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REDUCTION OF RAFFINOSE OLIGOSACCHARIDES IN RED GRAM FLOUR BY MICROBIAL α-GALACTOSIDASE

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SUMMARY

The effect of crude α-galactosidase from *Aspergillus oryzae, Gibberella fujikuroi* and *Lactobacillus brevis* in reducing the raffinose, stachyose and verbascose content in red gram flour was studied. The optimum pH for α-galactosidase was found to be 4.8 for *A. oryzae,* 5.0 for *G. fujikuroi* and *L. brevis*, while the optimum temperature of enzyme activity was 55°C for *A. oryzae*, 60°C for *G. fujikuroi* and 40°C for *L. brevis.* The specific activities of α-galactosidase from *A. oryzae, G. fujikuroi* and *L. brevis* were 1.54, 0.73 and 0.93 unit mg^{-1} protein respectively. These enzymes were thermostable when incubated at temperature ranges of 40-60°C for *A. oryzae,* 40-65°C for *G. fujikuroi* and 35-45°C for *L. brevis*. The optimum conditions for removing the raffinose, stachyose and verbascose were obtained by incubating red gram flour with 50 ml of crude microbial α-galactosidase extract (0.67, 0.55 and 0.67 units ml^{-1} for *A. oryzae, G. fujikuroi* and *L. brevis* respectively) for 3 hr at the optimum conditions of each strain. Crude *A. oryzae* and *G. fujikuroi* α-galactosidase reduced the raffinose oligosaccharide content in red gram flour by 100%, while crude α-galactosidase treatment from *L. brevis* reduced the raffinose content by 70.20%, stachyose 58.14% and verbascose by 71.56%.

***Key words*: *Aspergillus oryzae, Gibberella fujikuroi, Lactobacillus brevis,* microbial α-galactosidase, raffinose, red gram flour, stachyose**

INTRODUCTION

Red gram (*Cajanus cajan*. L) (pigeonpea) is one of the important grain legumes, which are grown and consumed in the tropics and semiarid parts of the world (Singh 1988). Grain legumes, including red gram, are a rich source of protein, vitamins, especially the B-complex, and minerals such as calcium and iron (Meiner *et al*. 1976). Red gram has been shown to contain many antinutritional factors such as trypsin and chymotrypsin inhibitors, polyphenols and raffinose family oligosaccharides (Singh 1988). These galacto-oligosaccharides constitute 53% of the total soluble sugars. These sugars contain one, two or three galactose units joined to sucrose by α-1,6 linkages. Owing to the absence of an α-1,6-galactosidase enzyme capable of hydrolyzing the α-1,6-galactosidic linkage, these oligosaccharides accumulate in the lower intestine and undergo anaerobic fermentation by bacteria, which may result in the production of flatus gases (H_2, CO_2 and small amounts of CH_4), abdominal rumbling, diarrhoea and discomfort (Flaming 1981, Price *et al*. 1988, Liener 1994). Therefore, to utilize legumes as more acceptable source of inexpensive protein, it is desirable to reduce the flatulence inducing agents.

Soaking, cooking, germination and fermentation have been suggested techniques to reduce the α-galactosides and improve the digestibility of available carbohydrates in a variety of legumes (Reddy and Salunkhe 1980). A

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variety of microorganisms have been reported to produce α-galactosidase. Thananunkul et al. (1976) have tested the ability of *M. vinacea* mycelium in three different forms (undisrupted, mycelial homogenate and entrapped) for the hydrolysis of raffinose and stachyose present in soymilk, but much information regarding the effects of enzymatic processing on the levels of raffinose family oligosaccharides such as raffinose, stachyose, verbascose and oligosaccharides in newly developed cultivars of red gram is not available.

To utilize legumes as more acceptable source of protein, it is necessary to reduce the flatus inducing agents. However information on the oligosaccharides and use of crude α-galactosidase of red gram seeds appears to be lacking. Hence in the present study an attempt has been made to examine the effect of crude α-galactosidase from different microorganisms for the hydrolysis of raffinose family sugars from red gram.

MATERIALS AND METHODS

The different varieties of red gram cultivars grown locally were obtained from Pulse Research Station, Gulbarga (ICAR). The seeds of uniform size and weight were picked and stored in plastic bottles at room temperature (35 ± 2°C) during the course of the study.

