Production of α-Galactosidase from *Aspergillus awamori*: Properties and Action on Para-Nitrophenyl α-D-Galacto-pyranoside and Galacto-Oligosaccharides of Soy Milk

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ABSTRACT

Soybean products including soy milk can provide needed protein for human consumption. Soybean flour contains raffinose and stachyose considered responsible for flatulence often associated with soy products. A partially purified preparation of α-galactosidase and invertase, prepared from *Aspergillus awamori* NRRL 4869 cultured on wheat bran, hydrolyzes the oligosaccharides of soy milk. The α-galactosidase exhibited its maximum activity at pH 5.0 and was stable in that pH range. The optimum temperature was 50 C; however, 8% of α-galactosidase activity was lost after 15 min at 55 C, and complete inactivation was observed after 14 min at 70 C. Parachloromercuribenzoate, $AgNO_3$, $HgCl_2$, and iodine exhibited strong inhibitory effects on the enzymes. Km values of the α-galactosidase with melibiose and raffinose as substrates were 3.0 and 3.6 x 10^{-2} M, respectively, and the molecular weights of both enzymes were estimated to be about 130,000 on the basis of Sephadex and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

INTRODUCTION

A large portion of the world population is undernourished because of insufficient protein in the diet. Soybeans and derivatives of soybeans are being utilized increasingly to alleviate this problem. However, the discomfort of flatulence associated with ingestion of soy products must be dealt with to produce protein acceptable for humans. The galacto-oligosaccharides raffinose and stachyose are believed responsible for the discomfort from eating certain legumes, including soybeans (1,2). These flatulence substances can be removed by water soaking, alcohol extraction, preparation of protein isolates, and enzyme digestion (1).

The present paper describes preparation, partial purification, and characterization of α-galactosidase and invertase from *Aspergillus awamori*. This information could be useful in providing an economical process for removing raffinose and stachyose from soy products.

EXPERIMENTAL PROCEDURES

Materials

Substrates used in the present studies were obtained from the following sources: galactose and melibiose, Aldrich Chemical Company, Inc. (Milwaukee, WI); glucose and sucrose, Difco Laboratories (Detroit, MI); raffinose, Eastman Chemical Company (Kingsport, TN); fructose, Fisher Scientific Company (Fairlawn, NJ); and PNPG (para-nitrophenyl α-D-galactopyranoside), Sigma Chemical Company (St. Louis, MO).

Methods

Preparation of α-galactosidase and invertase: Enzyme was produced by the procedure of Smiley et al. (3). By our modification, 71 g of wheat bran was added to tared 2800-ml Fernbach flasks, and the flasks then were autoclaved 30 min at 130 C. Fifty-seven milliliters of distilled water was added to each flask. The flasks were sealed with dairy filter, pipe cleaner, and aluminum foil and then were shaken on a New Brunswick table shaker at 300 rpm 1 hr. Foil covers were removed, and flasks were autoclaved 20 min at 121 C and slow exhaust. Foil covers were immediately replaced. Under these conditions the moisture was about 50%. The bran medium was inoculated with a spore

TABLE I

Effect of Metal Ions and Organic Acids on α-Galactosidase and Invertase

Inhibitors	Final (M) concentration	Relative activity	
		α-Galactosidase (%)	Invertase (%)
None		100	100
Lead acetate	2.5 x 10^{-5}	100	100
Lead acetate	1.25 x 10^{-4}	97	100
Silver nitrate	2.5 x 10^{-5}	17	32
Silver nitrate	1.25 x 10^{-4}	0	18
Silver nitrate	2.5 x 10^{-4}	0	9
Mercuric chloride	2.5 x 10^{-5}	0	0
p-Chloromercuribenzoate	2.5 x 10^{-4}	4	60
Magnesium chloride	2.5 x 10^{-5}	91	95
Magnesium chloride	1.25 x 10^{-4}	89	89
Magnesium chloride	2.5 x 10^{-4}	75	67
Manganous sulfate	2.5 x 10^{-5}	100	100
Manganous sulfate	1.25 x 10^{-4}	88	100
Manganous sulfate	2.5 x 10^{-4}	88	100
Potassium iodide	2.5 x 10^{-5}	100	93
Potassium iodide	1.25 x 10^{-4}	100	89
Potassium iodide	2.5 x 10^{-4}	98	82
p-Hydroxybenzoic acid	2.5 x 10^{-4}	100	100
Sodium benzoate	2.5 x 10^{-5}	100	100
Sodium benzoate	1.25 x 10^{-4}	89	100
Sodium benzoate	2.5 x 10^{-4}	89	95
Sodium citrate	2.5 x 10^{-5}	100	93
Sodium citrate	1.25 x 10^{-4}	100	89
Sodium citrate	2.5 x 10^{-4}	98	82

