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Hydrolysis of oligosaccharides in soybean flour by soybean α-galactosidase

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Abstract

Raffinose oligosaccharides (ROs) make up a substantial part (40%) of the soluble sugars found in soybean seeds and are responsible for flatulence after the ingestion of soybean and other legumes. Consequently, soy-based foods would find a broader approval if the ROs were removed from soybean products or hydrolysed by α-galactosidases. During soybean seed germination, of content the ROs decrease substantially, while the α-galactosidase activity increases. α-Galactosidase was partially purified from germinating seeds by partition in an aqueous two-phase system and ion-exchange chromatography. The enzyme preparation presented maximal activities against ρ-nitrophenyl-α-D-galactopyranoside (ρNPGal) at 60 °C and a pH of 5.0 and the $K_{M\,app}$ values for ρNPGal, melibiose, and raffinose of the enzyme preparation were 0.33, 0.42, and 6.01 mM, respectively. The enzyme was highly inhibited by SDS, copper, and galactose. Hydrolysis of soybean flour ROs by enzyme preparation reduced the stachyose and raffinose contents by 72.3% and 89.2%, respectively, after incubation for 6 h at 40 °C.
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1. Introduction

Soybean (*Glycine max*) is an excellent food and feed source. Soybean differs from grain legumes in its high amounts of proteins and due to its well-balanced amino acid pattern (De, 1971). However, considerable amounts of flatulence-causing raffinose oligosaccharides (ROs) in soybean-based foods limit their biological value and acceptability. Raffinose and stachyose (α-galactosyl derivates of sucrose) are the major oligosaccharides associated with flatulence production (De Lumen, 1992). Since monogastric animals and human beings do not have the α-galactosidase enzyme that cleaves the α-1,6 galactosyl linkage in their digestive tract, the intact oligosaccharide is not absorbed. These oligosaccharides accumulate in the large intestine, where anaerobic microorganisms ferment them and lead to flatus formation (Steggerda, 1968). A reduction of the ROs level is therefore highly desirable, so that soybean could be used as a more acceptable source of inexpensive proteins. Many attempts have been made to reduce the ROs content in legumes by hulling, soaking, cooking, gamma irradiation,

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germination, and microbial or plant α-galactosidase treatments (Guimarães, De Rezende, Moreira, Barros, & Felix, 2001; Machaiah & Pednekar, 2002; Mulimani & Devendra, 1998; Sanni, Onilude, & Ogundoye, 1997; Scalabrini, Rossi, Spettoli, & Matteuzzi, 1998). α-Galactosidase enzymes are widespread in microorganisms, plants, and animals (Dey & Pridham, 1972). Generally, α-galactosidase can hydrolyze a variety of simple α-D-galactosides as well as more complex molecules, such as oligosaccharides and polysaccharides. Soybean α-galactosidase has been proposed to reduce ROs in soymeal and soymilk (Guimarães et al., 2001; Porter, Herrmann, & Ladish, 1990). However, no large-scale enzymatic process for this purpose has been carried out with this enzyme. In the present investigation, the ROs content and α-galactosidase activity in soybean seeds were determined during germination and the conditions for enzymatic ROs hydrolysis by soybean α-galactosidase tested in soybean flour.

2. Materials and methods

2.1. Soybean germination conditions, extraction, and HPLC analysis of soluble sugars

Soybean seeds (*Glycine max* L. Merr. cv. CAC-1) were placed on a water-soaked paper filter to germinate (Germitest) for 120 h, at 27 °C, and then frozen at −20 °C. Lyophilized soybean seed samples were ground and freed from fat with petroleum ether. The soluble sugars of 20 mg seed powder were extracted with 80% aqueous-ethanol (v/v) according to Saravitz, Pharr, and Carter (1987). The solvent was evaporated at 40 °C and the sugars resuspended in 1 ml water, and then analyzed by HPLC on a Shimadzu series 10A chromatograph. For this purpose, an analytical column [aminopropil (–NH_2)] was used, eluted with an acetonitrile–water isocratic mixture (70:30 v/v). The individual sugars were automatically identified and quantified by comparison with the retention periods and standard sugar concentrations. Gentiobiose was used as an internal standard because it does not interfere with the other sugars and is not found in soybean seeds.