Production of crude microbial α-galactosidase:

Aspergillus oryzae and *Gibberella fujikuroi* were obtained from Biochemistry department's culture collection and *Lactobacillus brevis* (NCIM 2090) were supplied by the National Chemical Laboratory (NCL) Pune, India. *A. oryzae* was maintained on potato-dextrose-agar (PDA) slants. The organisms were grown in a medium containing the following composition (g/l): peptone, 15; yeast extract, 5; $NaNO_3$, 2; KH_2PO_4, 5; $MgSO_4$, 0.5; KCl, 0.5; galactose, 2 and one litre distilled water. The pH of the culture medium was adjusted to 6.0. Batch, submerged fermentation was carried out for the production of α-galactosidase from *A. oryzae.* Spores were inoculated into 80 ml of the above medium in Erlenmeyer flasks (500 ml capacity) incubated at 28 ± 1°C on a rotary shaker at 250 rpm for 5 days. After incubation, the mycelia were removed by filtration through Whatmann No.1 filter paper, and the culture filtrate thus obtained was used as source of enzyme. Solid ammonium sulfate was added to the culture filtrate at 4°C up to 20% saturation and the mixture was kept in refrigerator for 12 h. At the end of 12 h, the precipitate was collected by centrifugation at 12000xg for 15 min and precipitate thus obtained was discarded. To the supernatant, ammonium sulfate was added up to 80% saturation and the mixture was kept aside at 4°C for 4 h. Then the mixture was centrifuged at 15000xg for 30 min. The precipitate collected was dissolved in acetate buffer (25 mM, pH 5.0) and dialyzed for 12 h using acetate buffer (10 mM, pH 5.0). The dialyzate was used for further studies.

The *G. fujikuroi* was grown in a mineral salts medium and it contained the following composition: galactose, 20 g; $(NH4)_2NO_3$, 0.48 g; KH_2PO_4, 5 g and $MgSO_4$, 1 g per litre of distilled water. A trace elements solution (2 ml) was added and it contained $FeSO_4$, 0.1 g; $CuSO_4$, 0.015 g; $ZnSO_4$, 0.16 g, $MnSO_4$, 0.01 g and ammonium molybdate, 0.1 g in one litre of distilled water. To obtain conidia, cultures were grown on a special carbon-limiting medium. A conidial suspension was obtained by passing sterile distilled water over sporulated mycelia and 2 ml of conidial suspension (approximately $2x10^6$ micro conidia) was inoculated into 50 ml of the above medium. Batch, submerged fermentations were carried out in Erlenmeyer flasks (250 ml) at 25 ± 1°C and flasks were placed on orbital shaker (160 rpm) for 5 days. At the end of the 5th day, mycelia were collected by filtration through Whatman No.1 filter paper, washed twice with distilled water and stored at –5°C. Each pad was suspended in 0.2 M acetate buffer (pH 5.0) and disrupted with ultrasonicator (Sonics and Materials Inc. Danbury, Connecticut, USA) in an ice bath between 0-4°C for 1 min at full strength and repeated for six times. The sonicated sample was centrifuged at 10000 x g and 4°C for 20 min and the clear supernatant was used as enzyme source.

The strain *L. brevis* was grown in MRS broth without glucose and supplemented with 2% (w/v) maltose. The MRS broth had the following composition (g/l): peptone, 10; meat extract, 10; yeast extract, 5; maltose, 20; Tween 80, 1; KH_2PO_4, sodium acetate, 5; triammonium citrate, 2; $MgSO_4$ $7H_2O$, 0.2 and $MnSO_4$

$7HO_2$, 0.05. The ingredients were dissolved in distilled water; pH was adjusted to 6.3 before autoclaving and sterilized at 121°C for 15 min.

