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Purification and Characterization of Guar Galactomannan Degrading α-Galactosidase from *Aspergillus oryzae* DR-5

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Abstract α-Galactosidase from *A. oryzae* DR-5 was induced in the presence of melibiose, raffinose, galactose, and locust bean galactomannan. The enzyme was purified to homogeneity by precipitation with acetone followed by ion-exchange chromatography using DEAE-Sephacel. The purified enzyme showed a single band in both nondenaturing-PAGE and SDS-PAGE. The enzyme was a glycoprotein in nature by activity staining. The molecular weight of the purified enzyme was 93–95 kDa by SDS-PAGE. The enzyme exhibited the optimum pH and temperature at 4.7 and 60°C, respectively. α-Galactosidase activity was strongly inhibited by Ag^{2+}, Hg^{2+}, Cu^{2+}, and galactose. EDTA, 1,10-phenanthraline, and PMSF did not inhibit the enzyme activity, whereas N-bromosuccinimide completely inhibited enzyme activity. Investigation by TLC showed complete hydrolysis of stachyose and raffinose in soymilk in 3 h at pH 5.0 and 50°C.

Key words: α-Galactosidase, *Aspergillus oryzae*, galactomannan

α-D-Galactosidase has a number of industrial applications. In the beet sugar industry, α-galactosidase helps to increase the yield of sucrose by hydrolyzing raffinose, which inhibits the sucrose crystallization if it exceeds 5% in the sugar syrup. In Japan, α-galactosidase from *Mortierella vinacea* is used in beet sugar refining [17, 22]. In the food processing industry, α-galactosidase finds an extensive use in the decomposition of flatulence-causing raffinose-family sugars in soymilk and feed additives [18]. Raffinose is widely distributed in legume seeds [5]. Human digestive juice lacks α-galactosidase, therefore raffinose-family sugars can escape digestion. Microorganisms present in the large intestine ferment these sugars to flatus (gas production) [19]. In pulp and paper industry, α-galactosidase in combination with xylanase and mannanase enhances the softwood pulp's bleachability [3]. Galactomannans are the storage polysaccharides, which consist of a β-1,4-linked mannan backbone to which galactose residues are attached in a α-1,6-linkage. Galactomannan is widely used in paper, food, cosmetic, and other allied industries. Galactomannan (galactose/mannose, G/M, 1:4) from locust bean is preferred in food industry because it has good gel promoting properties [2]. Guar galactomannan is an alternative candidate for locust bean galactomannan, because guar is economical and readily available. Quantitative removal of galactose residues in guar galactomannan (galactose/mannose, G/M, 1:2) by α-galactosidase led to intended gel-promoting properties suitable for food industries [2, 6].

For industrial use, larger amounts of α-galactosidase are used for intended applications. Cloning and expression of the gene in different hosts were carried out for large-scale production of the enzyme. However, traditional screening for microorganisms, immobilization of whole cells, and optimization of culture conditions for the enzyme production are used as alternatives before biochemical engineers are required to produce larger amounts of enzyme needed for industrial use. The removal of α-D-galactose moieties from guar galactomannan by α-galactosidase has been reported for a limited number of microbial sources [6, 24]. *Aspergillus oryzae* DR-5, which was isolated from soil, has the capacity to degrade highly viscous guar galactomannan. This fungal strain secretes higher levels of α-galactosidase and lower levels of protease. The purpose of the present investigation is to study the purification and properties of α-galactosidase.

One of the isolates that showed luxuriant growth on guar galactomannan as a carbon source was selected and identified as *Aspergillus oryzae* by the Indian Type Culture Collection (ITCC) staff, IARI, New Delhi. To study the effect of sugars on the induction, individual sugar (2%, w/v) was dissolved in distilled water, sterilized, and added prior to inoculation. After autoclaving, the flasks with the containing spores (2×10^6) of *A. oryzae* DR-5 were

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inoculated. Batch submerged fermentations were carried out for five days on an orbital shaker at 37°C and 130 rpm. At the end of the fifth day, the mycelium was separated from the culture medium by filtration using the Whatmann filter paper No.1 and stored at 4°C until further use.