2.2. Enzyme assay

α-Galactosidase was assayed in a reaction system containing 650–750 μl of 0.1 M sodium acetate buffer, pH 5.0; 0–100 μl enzyme preparation; and 250 μl of 2 mM ρ-nitrophenyl-α-D-galactopyranoside (ρNPGal) or other synthetic substrates. The reaction was run for 15 min at 37 °C and stopped by the addition of 1 ml of 0.5 M sodium carbonate. One enzyme unit (U) was defined as the amount of protein required to produce one μmol of ρ-nitrophenol per min. The activities against raffinose, stachyose and sucrose were assayed for 20 min at 37 °C using a reaction mixture containing 500 μl of 0.1 mM sodium acetate buffer; pH 5.0; 100 μl of enzyme extract; and 400 μl of a 0.1 M substrate solution. The produced amount of reducing sugar was determined by adding 1 ml of 3,5-dinitrosalicylate reagent according to Miller (1956). The activities against melibiose, lactose, and maltose were assessed under the same reaction conditions and substrate concentrations used for raffinose, stachyose, and sucrose. In this case, the glucose yield was determined by the glucose–oxidase method (Bergmeyer & Bernt, 1974). The data presented for all α-galactosidase activity determinations are mean values of triplicate assays, in which the standard deviations always lay under 10%.

2.3. Protein determination

Protein quantities in the enzymatic extract were determined by the Coomassie Blue binding method (Bradford, 1976) with bovine albumin serum as standard.

2.4. α-Galactosidase purification

The pre-germinated seeds during 60 h were frozen (423 g fresh weight), powdered in a blender, resuspended in 870 ml of 0.1 M citric acid containing 0.05 M sodium phosphate with a pH of 5.0, and incubated under agitation for 1 h at 4 °C. The suspension was filtered through cheesecloth and the filtrate centrifuged (15,300*g*) for 40 min at 4 °C. The supernatant was used as a crude enzyme preparation. α-Galactosidase was partially purified by separation on ATPS (Hustedt, Kroner, & Kula, 1985) followed by ion exchange chromatography. The ATPS preparation was used as described by Guimarães et al. (2001) with 12% NaCl. The upper phase resulting from the ATPS containing the enzyme was dialyzed overnight against 50 mM sodium acetate buffer, pH 4.0, and loaded onto a CM-Sepharose Fast Flow column (25.5 × 1.5 cm) equilibrated with 0.1 M sodium acetate buffer, pH 4.0. The proteins were eluted at 4 °C at a flow rate of 42 ml/h, with a linear gradient formed by 300 ml of the acetate buffer and 300 ml of the same buffer containing 0.8 M NaCl. Five-ml fractions were collected. Fractions containing α-galactosidase activity were pooled and concentrated by ultrafiltration through a PM-30 membrane filter (Amicon).

2.5. Enzyme characterization

Kinetic experiments were performed at 37 °C and pH 5.0. The Michaelis–Menten constant (K_m) and the V_{max} for substrate hydrolysis were calculated by the Michaelis–Menten plot. The substrate concentrations, ex-

pressed in mM, were 0.025, 0.05, 0.1, 0.15, 0.25, 0.5, 1.0, and 1.5 in the case of ρNPGal; 0.1, 0.2, 0.5, 1.0, 1.5, 2.0, 3.0, and 5.0 for melibiose; and 0.25, 0.5, 1.5, 2.5, 5.0, 10.0, and 20.0 for raffinose. The effect of ions, simple sugars, and reducing agents on the enzyme activity was tested by the standard assay with enzyme samples pre-incubated with each of the compounds tested for 15 min at 37 °C.

2.6. Treatment of soybean flour with α-galactosidase

Commercial fat-free soybean flour was mixed with deionized water (1:10 w/v) and the samples (1.2 g) were incubated with 5 U of partially purified α-galactosidase for zero, 2, 4, and 6 h under shaking (100 rpm) at 40 °C. Each reaction mixture was lyophilized and the soluble sugars were extracted from 30 mg of dried powder with 80% aqueous-ethanol (v/v) according to Saravitz et al. (1987). The solvent was evaporated at 40 °C and the sugars resuspended in 1.2 ml water and analyzed by HPLC as described before.