Batch submerged fermentation was carried out for the cultivation of *L. brevis* in Erlenmeyer flasks (250 ml) added with 50 ml of the medium. The flasks were inoculated with 24 h old grown *L. brevis* ($2x10^9$ colony forming units, cfu ml^{-1}). The flasks were incubated statically at 37°C for 60 h. The cells were harvested at the end of 60 h by centrifugation at 12000xg at 4°C for 15 min, and the cell pellet was twice washed with 50 mM McIlvaine buffer (Na_2HPO_4 and citric acid, pH 6.0). The cells were disintegrated using Ultrasonicator (Sonics and Materials Inc. Danbury, Connecticut, USA) at full strength in an ice bath for 30 seconds (repeated for 10 times). The cell lysate was centrifuged at 12000xg at 4°C for 30 min. The supernatant thus obtained after centrifugation was dialyzed against McIlvaine buffer (pH 6.0, 0.05 M) for 48 h with intermittent change in the buffer at every 12 h time intervals. At the end of 48 h of dialysis, dialyzate was centrifuged at 12000xg at 4°C for 1 h and the supernatant was used as crude α-galactosidase. For control experiment, buffer was added to red gram flour in place of enzyme.

Determination of enzyme activity: α-Galactosidase activity was determined by the method of Dey and Pridham (1972) with the following modifications. The reaction mixture consisted of 100 µl of 10 mM p-nitrophenyl-α-galactopyranoside (PNPG) in water, 800 µl of 0.2 M acetate buffer (pH 5.0) and 100 µl of suitably diluted enzyme. The reaction mixture was incubated at 37°C for 15 min, terminated by the addition of 0.2 M sodium carbonate solution (3 ml) and the liberated p-nitrophenol was measured at 405 nm. One unit of α-galactosidase activity is defined as the amount of enzyme required to release 1µmol of p-nitrophenol per min under the above conditions. Protein concentrations were determined by the method as described by Lowry *et al.* (1951) using BSA as standard.

Determination of optimal pH and temperature: The optimal pH for α-galactosidase activity was determined in the assay mixture over the pH range 2.0–7.5. The optimal temperature of enzyme activity was determined by incubating the assay mixture for 15 min at a temperature ranging from 10-80°C.

Treatment of red gram flour with crude microbial α-galactosidase: Red gram cultivars (Local-1 and Local-2) used in the present study were obtained from a local market. All samples of red gram used in the present study are grown in the year 2000-2001. The dried seeds were cleaned and any broken or immature seeds were removed. The seeds were stored in a plastic container at 37^0C until processing. The red gram flour (5 g) was taken in a 100 ml glass beaker and treated with 20 ml of crude α–galactosidase preparations. In control experiments, 20 ml of buffer was added to flour (5 g) in place of enzyme solution. The enzymatic treatment was carried out at optimum conditions of pH and temperature for 3 hr on an orbital rotary shaker (125 rpm). After incubation, the treated red gram paste were dried in a hot-air oven at 50°C for 36 h. Dried flour were then milled to flour in flourmill (400 µm sieve). Extraction, separation and estimation of oligosaccharides in the enzyme treated red gram flour was followed by the method of Tanaka *et al.* (1975).

Determination of oligosaccharide content: Standard oligosaccharides were purchased from Sigma Chemical, Co, St, Louis, MO, USA. All other reagents were of analytical grade. The oligosaccharides in the red gram flour were extracted, isolated and determined by the method of Tanaka *et al.* (1975). Five grams of flour were put in a 250 ml Erlenmeyer flask was mixed with 50 ml of 70% (v/v) aqueous ethanol. The flask was placed on orbital shaker (Steelmet Industries, India. 1994) 120 rpm for 12 hr. The content of the flask were filtered through Whatman No.1 filter paper. The filtrate was evaporated in a rotary vacuum evaporator (Heidolph, Laborota-4000, Germany) at 40°C. After evaporation, the residue was dissolved in 5 ml distilled water to obtain concentrated sugar syrup. Five microlitre of the syrup was loaded onto TLC-plates coated with cellulose powder G. The plates were kept for 4 hr in a chromatographic chamber containing n-propanol: ethyl acetate: water (6:1:3) as the solvent system. After development, the plates were sprayed with 1% a-nephthol in ethyl acetate containing 10% orthophosphoric acid to visualize sugar spots. The corresponding to the standards for raffinose, stachyose

and verbascose were scraped into a test tube and 2 ml of distilled water was added. The contents of the test tube were filtered through Whatman No.1 filter paper and 1 ml of filtrate was used to estimate the individual oligosaccharides according to the Tanaka *et al*. (1975).

