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ORIGINAL PAPER

Enzymatic removal of flatulence-inducing sugars in chickpea milk using free and polyvinyl alcohol immobilized α-galactosidase from *Aspergillus oryzae*

Aravind Goud G. Patil · Naganagoud V. Kote · Veerappa Mulimani

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Abstract The treatment of chickpea milk was carried out in batch, repeated batch and continuous reaction by soluble and polyvinyl alcohol (PVA) immobilized *Aspergillus oryzae* α-galactosidase for the removal of raffinose family oligosaccharides (RFOs). In the batch mode of treatment 96 and 92% of RFOs hydrolysis was observed by soluble and immobilized enzyme, respectively. In repeated batch experiments, immobilized enzyme showed 70% RFOs hydrolysis up to sixth cycle. Polyvinyl alcohol immobilized α-galactosidase in fluidized bed reactor showed highest reduction of 94% at a flow rate of 30 ml/h. The results obtained from the present study are very interesting for industrial use of PVA-immobilized enzyme.

Keywords Raffinose family oligosaccharides · α-Galactosidase · Fluidized bed reactor · Polyvinyl alcohol · Chickpea milk

Introduction

Legumes and cereals are good and relatively inexpensive source of proteins. Chickpea (*Cicer arientium*) is the fifth major important legume in the world on the basis of total grain product. Chickpea has one of the highest nutritional composition of daily edible legumes, on an average chickpea contain 23% of protein, 64% of total carbohydrates, 6% fats and some amounts of minerals such as calcium, magnesium, phosphorus, zinc and iron [3, 6]. Chickpea is mainly used for human consumption and small amount for animal feed, and is also used as herbal medicine, cosmetics. However, the presence of undesirable components (antinutritional factors) present in chickpea limits its wider use. Chickpea contains RFOs such as stachyose, raffinose, and small amount of verbascose. The human consumption of chickpea is hampered due to the lack of ability of the human intestinal tract to synthesize the enzyme α-galactosidase, which is necessary to hydrolyze oligosaccharides containing α-galactosidic linkages. The RFOs are relatively large molecules and are hence not resorbed by the intestinal wall. Hence the oligosaccharides enter the lower intestine; the microfloura present in the lower intestine hydrolyzed the oligosaccharides into carbon dioxide, hydrogen and, to lesser extent methane. Production of these gases which leads to flatulence with symptoms of nausea, cramps, diarrhea, abdominal rumbling, and social discomfort associated with the ejection of rectal gas [1]. Traditional food practices such as soaking, cooking, roasting, frying have been suggested by several investigators to reduce the level of galactosyl oligosaccharides in legumes [7, 12]. Unfortunately, none of the above methods were able to eliminate the RFOs completely from legumes. Enzymatic processing by α-galactosidase showed maximum extent of removal of RFOs from legumes [5, 8]. Although satisfactory hydrolysis was obtained by soluble enzyme, single usage of soluble enzyme appears uneconomical and processed chickpea milk has the possibility to contain unwanted protein. Immobilization of α-galactosidase leads to more economically viable process with added advantage of reusability, prevention of contamination, use in bioreactor and cost effectiveness, The poly-vinyl alcohol is non-toxic, biocompatible, low cost, highly stable, and resistant to biological degradation and its rehological property can be exploited to use in

A. G. G. Patil · N. V. Kote · V. Mulimani (✉)
Department of Biochemistry,
Gulbarga University, Gulbarga, Karnataka 585 106, India
e-mail: v_h_mulimani@rediffmail.com

bioreactors [4]. In the present investigation, we report the removal of RFOs content in chickpea milk by free and poly-vinyl alcohol immobilized α-galactosidase from *Aspergillus oryzae*.

Materials and methods

Chemicals

Galactose, sucrose, raffinose, stachyose and paranitrophenyl-α-D-galactopyranoside (PNPG) were procured from Sigma Chemicals; polyvinyl alcohol, sodium alginate, glutaraldehyde 25% (w/v), calcium chloride, boric acid were from S.D. Fine Chemicals, and all other chemicals used were of analytical grade (Qualigens and S. D. Fine Chemicals) Chickpea seeds were obtained from Indian Agricultural Research Institute (IARI), Gulbarga, India.