2.7. Properties of soybean flour

The amount of protein and oil in commercial soybean flour were determined by standard Kjeldahl (Association of Official Analytical Chemists, 1975) and Soxhlet procedures, respectively.

3. Results and discussion

Raffinose, stachyose, and sucrose contents in germinating seeds decreased from maximum values at the beginning of the germination period to very low amounts according to the increasing germination time (Fig. 1). The fructose content, on the other hand, increased, confirming the hydrolysis of the fructose-containing oligosaccharides. Accordingly, the α-galactosidase activity against the substrate (ρ-nitrophenyl-α-D-galactopyranoside) ρNPGal in the germinating seeds increased along the germination period and reached its activity peak after 48–60 h (not shown). These observations confirm that ROs content is reduced during germination by the increase on α-galactosidase activity. ROs degradation during germination by endogenously synthesized α-galactosidase is also well-documented (Guimarães et al., 2001; Machaiah & Pednekar, 2002; Mansour & Khalil, 1998; Modi, McDonald, & Streeter, 2000). ROs are accumulated in soybean seeds during maturation until desiccation, when all enzymes involved in the ROs synthesis are active. ROs are found in the cotyledons of mature soybeans, located in the cytosol, while hydrolytic enzymes such as α-galactosidase are located in the protein bodies. The hydration of seeds during imbibition possibly induces α-galactosidase activity, leading to a ROs breakdown, which supplies the germinative energy (Herman & Shannon, 1985). Germination improved the nutritive value of legumes due to the formation of enzymes, including α-galactosidase, which could eliminate or reduce the level of anti nutritional factors in seeds. Bau, Villaume, and Méjean (2000) observed that germination gave rise to a substantial increase in the content of saponin, oestrogenic compounds, lecithin, and almost all phytosterols in soybean seeds. On the other hand, the duration of the germination process greatly influences the nutritional value and flavour of seeds. Germination may produce undesired compositional changes due to the initiation of metabolic processes and rootlet development (Naczk, Amarowicz, & Shahidi, 1997).

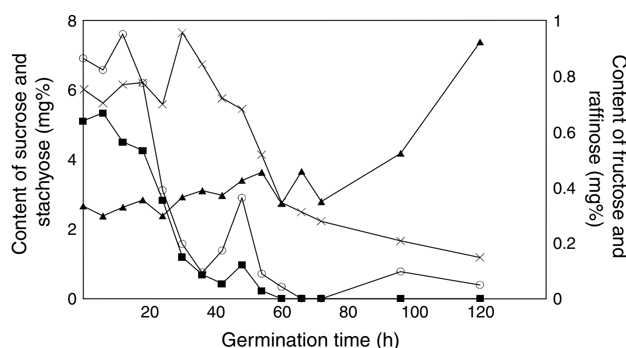


Fig. 1. Contents (mg%) of raffinose (○), stachyose (■), fructose (▲), and sucrose (×) in germinating soybean seeds.

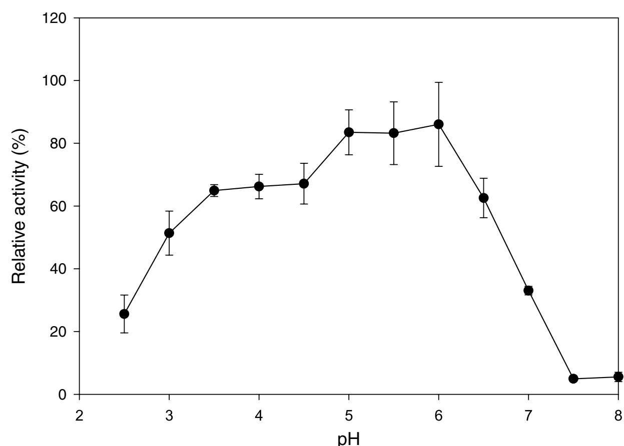
Results of the partial purification of α-galactosidase from germinating soybean seeds are summarized in Table 1. Most of the α-galactosidase activity present in the crude seed extract was observed in the upper phase of a two-phase aqueous system (ATPS). The chromatography of the resulting ATPS enzyme sample on a CM-Sepharose column indicated out one protein fraction with α-galactosidase activity. Soybean α-galactosidase was purified 26-fold with a recovery of 25.6% of the enzyme activity. No invertase activity was detected in the final enzyme preparation.

