α -Galactosidase from Alfalfa Seeds (*Medicago sativa* L.)

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An α -galactosidase from alfalfa seeds was purified 140-fold by ammonium sulfate fractionation, and column chromatography on Sephadex G-100, DEAE- and CM-Sephadex. Polyacrylamide-gel electrophoresis of the purified enzyme showed a single protein band. The molecular weight was estimated to be approximately 57,000 by gel-filtration. The purified enzyme hydrolyzed *p*-nitrophenyl α -D-galactoside more rapidly than raffinose. The maximal enzyme activities were obtained at pH 4.0 and 5.5 for *p*-nitrophenyl α -D-galactoside and at 4.5 for raffinose. The enzyme was shown to be inhibited by Hg^{2+} and Ag^+ ions, and D-galactose.

Oligosaccharides containing an α -D-galactosyl residue in their structure are widely distributed in the plant kingdom. Such oligosaccharides as raffinose and stachyose are considered to be one of the principal factors of gastrointestinal distress and flatulence observed when legume seeds are consumed by mammals.^{1~3)} α -Galactosidase (α -D-galactoside galactohydrolase: EC 3.2.1.22) which can hydrolyze these oligosaccharides has been shown to occur not only in plants but also in microorganisms and animals,^{4~6)} and is known to be important in the metabolism of raffinose and stachyose.^{4,7~9)} The enzymes have been isolated from several sources, and examined for the mode of action and physiological significance,⁶⁾ and their use for reducing raffinose and stachyose in foods prior to their consumption.^{10~13)}

Alfalfa (*Medicago sativa* L.) is one of the major pasture crops belonging to the legume family, and occasionally causes flatulence in livestock fed on it. The presence of α -galactosidase in alfalfa seeds has been demonstrated by Courtois *et al.*^{14,15)} However, further detailed studies have not been reported. The present study was designed to investigate in detail the general properties and enzymatic function of α -galactosidase from alfalfa seeds.

MATERIALS AND METHODS

Alfalfa seeds (common lucerne, *Medicago sativa* L.)

were obtained from Yukijirushi Seed Co. *p*-Nitrophenyl α -D- and β -D-galactopyranoside were obtained from Senn Chemicals Co. Sephadex G-100, DEAE-Sephadex A-50 and CM-Sephadex C-50 were from Pharmacia Fine Chemicals. Bovine serum albumin and ovalbumin were purchased from Sigma Chemicals Co. Myoglobin and cytochrome C were obtained from Schwarz-Mann Co. β -Lactoglobulin was prepared from bovine milk according to the method of Aschaffenburg and Drewry.¹⁶⁾ Other reagents used were purchased from Wako Pure Chemicals Co. All the chemicals were of analytical grade.

Enzyme assays. *p*-Nitrophenyl α -D-galactopyranoside was used as substrate for enzyme assays. The enzyme solution (0.1 ml) in McIlvaine buffer, pH 5.5, was incubated at 30°C with 2.5 mM substrate solution (0.4 ml) in the same buffer for 15 min. The reaction was stopped by adding 0.1 M Na_2CO_3 (5.0 ml), and the yellow colour of *p*-nitrophenol thus developed was measured at 405 nm. α -Glucosidase (α -D-glucoside glucohydrolase, EC 3.2.1.20), β -glucosidase (β -D-glucoside glucohydrolase, EC 3.2.1.21) and β -galactosidase (β -D-galactoside galactohydrolase, EC 3.2.1.23) were assayed with the corresponding *p*-nitrophenylglycosides as substrate under conditions identical with those used for α -galactosidase. The hydrolysis of raffinose was conducted under the same conditions and followed by determining the liberated galactose by the method of Somogyi¹⁷⁾ at 560 nm.

One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μ M of substrate/min under the conditions specified above. Specific activity was expressed as units/mg of protein.

Protein determination. The protein determinations were made using the method of Lowry *et al.*¹⁸⁾ with crystalline bovine serum albumin as a standard.