Chilled acetone at 20°C was added to the above culture filtrate in a ratio of 1:1.5% (v/v), stirred well, and the mixture was kept at 4°C for 12 h. The precipitate was collected by centrifugation at 12,000 ×*g* for 15 min, dissolved in a minimum amount of Tris-HCl buffer (0.05 M, pH 7.3), and dialyzed. The dialyzate was centrifuged at 14,000 ×*g* and 10°C for 45 min to remove any precipitate that existed. The dialyzed α-galactosidase preparation was loaded onto a column (2×13 cm) of DEAE-Sephacel, previously equilibrated with Tris-HCl buffer (50 mM, pH 7.3). The column was washed thoroughly with the same buffer until the effluent O.D. at 280 nm was attained near zero. The bound proteins were eluted with the same buffer in a NaCl linear gradient from 0 to 0.5 M at a flow rate of 30 ml h^{-1}. The fractions having high α-galactosidase activity were pooled and concentrated in a lyophilizer. The concentrated enzyme solution was dialyzed against acetate buffer (50 mM, pH 4.5) at 4°C for 12 h with intermittent changes in the same buffer, and centrifuged. The α-galactosidase activity was assayed by using a reaction mixture containing 100 µl of 10 mM 4-nitrophenyl-α-D-galactopyranoside (PNPG) in distilled water, 800 µl of acetate buffer (50 mM, pH 4.7), and 100 µl of appropriately diluted enzyme that was incubated at 37°C for 15 min. The reaction was terminated by the addition of 3 ml 0.2 M sodium carbonate and the liberated p-nitrophenol was measured at 405 nm. One unit of enzyme activity is defined as the amount of enzyme which liberated one µmol of p-nitrophenol per min under the assay conditions.

The effect of pH on the enzyme was measured at 37°C in the range from 3.5 to 9.5. The effect of temperature was studied in the range of 20°C- 70°C by using acetate buffer (100 mM, pH 4.7). The enhancement or inhibitory effects of metals, sugars, and some chemical agents were investigated by preincubating the mixture containing 75 µl of acetate buffer (100 mM, pH 4.7), 25 µl of appropriately diluted purified enzyme solution, and 100 µl of the indicated concentration of each sugar or metal ion or chemical agents (Table 2) for 30 min at 37°C. Residual activity was assayed at 37°C by adding 100 µl of PNPG solution (10 mM in water). Continuous nondenaturating gel (7.5%) electrophoresis (native-PAGE) and SDS-PAGE (7.5%) were carried out using slab gels by applying the methods of Laemmli [10]. Proteins in the gels were stained with Coomassie Brilliant Blue R-250. SDS-PAGE molecular weight marker proteins were used to estimate the molecular weight of the purified enzyme. Schiff's reagent was used for the detection of the glycoprotein nature of purified α-galactosidase. Protein was assayed by the Folin Ciocalteu method with BSA as a standard. Soymilk was prepared using the method of Mulimani and Ramalingam [13]. To 50 ml of soymilk, 3 ml of purified enzyme (20 unit) were added and incubated for 3 h at pH 5.0 and 50°C. At regular time intervals, 2 ml of reaction mixture was taken out and placed in boiling water for 5 min to arrest the reaction. Separation, extraction, and estimation of raffinose family oligosaccharides in soymilk before and after enzyme treatment were carried out by the method of Tanaka *et al.* [20].