Substantial activity against ρNPGal was observed for partially purified enzyme preparations in a temperature range of 40–55 °C and pH range of 3.5–6.0 (Fig. 2). Maximal substrate hydrolysis by the enzyme was achieved at 60 °C, while the optimum pH was 5.0. These optimum pH and temperature values are close to those determined for ρNPGal hydrolysis: by the partially purified preparation of α-galactosidase from *Cassia sericea* (Mulimani & Devendra, 1998); by the crude preparation of α-galactosidase from germinating soybean seeds (Cruz & Silva, 1986) and from germinating guar (Shivanna, Ramakrishna, & Ramadoss, 1989); and by purified α-galactosidase from soybean seeds (Guimarães et al., 2001).

The $K_{M\,app}$ and V_{max} values calculated by the Michaelis–Menten plot for NPGal hydrolysis by the

Table 1
Summary of the steps of α-galactosidase purification from germinating soybean seeds

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	1388	103.3	0.07	1	100
Upper layer of ATPS	147	35.2	0.23	3.28	34.0
Dialyzed sample	108	34.2	0.31	4.42	33.1
CM-sepharose	29.6	26.4	0.89	12.7	25.6



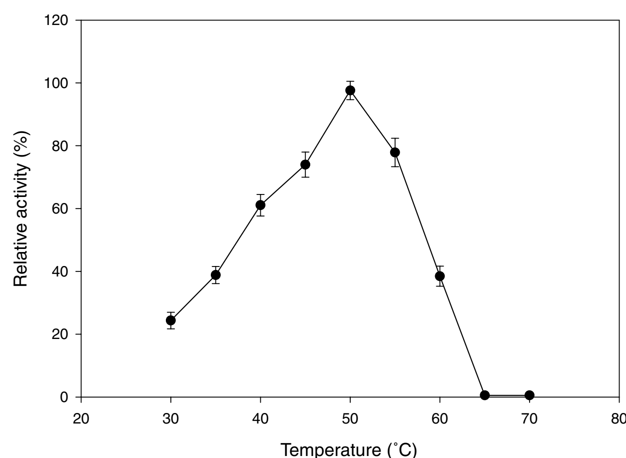


Fig. 2. Influence of pH and temperature on the activity of α-galactosidase from germinating soybean seeds.

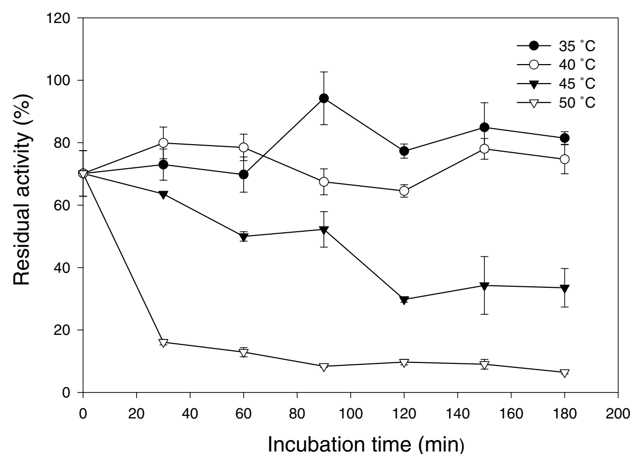


Fig. 3. Temperature influences on the stability of α-galactosidase from germinating soybean seeds. Enzyme preparation was pre-incubated at 35 °C (●), 40 °C (○), 45 °C (▼), and 50 °C (▽) for up to 3 h and the assays run at 37 °C.