Inhibition study. α -Galactosidase, 0.1 ml (6.4 μ g

enzyme), was incubated at 30°C with 0.2 ml of inhibitor containing 25 mM of carbohydrates or 2.5 mM of metal salts for 30 min before introducing 0.2 ml of 5 mM *p*-nitrophenyl α -D-galactoside. Incubation was continued at 30°C for an additional 15 min. The reaction was stopped by adding 5 ml of 0.1 M Na_2CO_3 . Inhibitor, enzyme and substrate were used dissolved in McIlvaine buffer, pH 5.5.

Molecular weight estimation. The molecular weight of the enzyme was estimated by gel filtration at 4°C on a column (2.5 $\times$ 82 cm) of Sephadex G-100 according to the method of Andrews.¹⁹⁾ The eluting buffer was McIlvaine buffer, pH 6.0, and elution was at 20 ml/hr. The column was calibrated with protein standards of bovine serum albumin (67,000), ovalbumin (45,000), β -lactoglobulin (36,500), myoglobin (17,800) and cytochrome C (12,400).

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis was carried out using the buffer system described by Koehler²⁰⁾ in a horizontal tray (17 $\times$ 8 $\times$ 0.3 cm) with 8% (w/v) polyacrylamide-gel at pH 8.5. The gel was run at room temperature at 10 mA for 4 hr. After running, the gel was stained with 0.2% (w/v) Amido Black 10B in a mixture of acetic acid: water: methanol (1: 5: 4).

RESULTS

Purification of α -galactosidase from alfalfa seeds

All extraction and purification operations were carried out at approximately 4°C unless otherwise specified.

Step 1: Extraction of the enzyme. Alfalfa seeds were milled in a laboratory mill. The pulverized seeds (300 g) were suspended in 4,000 ml of McIlvaine buffer, pH 5.5, left overnight and the supernatant solution was then collected by centrifugation for 30 min at 7,000 $\times g$.

Step 2: Ammonium sulphate fractionation and dialysis. To the above supernatant was gradually added solid ammonium sulphate (1,420 g/liter) up to 70% saturation with continuous stirring. The mixture was centrifuged for 30 min at 7,000 $\times g$. The precipitate was dissolved in a small quantity of water and dialyzed against McIlvaine buffer, pH 3.5. The precipitate formed was removed by centrifugation. The clear supernatant (1,250 ml) was adjusted to pH 6.0 with 1.0 N NaOH, and then concentrated in a cellophane tube covered by polyethylene glycol 20,000.

Step 3: DEAE-Sephadex chromatography. The above solution was thoroughly equilibrated with 0.02 M phosphate buffer, pH 7.7, by dialysis and applied to a column of DEAE-Sephadex A-50 (3.5 $\times$ 30 cm), previously equilibrated with the same buffer. The column was eluted with 1.4 liters of a linear gradient between 0.02 M phosphate buffer, pH 7.7, and

FIG. 1. Elution Profile of α -Galactosidase from DEAE-Sephadex A-50 (Step 3).

Enzyme from step 2 was applied to a DEAE-Sephadex A-50 column (3.5 $\times$ 30 cm) equilibrated with 0.02 M phosphate buffer, pH 7.7. The column was eluted with 1.4 liter of a linear gradient between 0.02 M phosphate buffer, pH 7.7, and 0.3 M NaCl in the same buffer (---). The column was operated at a flow rate of 50 ml/hr, and fractions of 10 ml were collected. ●, E_{280} ; ○, α -galactosidase activity.

0.3 M NaCl in the same buffer. An elution profile is shown in Fig. 1. Active fractions were pooled together (290 ml) and concentrated with polyethylene glycol 20,000 in a cellophane tube. The solution was dialyzed against McIlvaine buffer, pH 6.0.

Step 4: Sephadex G-100 gel filtration. The above solution was passed through a Sephadex G-100 column (3.5×72 cm), equilibrated previously with McIlvaine buffer, pH 6.0. An elution pattern is shown in Fig. 2. The frac-

FIG. 2. Chromatography of α -Galactosidase on Sephadex G-100 (Step 4).

Enzyme from the DEAE-Sephadex chromatography (Step 3) was applied to a Sephadex G-100 column (3.5×72 cm) equilibrated with McIlvaine buffer, pH 6.0. The column was eluted with the same buffer at a flow rate of 30 ml/hr, and fractions of 10 ml were collected. ●, E_{280} ; ○, enzyme activity.