Aspergillus oryzae secreted extracellular α-galactosidase when it was grown on locust bean galactomannan as a carbon source. Galactose, melibiose, and raffinose also induced the secretion of α-galactosidase, but to a lesser extent when compared with guar galactomannan as a carbon source (data not shown). On the contrary, *A. oryzae* failed to produce α-galactosidase in a medium containing glucose as a carbon source. Glucose is known to repress the secretion of a variety of fungal hydrolytic enzymes, commonly known as catabolite repression [12]. The concentration of cAMP, a secondary messenger, is lowered in eukaryotic cells in the presence of glucose to prevent the wasteful duplication of energy-producing enzyme systems [23]. *Penicillium ochrochloron* secreted α-galactosidase when the fungus was grown in a medium with guar galactomannan as a carbon source [6]. The presence of galactomannan in the culture medium induced extracellular α-galactosidase secretion by *A. tamarii* [4]. Multiple forms of the α-galactosidase of *A. niger* were produced when the fungus was grown in a medium containing locust bean galactomannan as the sole carbon source [1].

A summary of the purification procedure is shown in Table 1. α-Galactosidase was purified by acetone precipitation of the culture filtrate, followed by anion-exchange (DEAE-Sephacel) chromatography. Fractionation of the enzyme by chromatography on DEAE-Sephacel showed the presence of a single peak of α-galactosidase activity (Fig. 1). Finally, the yield of protein was 7.5 mg for 1,100 ml of culture

Table 1. Purification of α-galactosidase from *Aspergillus oryzae* DR-5.

Purification step	Total volume (ml)	Total enzyme (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Crude extract	1100	1131	160	7.06	100	1
Acetone precipitation	62	232	19	12.21	20.5	1.7
DEAE-Sephacel chromatography	5	130	4.5	28.90	11.5	4.1

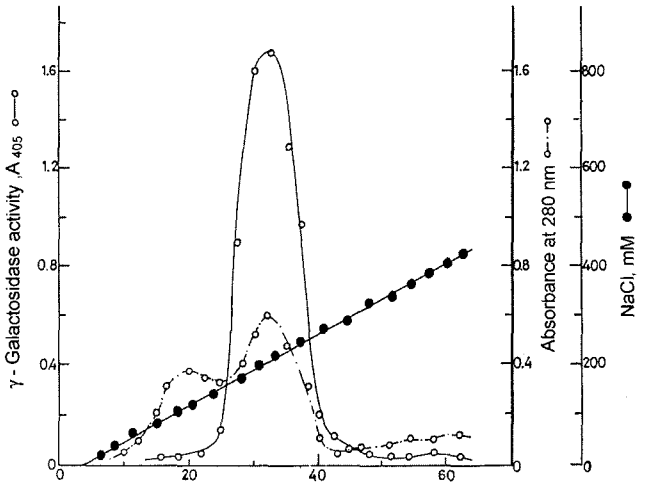

Fig. 1. Elution profile of DEAE-Sephacel column chromatography (2×13 cm) of α-galactosidase from *Aspergillus oryzae* DR-5.
(○—○) α-galactosidase, (○···○) 280 nm, and (●—●) NaCl.

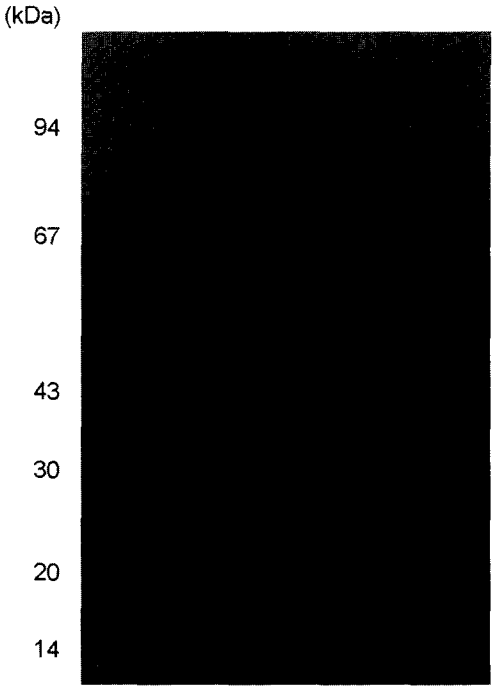

Fig. 2. SDS-PAGE of purified α-galactosidase.
SDS-PAGE (7.5%) was carried out and proteins in the gels were stained with Coomassie Brilliant Blue R-250. Lanes: 1, molecular size markers; and 2, purified α-galactosidase.