enzyme preparation were 0.33 mM and 2.91 μmol min^{-1} ml^{-1}, respectively. For the hydrolysis of melibiose, these values were 0.42 mM and 0.17 μmol min^{-1} ml^{-1}, respectively. They are comparable to the values determined for ρNPGal hydrolysis by a crude (Cruz & Silva, 1986) and purified (Guimarães et al., 2001) α-galactosidase from germinating soybean seeds and for melibiose hydrolysis by α-galactosidase II from *Aspergillus niger* (Ademark, Larsso, Tjerneld, & Stalbrand, 2001). The $K_{M\,app}$ and V_{max} values for raffinose hydrolysis were 6.01 mM and 3.32 μmol min^{-1} ml^{-1}, respectively. The $K_{M\,app}$ value is close to the one determined for α-galactosidase purified from dry (Porter et al., 1990) and germinating soybean seeds (Guimarães et al., 2001) and from α-galactosidase from *Penicillium* sp. 23 (Varbanets, Malanchuk, Buglova, & Kuhlmann, 2001). It is, however, much lower than those reported for raffinose hydrolysis by enzymes from *Torulaspora delbrueckii* IFO 1255 (Oda & Tonomura, 1996) and *Aspergillus fumigatus* (De Rezende & Felix, 1999).

The partially purified α-galactosidase was slightly thermostable. No detectable activity was lost after incubation for 3 h at 35–40 °C. The enzyme retained about 40% of its original activity after incubation for 3 h at 45 °C, but 78% of the activity was lost following 30 min of incubation at 50 °C (Fig. 3).

The activity of the α-galactosidase preparation against several substrates is shown in Table 2. Under the experimental conditions used, the enzyme was more effective to hydrolyze ρNPGal, followed by raffinose, stachyose, and melibiose. Synthetic substrates containing *β*-linkages or containing xylose ρNP-X, arabinose ρNP-A, and mannose ρNP-M were not hydrolyzed by the enzyme preparation (Table 2).

The α-galactosidase preparation showed distinct sensitivities to simple sugars and mono and divalent ions (Fig. 4). The enzyme presented very low or no inhibition by sodium, β-mercaptoethanol, sucrose, potassium, magnesium, iodacetamide, glucose, and EDTA, but was highly inhibited by SDS, copper and galactose. The enzyme was only partially inhibited by calcium,

Table 2
Hydrolysis of several substrates with α-galactosidase from germinating soybean seeds

Substrate	Concentration (mM)	Relative activity[a] (%) ± SD
ρNP-α-Gal[b]	0.5	100 ± 6.5
ρNP-β-Gal[b]	0.5	0
*o*NP-β-Gal[b]	0.5	0
ρNP-α-Glc[b]	0.5	0
ρNP-α-Xyl[b]	0.5	0
ρNP-α-Ara[b]	0.5	0
ρNP-α-Man[b]	0.5	0
Raffinose	40	113.0 ± 4.4
Stachyose	40	50.78 ± 3.0
Melibiose	40	7.9 ± 0.81
Sucrose	40	7.78 ± 0.19
Maltose	40	5.53 ± 4.00
Lactose	40	4.63 ± 0.72

[a] Relative activities were calculated in relation to ρNP-α-Gal activity , that was considered as 100%.
[b] ρNP-α-Gal, *para*-nitrophenyl-β-D-galactopyranoside; ρNP-β-Gal, *para*-nitrophenyl-β-D-galactopyranoside; *o*NP-β-Gal, *ortho*-nitrophenyl-β-D-galactopyranoside; ρNP-α-Glc, *para*-nitrophenyl-α-D-glicopyranoside; ρNP-α-Xyl, *para*-nitrophenyl-α-D-xylopyranoside; ρNP-α-Ara, *para*-nitrophenyl-α-D-arabinopyranoside; ρNP-α-Man, *para*-nitrophenyl-α-D-mannopyranoside.