Fungal culture

The fungal culture *A. oryzae*, which is capable of producing extra cellular α-galactosidase, was isolated from soil sample [9]. It was maintained on PDA (potato dextrose agar) slants and stored at 4°C.

Preparation of ammonium sulphate precipitated α-galactosidase from Aspergillus oryzae

The active culture was produced through submerged cultivation of *A. oryzae* in a chemically defined medium, [9].The crude α-galactosidase was precipitated with 80% ammonium sulphate and kept overnight in 4°C. The precipitated α galactosidase was recovered at 12,085.2×*g* for 10 min at 4°C. The precipitate obtained was dissolved in a minimum amount of 0.2 M acetate buffer pH 4.8, dialyzed against deionized water.

Immobilization of α-galactosidase in polyvinyl alcohol

Polyvinyl alcohol 16% (w/v), sodium alginate 4% (w/v) were dissolved in sterilized distilled water and to this partially purified α-galactosidase known unit of enzyme was added and mixed thoroughly and then passed through a needle into a saturated boric acid solution with 2% (w/v) calcium chloride. The composite beads formed allowed to stand in the same solution for 48 h in 4°C to complete the solidification process. The beads were washed several times with sterilized distilled water. The formed beads were treated with 0.6% (w/v) glutaraldehyde at 4°C for 1 h. They were washed with sterilized distilled water to remove excess glutaraldehyde and kept in 0.2 M acetate buffer (pH 4.8).

Enzyme assay

α-Galactosidase assays were carried out according to Dey and Pridham [2]. One milliliter of reaction mixture contained 0.1 ml suitably diluted enzyme + 0.8 ml of 0.2 M sodium acetate buffer (pH 4.8) + 0.1 ml of 2.0 mM PNPG and was incubated at 37°C for 15 min. The reaction was arrested by adding a 3 ml of 0.2 M sodium carbonate solution, and the absorbance was read at 405 nm in a spectrophotometer (Elico Ltd). The immobilized enzyme was assayed in the same manner as that of soluble enzyme except that appropriate quantity of immobilized beads were used in place of soluble enzyme for assay. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μmol of para-nitrophenol from PNPG per minute under standard assay conditions.

Preparation of chickpea milk

Chickpea were ground to flour and defatted with hexane (1:1 w/v) the fat free soybean flour was then suspended in 15 volumes of distilled water and heated to boiling. The undissolved residue were separated from the chickpea milk by centrifugation for 5 minutes at 5,000 rpm. The supernatant which contains the chickpea milk was then stored at 4°C for short period until further use.

Estimation of oligosaccharides

Chickpea milk (15 ml) was poured into 35 ml of absolute ethyl alcohol and centrifuged at 1933.63×*g* for 15 min at 37°C. The centrifugate was concentrated and dissolved in 15 ml of distilled water. The amount of sucrose, raffinose and stachyose was estimated according to the method of Tanaka et al. [11].

Separation of oligosaccharides by HPLC

HPLC analysis were performed with a Shimadzu (Shimadzu Corporation) equipped with Lc 10 ATVP pump and refractive index detector. Sample injection was via Rheodyne injector equipped with 20-μl sample loop. Carbohydrates were separated on a phenomenex Bond 120 clone 10-μ CHO column The mobile phase consisting of acetonitrile:water (70:30, v/v) was used for separation. Flow rate was fixed at 1 ml/min. Chromatographic data were collected and plotted using class VP-6.1 software. Peak identification of the chromatographs was done by comparing the retention times with those of standards.