filtrate. α-Galactosidase was purified 4-folds from culture filtrate to give a 11.5% yield. After two simple purification steps, native-PAGE of the final enzyme preparation showed a single protein band. The purified enzyme was also homogeneous as seen by SDS-PAGE (Fig. 2). A single protein band was also found following the native-PAGE. The results from native-PAGE and SDS-PAGE suggested that the α-galactosidase might be a single polypeptide in nature. The molecular weight of the enzyme by SDS-PAGE was estimated to be between 63-65 kDa. A single broad band of protein having α-galactosidase in native-PAGE stained with Schiff's reagent indicated that the enzyme is a glycoprotein (Fig. 3A). Because of this biochemical property, the enzyme probably migrates as a broad protein band even when a small amount of enzyme was used in SDS-PAGE. Multiple α-galactosidases (α-Gal I to IV) from *A. niger* have been reported [1]. α-Gal I was a

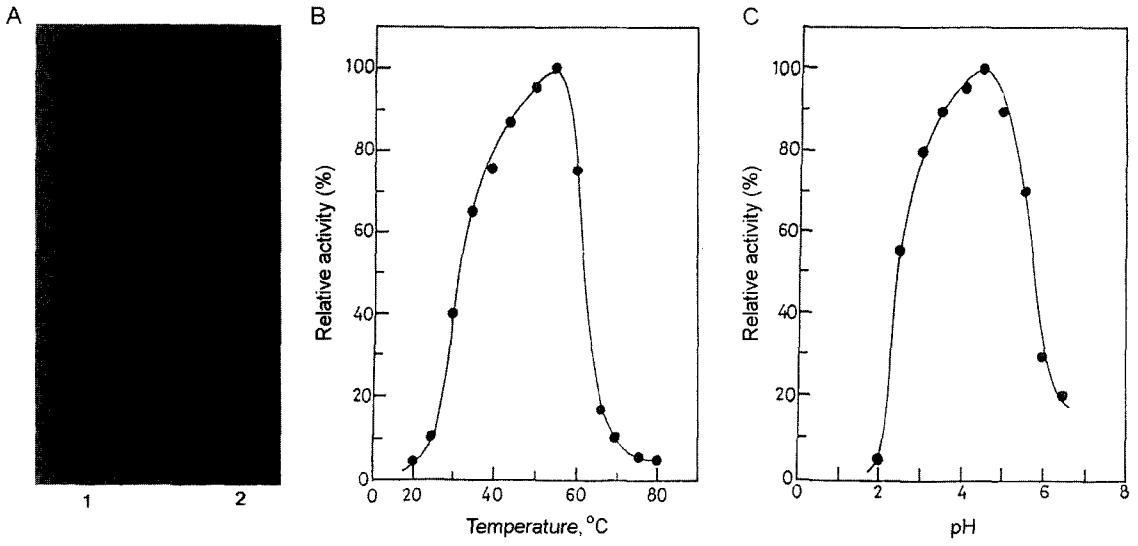

Fig. 3. A. Nondenaturating PAGE of purified α-galactosidase.
Nondenaturating PAGE (7.5%) was carried out for the detection of the glycoprotein nature of α-galactosidase using Schiff's reagent. Lanes: 1, 10 μg of enzyme; and 2, 25 μg of purified protein. **B** and **C**. Effect of temperature and pH on activity of α-galactosidase from *A. oryzae* DR-5.

tetramer composed of four 94-kDa subunits and α-Gal II-IV were dimers with monomeric molecular weight of 64 kDa [1]. The oligomeric state of α-galactosidase I from *A. niger* consists of a large tetramer having molecular weight of 350 kDa [1]. Being a large tetramer, α-galactosidase I (α-Gal I) failed to release galactose moieties attached to internal mannoses of the guar galactomannan main chain. Many of the α-galactosidases in family 36 are large enzymes with trimeric or tetrameric structure [1, 4]. On the contrary, α-galactosidases (α-Gal II-IV) from *A. niger* and *M. vinacea* belong to the 27th family of glycosyl hydrolase [1, 17]. α-Galactosidases that belong to the 27th family are usually smaller and a few of them are monomers [1].