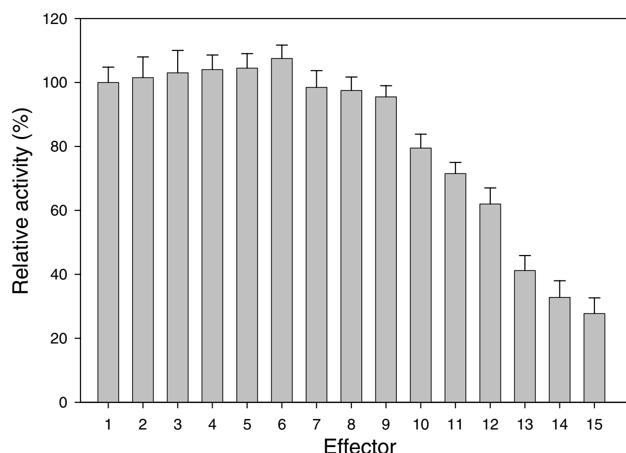


Fig. 4. Effect of NaCl (2); β-mercaptoethanol (3); sucrose (4); KCl (5); $MgCl_2$ (6); Iodacetamide (7); D-Glucose (8); EDTA (9); $CaCl_2$ (10); melibiose (11); raffinose (12); SDS (13); $CuSO_4$ (14); and D-galactose (15) on α-galactosidase from germinating soybean seeds, and (1) without effector. The final concentration of SDS was 1 mM. Final concentrations of all the other effectors were 2 mM.

melibiose, and raffinose. It has been suggested that α-galactosidase is not a metalloenzyme and the sulfhydryl groups do not take part in catalysis (lack of the inhibitor effects under treatment of enzyme by EDTA and iodacetamide, respectively). This is in agreement with the results reported for the α-galactosidase isolated from the culture liquid of *Penicillium* sp. 23 (Varbanets et al., 2001).

The potential of soybean α-galactosidase to hydrolyze the oligosaccharides present in fat-free soybean flour was tested. The soybean flour contained 32.3% sugar, 59.5% protein, 0.04% oil, and 8.2% ash. The partially purified α-galactosidase from germinating soybean seeds reduced the stachyose and raffinose content in soybean flour by 72.3% and 89.2%, respectively, after incubation for 6 h at 40 °C (Table 3). No oligosaccharide hydrolysis was detected in control tubes where the enzyme extract had been replaced by water. As the extract showed no invertase activity, these results indicate that soybean α-galactosidase acts on the oligosaccharides present in the soybean flour extract. Several food scientists have suggested the possibility to improve the nutritional value of soybean flour and soymilk by reducing the ROs content. Microbial α-galactosidases were used to degrade ROs in soymilk by Thananunkul, Tananka, Chichester, and Lee (1976), Cruz, Batistela, and Wosiacki (1981), Cruz and Park (1982), Mulimani and Ramalingam (1995), Kotwal, Gote, Sainkar, Khan, and Khire (1998), Scalabrini et al. (1998) and Thippeswamy and Mulimani (2002). Mulimani, Thippeswamy, and Ramalingam (1997) obtained promising results at removing ROs from soybean flour by crude α-galactosidase from guar seed. Guimarães et al. (2001) reported that the soybean seed α-galactosidase or the corresponding genes can be used to establish a process to hydrolyze ROs and improve the nutritional value of soymilk. Nevertheless, there is no biotechnological process available for the enzymatic treatment of soymilk or soybean flour with native or recombinant soybean enzymes. Since the enzymes used for this purpose are generally of microbial origin, they present the disadvantage of not having the GRAS (generally regarded as safe) status.

Table 3
Hydrolysis of oligosaccharides present in soybean flour by α-galactosidase from germinating soybean seeds

Hydrolysis time (h)	Raffinose content (%) ± SD	Stachyose content (%) ± SD
0	1.29 ± 0.09	5.0 ± 0.30
2	0.22 ± 0.01	2.2 ± 0.11
4	0.21 ± 0.01	1.72 ± 0.09
6	0.13 ± 0.01	1.38 ± 0.08

4. Conclusions

In the present work, we evaluated the germination effect on the ROs content reduction in soybean seed and tested the use of α-galactosidase from germinating soybean seeds for the hydrolysis of oligosaccharides found in soybean flour. The two treatments were efficient to remove the ROs content. The study of the enzyme properties showed that α-galactosidase from soybean seed is of potential interest to convert ROs in soybean flour to digestible sugars. The enzyme may be a better choice than α-galactosidase of microbial origin because, unlike

the homologous enzyme from microbial sources, soybean α-galactosidase is uniquely suited for the high protein and strongly buffered environment of soybean flour. Data from our studies indicate that this soybean enzyme will have practical applications to reduce flatulence caused by soybean flour consumption.

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