 ml of 40% $(NH_4)_2SO_4$ in 0.01 M McIlvaine buffer (pH 5.5) and eluted with a 2:1 gradient consisting of 300 ml of 0.01 M McIlvaine buffer (pH 5.5) and 150 ml of the same buffer containing 0.05 M perchlorate (pH 5.5). The flow rate was 30 ml/hr and 10 ml fractions were collected. The protein concn of the fractions was measured at 280 nm and their α-galactosidase activity was assayed at 405 nm. The fractions showing high enzyme activity were pooled and dialysed against 0.01 M McIlvaine buffer (pH 5.5). Perchlorate concn in the fractions was calculated from the gradient. The column was regenerated by washing with one column vol. each of McIlvaine buffer containing 1.5 M perchlorate (pH 5.5) followed by 0.01 M McIlvaine buffer (pH 5.5) for 20 hr.

Isoelectric focusing. Isoelectric focusing in polyacrylamide gels was carried out using LKB 2117 multiphor apparatus (LKB-Produckter AB, Bromma, Sweden). Commercial polyacrylamide gels for isoelectric focusing in the pH 3.5–9.5 range (Ampholine® PAG plates) were obtained from the same company. The gel was run at a constant power of 1 W/cm. Fixation and staining were done according to ref. [19].

Glycoprotein nature of α-galactosidase. The carbohydrate content of the purified α-galactosidase was determined by the method of ref. [19] using glucose as the standard.

Amino acid analysis. Amino acid analysis was carried out with a Durrum D-500 analyser. Samples were hydrolysed in 6 M HCl at 110° for 24 hr. The content of half cysteine was determined as cysteic acid after oxidation of the sample with performic acid. The tryptophan content was determined after hydrolysis in 3 M *p*-toluenesulphonic acid [20].

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