FIG. 3. Elution Profile of α -Galactosidase from CM-Sephadex C-50 (Step 5).

Enzyme from the gel-filtration (step 4) was applied to a CM-Sephadex C-50 column (1.5×12 cm) equilibrated with 0.1 M phosphate buffer, pH 6.3. The column was eluted with 200 ml of a linear gradient between 0.1 M phosphate buffer, pH 6.3, and 0.5 M NaCl in the same buffer (----). The column was operated at a flow rate of 20 ml/hr, and fractions of 10 ml were collected. ●, E_{280} ; ○, α -galactosidase activity; △, β -galactosidase activity.

tions containing enzyme activity were pooled. At this stage, the proportions of α -galactosidase and β -galactosidase activities were found to be 1:0.5. No α -glucosidase or β -galactosidase activities were detected.

Step 5: CM-Sephadex chromatography.

The above enzyme solution was dialyzed against 0.1 M phosphate buffer, pH 6.3 and applied to a CM-Sephadex C-50 column (1.5×12 cm) which had been equilibrated with the same buffer. Gradient elution was carried out with 100 ml of 0.1 M phosphate buffer, pH 6.3, and 100 ml of 0.5 M NaCl in the same buffer. The α - and β -galactosidases were separately eluted (Fig. 3). The α -galactosidase fractions (35 ml) were pooled and then dialyzed against 3.2 M ammonium sulphate. The resulting suspension of the enzyme was then kept in a refrigerator at 4°C.

The specific activities and the yields with the above purification steps are summarized in Table I. α -Galactosidase was purified 140-fold over the crude extract.

Homogeneity of the enzyme preparation

The purified α -galactosidase sample was free from β -galactosidase, α - and β -glucosidases. Only one protein band was detected on polyacrylamide-gel electrophoresis at pH 8.5 (Fig 4).

TABLE I. PURIFICATION OF α -GALACTOSIDASE FROM ALFALFA SEEDS

Step		Volume (ml)	Total units	Total protein (mg)	Specific activity (units/mg of protein)	Recovery (%)
1	Crude extract	3,000	762,000	30,300	25.1	100
2	Dialysis	1,250	471,000	6,440	73.1	61.8
3	DEAE-Sephadex	290	46,100	574	80.3	6.15
4	Sephadex G-100	80	21,500	16.8	1,280	2.82
5	CM-Sephadex	35	8,650	2.45	3,530	1.14

FIG. 4. Polyacrylamide Gel Electrophoresis of α -Galactosidase Preparation.

A sample was run in 8% gel at pH 8.5 for 4 hr at 10 mA in a horizontal tray (17×8×0.3 cm). The gel was stained with Amido Black 10B.

Molecular weight estimation

The molecular weight of the enzyme was estimated to be approximately 57,000 by gel filtration on Sephadex G-100 with standard proteins (Fig. 5).

FIG. 5. Molecular Weight Estimation of α -Galactosidase by Gel-Filtration on Sephadex G-100.

Standard proteins: 1, cytochrome C; 2, myoglobin; 3, β -lactoglobulin; 4, ovalbumin; 5, serum albumin. For details see MATERIALS AND METHODS.

Effect of pH and temperature on enzyme activity

The pH-activity curves of the α -galactosidase with *p*-nitrophenyl α -D-galactoside and raffinose as substrate are shown in Fig. 6. A well-

FIG. 6. Effect of pH on the Activity of α -Galactosidase with *p*-Nitrophenyl α -D-Galactopyranoside and Raffinose.

Standard assay conditions were used. The buffer used was McIlvaine buffer (pH 2.5~8). The amount of enzyme used: 8.1 μ g/0.1 ml for *p*-nitrophenyl α -D-galactopyranoside; 32.4 μ g/0.1 ml for raffinose. O, *p*-nitrophenyl α -D-galactopyranoside; ●, raffinose.

defined optimum near pH 4.5 was observed with raffinose, while two pH optima at pH 4.0 and 5.5 were observed when *p*-nitrophenyl α -D-galactoside was used. It has been indicated, in several cases, that α -galactosidases show multiple pH optima when a synthesized substrate was used.⁸⁾ The optimum temperature for enzyme activity measured at pH 5.5 with the standard assay method was at *ca.* 50°C.