RESULTS AND DISCUSSION

α-Galactosidase activity in different microbial sources is shown in Table 1. From the table, it is clear that *A. oryzae, G. fujikuroi* and *L. brevis* had a specific activity of about 1.52, 0.73 and 0.93 units ml^{-1}, respectively. The crude enzyme from *G. fujikuroi* had a lower total activity than those obtained for *A. oryzae* and *L. brevis*. The crude α-galactosidase from *G. fujikuroi* had a lower specific activity than that obtained for *A. oryzae* and *L. brevis*. Somiari and Balogh (1993) reported a specific activity of about 64 Units mg^{-1} proteins from *Aspergillus niger*. Mansour and Khalil (1998) reported α-galactosidase activity and total protein concentration in the culture filtrate obtained after the growth of *Aspergillu oryzae, Aspergillus niger* and *Cladosporium cladosporides*. They further reported that extracellular α-galactosidase from different fungal sources had an activity ranging from 130 to 289.34 units ml^{-1}. Garro *et al.* (1993) reported that different strains of *L. fermentum* had an activity ranging from 0.047 to 0.078 units. The levels of enzyme activity reported by Mansour and Khalil (1998) were higher than the values obtained in the present study and also those values reported by Garro *et al*. (1993) from different strains of *L. fermentum* (activity ranging from 0.047 to 0.078 units).

Table 1. α-Galactosidase activity of different microbial extract.

	A. oryzae	*G. fujikuroi*	*L. brevis*
Total activity (Unit ml^{-1})	0.67±0.10	0.56±0.09	0.67±0.11
Total protein (mg ml^{-1})	440±0.64	760±0.38	720±0.58
Specific activity (Unit mg^{-1})	1.52±0.05	0.73±0.03	0.93±0.07

Each value is average of triplicate determination, ± SD

The optimum temperature for α-galactosidase activity from different microbial source is shown in Fig. 1. α-Galactosidase from *A. oryzae, G. fujikuroi,* and *L. brevis* exhibited the optimum temperature at 55°C, 60°C, and 40°C, respectively. Cruz and Park (1982) reported that α-galactosidase from *A.. oryzae* exhibited an optimum temperature of 50°C for the hydrolysis of PNPG. Schuler *et al*. (1985) reported that α-galactosidase showed maximum activity at temperature 45°C. Somiari and Balogh (1993) reported that the optimum temperature of α-galactosidase activity was 50°C for *A. niger*. Garro *et al*. (1993) indicated that α-galactosidase from *L. fermentum* exhibited optimum temperature at 45°C for the hydrolysis of PNPG. Similar report have been reported by Mansour and Khalil (1998) for α-galactosidase from *C. cladosporides, A. oryzae* and *A. niger*. Ademark *et al.* (2001) reported that the multiple forms of α-galactosidases showed maximum activity at 60°C.

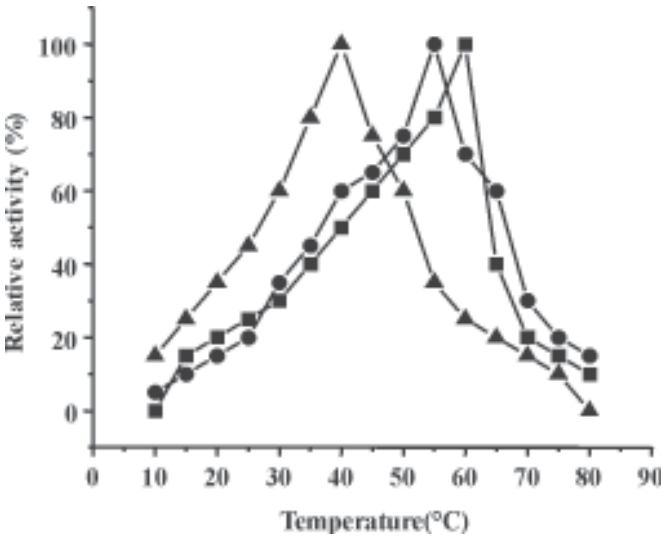

Fig. 1. Effects of temperature on the activity of different crude microbial α-galactosidase: (●) *A. oryzae,* (■) *G. fujikuroi* and (▲) *L. brevis*.