The purified α-galactosidases from *M. vinacea*, *Humicola* sp., and *Penicillium* sp. 23 were found to be glycoprotein in nature. α-Galactosidase from *Saccharomyces carlsbergensis* was also shown as a glycoprotein with a molecular weight of 280 kDa [11]. α-Galactosidase from thermophilic fungus *Thermomyces lanuginosus* was purified from a locust bean galactomannan-spent medium and the molecular mass of the purified enzyme was 57 kDa [15]. The molecular weight of the α-galactosidase from *Pycnoporus cinnabarinus* was reported to be approximately 210 kDa by gel filtration and the enzyme was composed of four identical subunits where each subunit was estimated to be 52 kDa by SDS-PAGE [14].

The purified α-galactosidase from *A. oryzae* was optimally active at pH 4.5- 5.0 with PNPG as a substrate in acetate buffer [Fig. 3B]. α-Galactosidases, which were produced by *Aspergillus* spp., and indeed by most fungi, have a pH optimal range of 4.5 to 5.5 [1, 7, 16]. The purified α-galactosidase from *A. oryzae* was optimally active at 60°C with PNPG as the substrate in acetate buffer (pH 4.7) [Fig. 3B]. The enzyme from *M. vinacea* had an optimal activity at 55°C [17]. α-Galactosidase from *A. nidulans* and *Humicola* sp. showed optimal activity at 50°C and 65°C, respectively [8, 16]. Four major α-galactosidase forms from *A. niger* had optimal activity at 60°C [1]. Table 2 shows the effect of metal ions on enzyme activity. Enzyme activity was completely inhibited by Hg^{2+}, indicating that tryptophan residue appears to play a significant role in the catalytic process of the α-galactosidase. Ag^{2+} and Cu^{2+} also strongly inhibited the enzyme action. K^{+} and Zn^{2+} had a slight inhibition on the enzyme activity. Table 2 shows the effect of inhibitors and some reagents on α-galactosidase activity. EDTA and 1,10-phenanthraline did not inhibit the α-galactosidase activity and this indicates that α-galactosidase is not a metalloenzyme. Inhibition of α-galactosidase from *A. oryzae* by N-bromosuccinimide also indicated the role of tryptophan at or near the active site of α-galactosidase. From Table 2, it is also evident that PMSF did not inhibit the α-galactosidase from *A. oryzae* and this indicates the absence of a serine group for enzyme activity. α-Galactosidase from *P. cinnabarinus* was strongly inhibited by Ag^{2+} and Hg^{2+}, whereas EDTA did not affect its enzyme activity [14]. α-Galactosidase from *M. pilosus* was strongly inhibited by Hg^{2+}, Cu^{2+}, and Hg^{2+} [17]. α-Galactosidase from *Humicola* sp. was strongly inhibited by Hg^{2+} [7]. Incubation of α-galactosidase from a *Humicola* sp. with N-bromosuccinimide inhibited the enzyme activity [9].

The effects of sugars on α-galactosidase are summarized in Table 3. Among the various sugar tested, mannose, galactose, lactose, and melibiose were recognized to be powerful inhibitors. Raffinose and stachyose also markedly inhibited enzyme activity. Fructose exerted a slight positive effect upon enzyme activity (Table 3). Galactose and melibiose were powerful inhibitors of α-galactosidase from *P. cinnabarinus* [14]. Glucose, maltose, and stachyose also

Table 2. Effect of metal ions, inhibitors, and some reagents on α-galactosidase from *Aspergillus oryzae* DR-5.