Effect of substrate concentration

Rates of hydrolysis of *p*-nitrophenyl α -D-galactoside and raffinose at different substrate concentrations are shown in Figs. 7 and 8. Simple Michaelis-Menten type kinetics were observed with raffinose, whereas inhibition of higher concentrations of substrate was observed with *p*-nitrophenyl α -D-galactoside. The *K_m*

FIG. 7. Effect of Substrate Concentration on the Initial Rate of Hydrolysis of *p*-Nitrophenyl α -D-galactopyranoside.

The enzyme (6.4 μ g/0.1 ml) was incubated under standard conditions with various amounts of substrate. A Lineweaver-Burk plot is given as an inset.

FIG. 8. Effect of Substrate Concentration on the Initial Rate of Hydrolysis of Raffinose.

The enzyme (38.0 μ g/0.1 ml) was incubated under standard conditions with various amounts of substrate. A Lineweaver-Burk plot is given as an inset.

values for *p*-nitrophenyl α -D-galactoside (determined from the linear parts of the plots) and raffinose in McIlvaine buffer, pH 5.5, at 30°C were calculated to be 5.13×10^{-4} M and 1.81×10^{-2} M, respectively.

Inhibition study

The effect of metal ions and carbohydrates on enzyme activity were examined, results are shown in Table II. α -Galactosidase was highly sensitive to heavy metal ions such as Ag^+

and Hg^{2+} . Co^{2+} and Fe^{2+} also inhibited the enzyme activity. D-Galactose was a powerful inhibitor, but other carbohydrates also had less inhibitory effects.

TABLE II. INHIBITION OF α -GALACTOSIDASE BY VARIOUS CARBOHYDRATES AND METAL SALTS

Inhibitor	Relative activity (%)
(Carbohydrates)	
None	100
Glucose	99
Galactose	36
Xylose	85
Fructose	91
Mannose	87
Arabinose	86
Lactose	84
Sucrose	91
Maltose	88
(Metal salts)	
CuCl_2	104
MgCl_2	103
MnCl_2	96
ZnCl_2	95
FeCl_2	84
CoCl_2	66
HgCl_2	7
AgNO_3	6

DISCUSSION

An α -galactosidase from alfalfa seeds was purified 140-fold. The greatest loss of enzyme activity occurred during the chromatography on DEAE-Sephadex A-50. Such a loss might be caused on exposure of the enzyme to a relatively higher pH value.

The purified enzyme showed a single elution peak on Sephadex G-100 gel filtration, and gave a single band when examined by polyacrylamide-gel electrophoresis. These results suggest that a single α -galactosidase is present in alfalfa seeds unlike coffee beans,²¹⁾ *Vicia faba* seeds,²²⁾ *Pisum sativum*²³⁾ and *Cucurbita pepo*²⁴⁾ in which multimolecular forms have been demonstrated. However, another form(s) of the enzyme may be involved in alfalfa seeds because the elution curve of α -galactosidase activity from DEAE-Sephadex did not show a single peak.

The α -galactosidase from alfalfa seeds was sensitive to Hg^{2+} and Ag^+ ions; this suggests that a sulfhydryl group or groups are necessary for enzyme activity. The enzyme was also inhibited by D-galactose. D-Galactose is known as a powerful and competitive inhibitor of α -galactosidases from several sources.^{22, 25~27)} The galactose from galactosyl sucrose derivatives such as raffinose and stachyose liberated by α -galactosidase-catalyzed hydrolysis would be expected to eventually inhibit further hydrolysis of the oligosaccharides. In the case of *Vicia faba* seeds,²⁸⁾ however, galactose is known to be converted into galactose 1-phosphate which is a very weak inhibitor of α -galactosidase, though such conversion has not been confirmed in alfalfa seeds.

This report shows that alfalfa seeds contain relatively high α -galactosidase activity, but the mechanism and the role in the metabolism of galactose-containing oligosaccharides remain unresolved.

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