α-Galactosidase from different microbial sources is shown in (Fig. 2). *G. fujikuroi* showed an optimum pH 5.0. Similarly, α-galactosidase from *A. oryzae and L. brevis* showed an optimum pH at 4.8 and 5.0 respectively. Schuler *et al.* (1985) reported that the optimum pH of enzyme activity for *L. fermentii* was in the range 4.5-5.0 at 37°C and 5.5-6.2 at 50°C. Garro *et al*. (1993) reported that α-galactosidase from *L.*

fermentum showed an optimum pH between 5.2 and 6.0. Shibuya *et al*. (1997) reported that α-galactosidase exhibited the optimum pH between 3.5-4.0 for the hydrolysis of PNPG. Ademark *et al*. (2001) reported multiple forms of α-galactosidase (α-gal I-IV) from *A. niger* and all four α-galactosidases exhibited maximum activity at pH 4.5 when assayed for 10 min.

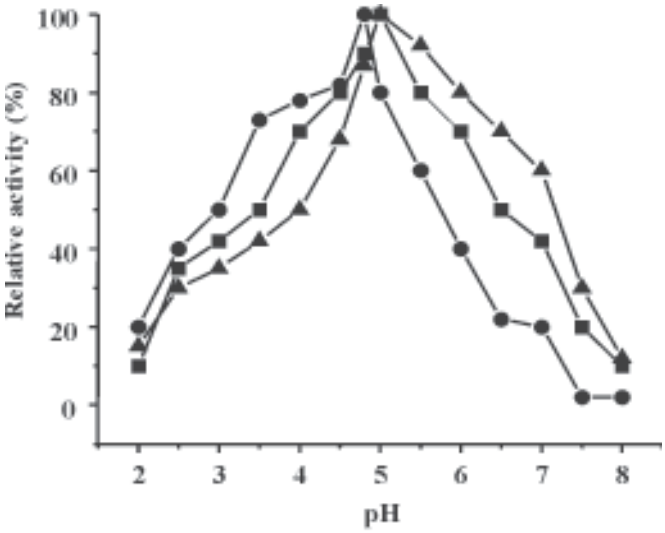

Fig. 2. Effects of pH on the activity of different crude microbial α-galactosidase: (●) *A. oryzae,* (■) *G. fujikuroi* and (▲) *L. brevis*.

α-Galactosidase treatment for 3 hr completely hydrolyzed the galacotosyl oligosaccharides, as evidenced by TLC analysis (Figure 3). From the figure it is clear that four spots are visible corresponding to authentic raffinose family sugars (A, B, C and D) (verbascose, stachyose, raffinose, and sucrose) were present in two cultivars of red gram (spot E and F) before α-galactosidase treatment. However, raffinose, stachyose and verbascose were completely degraded (spots G, H, I, J, K and L) after α-galactosidase treatment.

Crude α-galactosidase from *A. oryzae* and *G. fujikuroi* was effective in reducing the levels of raffinose family of sugars in two cultivars of red gram tested (Table 2). Incubation of α-galactosidase from *A. oryzae* and *G. fujikuroi* with red gram flour for 3 hr led to a completely reduction of raffinose, stachyose and verbascose. Processing of red gram flour with crude enzyme preparation from *A. oryzae* also led to a mean decrease of 45.49% for sucrose. The treatment of red gram flour with α-galactosidase from *G. fujikuroi* was