Metal ion	Concentration (mM)	Relative activity (%)
Control	-	100
KCl	5	80
$CaCl_2$	5	104
$ZnSO_4$	5	100
$FeCl_3$	5	95
$FeSO_4$	5	100
$CuSO_4$	10	0
$CdCl_2$	5	100
$MgSO_4$	5	100
$AgNO_3$	2.5	0
$AgNO_3$	5	0
$HgCl_2$	2.5	47
$HgCl_2$	5	42
1,10-Phenanthraline	5	100
PMSF	1	100
EDTA	5	100
N-Bromosuccinimide	1	70
N-Bromosuccinimide	5	0

Table 3. Effect of sugars on α-galactosidase from *A. oryzae* DR-5.

Sugar	Concentration (mM)	Relative activity (%)
Control	-	100
Arabinose	10	100
Ribose	10	90
Xylose	10	80
Glucose	10	70
Galactose	2.5	25
Galactose	5	0
Fructose	10	80
Maltose	10	100
Cellobiose	10	110
Lactose	10	70
Sucrose	10	100
Melibiose	10	25
Raffinose	10	80

markedly inhibited the enzyme activity, while xylose, arabinose, lactose, sucrose, and raffinose showed weak inhibition on α-galactosidase from *P. cinnabarinus* [14]. Galactose inhibited the purified α-galactosidase from *Humicola* sp. [9]. α-Galactosidase from *A. oryzae* hydrolyzed the raffinose and stachyose in soymilk (data not shown). The complete hydrolysis of the sugar took a longer duration (3 h), possibly due to the inhibition of α-galactosidase by the galactose that was liberated from raffinose and stachyose. It is also evident from Table 3 that purified α-galactosidase from *A. oryzae* was completely inhibited by galactose at 5 mM concentration. α-Galactosidase from *Cyamopsis tetragonolobus* and *Gibberella fugikuroi* hydrolyzed raffinose and stachyose in soymilk [13, 18]. Recently, immobilized α-galactosidase from *G. fujikuroi* was used to decompose raffinose and stachyose in soymilk [21].