TABLE II

Effect of Sugars, Iodine, and Urea on α-Galactosidase and Invertase

Inhibitors	Final (M) concentration	Relative activity α-Galactosidase (%)	Relative activity Invertase (%)
None	---	100	100
Glucose	1×10^{-2}	88	77
Fructose	1×10^{-2}	97	100
Galactose	1×10^{-2}	89	100
Glucose-fructose	2×10^{-2}[a]	100	100
Glucose-galactose	2×10^{-2}[a]	100	95
Galactose-fructose	2×10^{-2}[a]	84	97
Glucose-fructose-galactose	3×10^{-2}[a]	41	28
Iodine	2.5×10^{-5}	0	65
Iodine	1.25×10^{-4}	0	65
Iodine	2.5×10^{-4}		63
Urea	2.5×10^{-5}	100	96
Urea	1.25×10^{-4}	100	
Urea	2.5×10^{-4}	100	94

[a]Sums of individual monosaccharide concentrations.

TABLE III

Thermal Stability of α-Galactosidase[a]

Temperature	Time (min)	% Active
Room temperature, 25 C		100
30	15	98
40	15	98
50	15	98
55	15	92
60	15	92
70	15	0

[a]α-Galactosidase (0.6 U) in 2.00 ml.

suspension in a ratio of 0.2 ml spore suspension to 10 g of wheat bran containing 50% moisture. The spore suspension was prepared by washing a week-old (25 C incubation) malt agar slant (Difco) of NRRL 4869 with 3 ml sterile water. Aluminum foil covers were removed and incubation continued 7 days at 35 C as stationary cultures. At the end of 7 days α-galactosidase and invertase were extracted from the fermented bran by adding 710 ml of 2% NaCl solution to each flask. The flasks were emptied into a nalgene beaker and vigorously agitated about 15-30 sec. This slurry was returned to the flasks and agitated 1 hr at 245 rpm on a New Brunswick shaker. The combined slurry from ten flasks was then pressed to 500 psi through a canvas bag to retain the bran. Further removal of spores from the extract was accomplished by centrifugation on a Sharples TI at 10,000 rpm at 50 ml per min flow rate.

Purification of α-galactosidase and invertase: Dialysis to remove salts and other lower molecular weight substances was accomplished in an Amicon DC 30 Hollow Fiber dialysis-concentration system using a single cartridge for 4500 ml of extract. The centrifuged extract was recirculated at 10 liters per minute with distilled water pumped into the reservoir at adjusted rates to replenish the volume lost to ultrafiltration. Progress of dialysis was monitored by the loss of chloride ion ($AgNO_3$ indicator) and the increase in transmittance of the ultrafiltrate at 280 nm. After 75 min of dialysis, no further leaching of proteins smaller than 10,000 mw cut-off could be detected. Incoming water to the system was terminated to allow ultrafiltration to continue until the total extract volume was concentrated to ca. 1 liter. The recovered concentrate was assayed to demonstrate that there was negligible loss of total units of α-galactosidase activity. The extract was further purified by a gel filtration. Higher molecular weight substances including proteases were separated on Sephadex 75 and calcium phosphate gel according to Kawamura et al. (4).

Estimation of α-galactosidase and invertase activities: Enzyme activities were determined by the method of Suzuki et al. (5) using melibiose and sucrose as substrates for α-galactosidase and invertase, respectively. By this procedure the reaction mixture was composed as follows: 0.50 ml of 0.03 molar substrate, 0.50 ml 0.10 molar potassium phosphate buffer (pH 5.0), and 1 ml of the enzyme solution diluted to achieve 25-30% conversion of substrate. The reaction mixture was incubated 30 min at 40 C, and at the end of the incubation, the test tube was placed in a boiling water bath 5 min to inactivate enzyme. Glucose liberated was estimated by a modified glucose-oxidase procedure based on the method of Gruesbeck and Rase (6). Blanks were run in the same manner, using heat-inactivated enzymes. The amount of glucose in the blanks was subtracted from the respective sample. One unit (U) of enzyme was defined as the amount of enzyme which liberated 1 μmole of glucose per min under conditions of assay. Furthermore, when raffinose and stachyose are the substrates, the measurement of glucose formed is an index of α-galactosidase activity since α-galactosidase is much less active than invertase in this dual enzyme system (3).