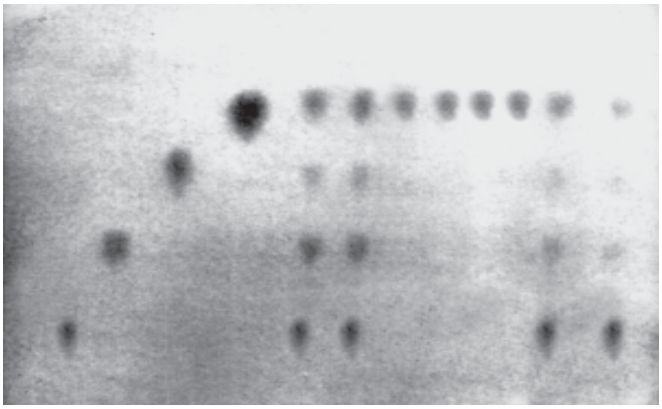

Fig. 3.Thin layer chromatographic pattern of separation of oligosaccharide from red gram flour after enzymatic treatment (30 ml). A. Verbascose, B. Stachyose, C. Raffinose, D. Sucrose, E. Local-1-before enzyme treatment, F. Local-2-before enzyme treatment, G. Local-1-after *A. oryzae* enzyme treatment, H. Local- 2-after *A. oryzae* enzyme treatment, I. Local-1-after *G. fujikuroi* enzyme treatment , J. Local-2- after *G. fujikuroi* enzyme treatment, K. Local-1-after *L. brevis* enzyme treatment, L. Local- 2-after *L. brevis* enzyme treatment

also effective for the removal of sucrose. The α-galactosidase treatment led to a mean decrease of 53.30% for sucrose.

Crude α-galactosidase from *L. brevis* extract treatment led to reduction in the levels of raffinose family sugars in two red gram varieties studied are given in Table 2. Reduction of stachyose was lower than that of raffinose and verbascose. Enzyme treatment from *L. brevis* led to a mean decrease of 70.20% for raffinose, 58.14% for stachyose and 71.56% for verbascose. Treatment of red gram flour with α-galactosidase showed incomplete reduction in the levels of raffinose, stachyose and verbascose. Crude extract from *L. brevis* also showed a mean decrease of 33.03% for sucrose.

Cruz and Park (1982) have reported that stachyose and raffinose was degraded by α-galactosidase and invertase into its constituents. Mulimani and Devindra (1998) have reported that hydrolysis of raffinose family sugars of red gram by α-galactosidse from *Casia ceresea*. There are several reports available in the literature on the use of α-galactosidase from plant and fungal sources for the removal of raffinose family sugars from soymilk and legume flours (Somiari and Balogh,

Table 2. Effect of α-galactosidase from *A. oryzae, G. fujikuroi* and *L. brevis* on the levels of α-galactosides and sucrose in red gram (g/100g dw basis)

Variety	Raw	Enzyme treated		
		A. oryzae	*G. fujikuroi*	*L. brevis*
		SUCROSE		
Local 1	2.44	1.34	1.22	1.68
Local 2	2.92	1.58	1.15	1.90
Mean±SD	2.68±0.33	1.46±0.16	1.18±0.04	1.79±0.15
		RAFFINOSE		
Local 1	1.26	ND	ND	0.40
Local 2	1.58	ND	ND	0.44
Mean±SD	1.42±0.22	ND	ND	0.42±0.02
		STACYOSE		
Local 1	1.72	ND	ND	0.86
Local 2	1.78	ND	ND	0.60
Mean±SD	1.75±0.22	ND	ND	0.73±0.18
		VERBASCOSE		
Local 1	4.80	ND	ND	1.60
Local 2	5.10	ND	ND	1.20
Mean±SD	4.95±0.21	ND	ND	1.40±0.28

Each value is average of triplicate determination, ± SD, ND: Not detected.

1993; Mulimani *et al* 1997). Somiari and Balogh (1993) have used crude preparations of α-galactosidase from *A. niger* for the removal of raffinose and stachyose content in cowpea flour. Mansour and Khalil (1998) have reported the use of α-galactosidase from *A. oryzae, A. niger* and *C. cladosporides* led to a total reduction in the levels of raffinose-family sugars in chickpea flour. Vladimir *et al*. (2000) have reported the use of an extracellular α-galactosidase from thermophilic fungus *Thermomyces lanuginoseus* is most effective aryl α-galactosides but also release galactopyranosyl residues from raffinose and stachyose.

Major limitation to the use of purified enzyme in bioprocessing is their high cost. The cost of bioprocessing can be brought down using crude enzyme preparation like fermentation broths for extracellular enzymes and cell homogenates or whole cells for intracellular enzymes.

From the results, it is concluded that crude α-galactosidase from *A. oryzae, G. fujikuroi*, and *L. brevis* hydrolyzed raffinose family of oligosaccharides in red gram flour, leading to removal of flatulence factors.

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