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REFERENCES

1. Ademark, P., M. Larsson, F. Tjerneld, and H. Stalbrand. 2001. Multiple α-galactosidases from *Aspergillus niger*: Purification, characterization and substrate specificities. *Enzyme Microbiol. Technol.* **29:** 441-448.
2. Bulpin, P. V., M. J. Gidley, R. Jeffcoat, and D. J. Underwood. 1990. Development of a biotechnological process for the modification of galactomannan polymers with plant α-galactosidase. *Carbohydr. Polym.* **12:** 155-168.
3. Clarke, J. H., K. Davidson, J. E. Rixon, J. R. Halstead, M. P. Fransen, H. J. Gilbert, and G. P. Hazlewood. 2000. A comparison of enzyme-aided bleaching of softwood paper pulp using combinations of xylanase, mannanase and α-galactosidase. *Appl. Microbiol. Biotechnol.* **53:** 661-667.
4. Civas, A., R. Eberhard, R. Le Dizet, and F. Petek. 1984. Glycosidases induced in *Aspergillus tamarii*. *Biochem. J.* **219:** 857-863.
5. Dey, P. M. 1985. Biochemistry of storage carbohydrates in green plants. *In*: *D-Galactose-Containing Oligosaccharides*. London: Academic Press.
6. Dey, P. M., S. Patel, and B. D. Brownleader. 1993. Induction of α-galactosidase in *P. ochrocholoron* by guar (*Cyamopsis tetragonoloba*) gum. *Biotechnol. Appl. Biochem.* **17:** 361-371.
7. Kotwal, S. M., M. I. Khan, J. M. Khire. 1995. Production of thermostable α-galactosidase from thermophilic fungus *Humicola* sp. *J. Ind. Microbiol.* **15:** 116-120.
8. Kotwal, S. M., M. M. Gote, M. I. Khan, and J. M. Khire. 1999. Induction, purification and characterization of constitutive intracellular α-galactosidase from the thermophilic fungus *Humicola* sp. *J. Ind. Microbiol. Biotechnol.* **23:** 661-667.
9. Kotwal, S. M., J. M. Khire, and M. I. Khan. 2000. Chemical modification of α-galactosidase from the thermophillic fungus *Humicola* sp. *J. Biochem. Mol. Biol. Biophys.* **4:** 65-72.
10. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.
11. Lazo, P. S., A. G. Ochoa, and S. Gascon. 1977. α-Galactosidase from *Saccharomyces carlsbergensis:* Cellular localization and purification of the external enzyme. *Eur. J. Biochem.* **77:** 375-382.
12. Martinez J. P., M. V. Elorza, D. Gozalbo, and S. Sentandreu. 1982. Regulation of α-galactosidase synthesis in *Saccharomyces cerevisiae* and effect of cerulenin on the secretion of this enzyme. *Biochim. Biophys. Acta* **716:** 158-168.
13. Mulimani, V. H. and Ramalingam. 1995. Enzymic hydrolysis of raffinose and stachyose in soymilk by crude α-galactosidase from *Gibberella fujikuroi*. *Biochem. Mol. Biol. Int.* **36:** 897-905.
14. Ohtakara, A., M. Mitsutomi, and Y. Uchida. 1984. Purification and enzymatic properties of α-galactosidase from *Pycnoporus cinnabarinus*. *Agric. Biol. Chem.* **48:** 1319-1327.
15. Puchart, V., M. Vrsanska, M. K. Bhat, and P. Biely. 2000. Purification and characterization of α-galactosidase from a thermophilic fungus *Thermomyces lanuginosus*. *Biochim. Biophys. Acta* **1524:** 27-37.
16. Rios, S., A. M. Pedregosa, I. F. Monistrol, and F. Laborda. 1993. Purification and molecular properties of an α-galactosidase synthesized and secreted by *Aspergillus nidulans*. *FEMS Microbiol. Lett.* **112:** 35-42.
17. Shibuya, H., H. Kobayashi, T. Sato, W. S. Kim, S. Yoshida, S. Kaneko, and I. Kusakabe. 1997. Purification, characterization and cDNA cloning of a novel α-galactosidase from *M. vinacea*. *Biosci. Biotechnol. Biochem.* **61:** 592-598.
18. Shivanna B. D., M. Ramakrishna, and C. S. Ramadoss. 1989. Enzymic hydrolysis of raffinose and stachyose in soymilk by α-galactosidase from germinating guar (*Cyamopsis tetragonolobus*). *Process Biochem* **24:** 197-201.
19. Steggerda, F. R., E. A. Richards, and J. J. Rackis. 1966. Effects of various soybean products on flatulence in the adult man. *Proc. Soc. Exp. Biol. Med.* **121:** 1235-1239.
20. Tanaka, M., D. Thananunkul, T. C. Lee, and C. O. Chichester. 1975. A simplified method for the quantitative determination of sucrose, raffinose and stachyose in legumes. *J. Food Sci.* **40:** 1087-1088.
21. Thippeswamy, S. and V. H. Mulimani. 2002. Enzymic degradation of raffinose family oligosaccharides in soymilk by immobilized α-galactosidase from *G. fujikuroi*. *Process Biochem.* **38:** 635-640.
22. Yamane, T. 1971. Decomposition of raffinose by α-galactosidase: An enzymatic reaction applied in the factory-process in Japanese beet sugar factories. *Sucr. Belge/Sugar Ind. Abstr.* **90:** 345-348.
23. Voet, D. and J. G. Voet. 1990. *Biochemistry*, pp 933-935. NewYork: John Wiley & Sons.
24. Zeilinger, S., D. Krristufek, I. Arisan-Atac, R. Hodits, and C. P. Kubicek. 1993. Conditions of formation, purification, and characterization of an α-galactosidase of *Trichoderma reesei* RUT C-30. *Appl. Environ. Microbiol.* **59:** 1347-1353.