Effect of inhibitors, temperature, and pH: Metal ions, organic acids, sugars, and other inhibitory substances were added to the assay system, and activities were obtained in the usual manner.

TABLE IV

Kinetics of α-Galactosidase

Substrate	PNPG	Melibiose	Raffinose
(M)	$2.6 \sim 8.2 \times 10^{-4}$	$2.9 \times 10^{-2} \sim 1.17 \times 10^{-1}$	$1.68 \times 10^{-2} \sim 1.0 \times 10^{-1}$
Enzyme unit/ml	0.112	0.60	0.60
Km (M)[a]	6.0×10^{-3}	3.0×10^{-2}	3.6×10^{-2}
Vmax[b]	3.0×10^{-7} (moles/min/5 ml)	5.6×10^{-7} (mole/min/2 ml)	8.6×10^{-7} (moles/min/2 ml)

[a]Km, Vmax obtained from initial velocities.

[b]Total volume = 2.0 ml (melibiose, raffinose) or 5.0 ml (PNPG).

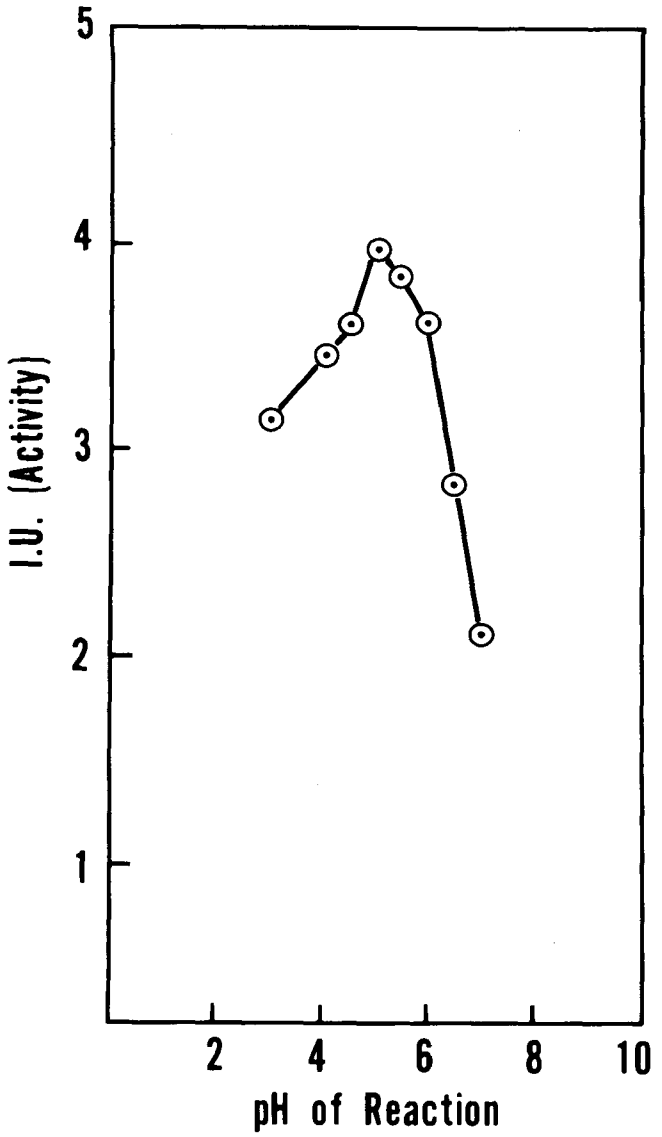

FIG. 1. Effect of pH on α-galactosidase activity.

The enzyme was subjected to temperatures ranging from 25-70 C for 15 min. α-Galactosidase activity was then determined.

The enzyme was assayed at pH values ranging from 3 to 7. The activity was measured by the usual method.

Kinetics of α-galactosidase: Hofstee and linear regression plots of α-galactosidase action on PNPG, melibiose, and raffinose were constructed. Apparent Michaelis constants (Km) and maximum velocities (Vmax) were determined from the plots.

RESULTS

Activity of α-Galactosidase and Invertase

Activities of 0.60 and 1.63 U per ml were obtained for α-galactosidase and invertase, respectively. After dialyzing 4500 ml of the above combined extract against tap water overnight and concentrating to 1000 ml, the activities were 2.8 and 7.2 U per ml for α-galactosidase and invertase, respectively.

The concentrates were stable for 5 months when stored at 10 C. Activity of dilute enzyme solutions decreased after a few days in the refrigerator.

Effect of Metal Ions and Organic Acids on α-Galactosidase and Invertase Activity

The results of inhibition studies are in Table I. The results indicate that α-galactosidase and invertase prepared from *Aspergillus awamori* NRRL 4869 are sensitive to certain metal ions but only slightly sensitive to the organic acids tested. p-Chloromercuribenzoate, silver nitrate, and mercuric chloride at 2.5 x 10^{-4} molar caused complete inhibition of α-galactosidase and strong inhibition of invertase.

Effect of Sugars, Iodine, and Urea on α-Galactosidase and Invertase Action

Glucose and galactose inhibited α-galactosidase to the same extent. Equal combined molar concentrations did not decrease α-galactosidase activity, but activity was decreased when combined concentrations were incubated with invertase (Table II). However, a combination of glucose, fructose, and galactose caused 60-70% depression of α-galactosidase and invertase activity. Iodine at 2.5 x 10^{-5} molar caused complete inhibition of α-galactosidase and 35% depression of invertase.

Thermal Stability of α-Galactosidase

α-Galactosidase lost 8% of its activity after 15 min at 55 C, and 100% inactivation was observed after 15 min at 70 C (Table III).

Kinetics of α-Galactosidase

The Km and Vmax values, determined by Hofstee plots of the action of α-galactosidase on melibiose or raffinose, demonstrated the same order of preference for both substrates (Table IV). Although invertase is present in the reaction mixture, it is believed not to interfere with the action of α-galactosidase (3,7). The assumption is supported by the fact that under similar conditions, α-galactosidase demonstrated the same order of preference for melibiose and raffinose. α-Galactosidase demonstrated one order of magnitude greater preference for the synthetic substrate PNPG as evidenced by Km when compared to the natural substrates melibiose and raffinose. Vmax values were of the same order of preference for all three substrates.

Effect of pH on α-Galactosidase Activity

The enzyme was assayed at pH values ranging from 3-7. The enzyme demonstrated a maximum activity at pH 5.0 (Fig. 1).

DISCUSSION

Use of α-galactosidase for the improvement of crystalline sucrose recovery from beet molasses was reported by Suzuki et al. (8); this research is apparently the first application in food processing. The were careful to avoid contamination with invertase in their enzyme preparation. However, in removing oligosaccharides from soy products including soy milk and soy whey, the presence of invertase in the preparation can be useful since it can hydrolyse sucrose and increase sweetness of the product (7). On the other hand, the proteases contribute to undesirable flavors. The large molecular weights of our α-galactosidase and invertase permit the separation of lower molecular weight substances including proteases by dialysis and molecular sieving methods. The α-galactosidase reported here appears quite similar to an α-galactosidase from *Aspergillus saitoi* reported by Sugimoto and Van Buren (7). Both enzymes have maximum activity at pH 5.0 and are inactivated at 70 C. Both appear to be an SH-enzyme type since they are inhibited by p-chloromercuric benzoate as well as by iodine, silver nitrate, and mercuric chloride at 2.5 x 10^{-4} molar. Both enzymes were slightly inhibited by galactose at 1 x 10^{-2} M. Sugimoto and Van Buren (7) reported a molecular weight of 290,000 for α-galactosidase prepared from *Aspergillus saitoi.* Tentative information using Sephadex and sodium dodecyl sulfate (SDS)-polyacryl-

amide gel electrophoresis (9) suggests that our α-galactosidase and invertase have molecular weights of about 130,000.

One advantage of our α-galactosidase-invertase system prepared from NRRL 4869 is that it might be used to remove raffinose and stachyose from soy products such as soy milk and soy whey. Another advantage is that α-galactosidase can be produced by culturing *Aspergillus awamori* NRRL 4869 on inexpensive wheat bran by stationary fermentation. Furthermore, the optimum pH for enzyme activity is that of soy milk. Also, differing from some α-galactosidases already reported (7), our enzyme is relatively insensitive to the presence of α-D-galactose in the reaction mixture.

Another advantage of our enzyme is its insensitivity to certain organic acids. This could be significant since soybeans contain organic acids in considerable amounts (10). Our α-galactosidase does, however, demonstrate inhibition by a combination of the monosaccharides galactose, glucose, and fructose at 3×10^{-2} M. This phenomenon is the subject of further investigation along with use of hollow fiber cartridges.

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