Treatment of chickpea milk by free and immobilized enzyme batch reaction

The batch reactions were performed (in triplicate) for both immobilized and soluble enzyme at different incubation periods. For the reaction involving soluble enzyme, around 10 ml of enzyme (5.21 U ml^{-1}) were added to 60 ml of chickpea milk in an Erlenmeyer flask (250 ml). For the immobilized enzyme (equivalent to activity of free enzyme), beads were added to 60 ml of chickpea milk. The hydrolysis reaction was carried out at 50°C in an incubator shaker (200 rpm) for different incubation periods of 3, 6, 9 and 12 h. After the incubation period, an aliquot of the reaction mixture was taken out. For the reaction involving soluble enzyme, reaction mixture was kept in a boiling water bath for 10 min to arrest the enzyme reaction. Afterwards the sample was analyzed for degradation of oligosaccharides. Control experiments were performed in the same manner with 0.2 M acetate buffer (pH 4.8) replacing the enzyme.

Repeated batch reactions were carried out (in triplicate) for immobilized enzyme at 50°C for 6 h incubation period. Chickpea milk was taken out and oligosaccharides concentration was determined. The beads were separated from filtration, washed with sterile distilled water and transferred into fresh batch of chickpea milk (60 ml) for 6-h incubation. The reaction was carried out under similar conditions for six repeated uses.

Design of fluidized reactor for the continuous degradation of raffinose family oligosaccharide sugars in chickpea milk

Fluidized bed reactor studies were carried out in jacketed glass column of 75 cm in length and 1.5 cm in diameter, with a bed volume of 150 ml and the void volume of 40 ml, respectively. The jacket temperature was maintained at 50°C using a water bath (Julabo). The chickpea milk feed solution, preheated at 50°C in water bath, was introduced from the bottom of the column through a peristaltic pump (Amersham Pharmacia Biotech) and the product was withdrawn from the top the column. α-Galactosidase immobilized on polyvinyl alcohol beads was packed in a column. The upward substrate stream fluidized the beads filled up in the column. Different flow rates of 30, 60, 90, 120 and 150 ml h^{-1} were used for the hydrolysis of oligosaccharides in chickpea milk. The outlet stream was continuously collected from the front end of the column in a container. The effluents were analyzed for the hydrolysis of oligosaccharides raffinose and stachyose.

Results and discussion

Chickpea milk initially contained 680 mg of total galactooligosaccharides per 100 ml, out of which 340 mg verbascose plus stachyose and 270 mg of raffinose were the main constituents. The crude α-galactosidase was subjected to 80% ammonium sulphate precipitation. The precipitate was dialyzed to remove salts. Partially purified *A. oryzae* α-galactosidase was physically entrapped in polyvinyl alcohol cross-linked with boric acid and treated with glutaraldehyde. The soluble and PVA-immobilized enzyme were incubated at 3, 6, 9, and 12 h, 3 and 6 h incubated soluble enzyme resulted in 82 and 86% hydrolysis of RFOs, whereas PVA-immobilized enzyme showed 70 and 82%, respectively. After 9 and 12 h of incubation the soluble enzyme resulted 88 and 96% hydrolysis of RFOs, whereas PVA-immobilized α-galactosidase showed 86 and 92% reduction of RFOs. Somiari and Balogh [10] have reported a reduction in the raffinose and stachyose content of 95 and 82%, respectively, by *A. niger* soluble α-galactosidase in cowpea flour. Mulimani and Ramlingam [8] have reported hydrolysis of 91% raffinose and 82% stachyose plus verbascose using *G. fujikuroi* crude α-galactosidase in chickpea flour. The operational stability of polyvinyl alcohol immobilized α-galactosidase was evaluated in repeated batch process and the enzyme was able to maintain good oligosaccharide reduction. The immobilized enzyme retained its well catalytic activity and leakage of entrapped enzyme was evidently not serious on subsequent uses, i.e., first, second, third, fourth, fifth, and sixth cycles the percent hydrolysis was found to be 88, 86, 82, 78, 76 and 70%, respectively. There was no drastic decrease in percent hydrolysis even after six uses. This could be due to boric acid treatment of polyvinyl alcohol beads, which prevent the leakage of enzyme. The use of an immobilized enzyme makes it economically feasible to operate in a fluidized bed reactor, which was designed to hydrolyze the RFOs present in chickpea milk by using PVA-immobilized α-galactosidase. Figure 1 shows the hydrolysis of RFOs in chickpea milk at different flow rates. At lower flow rate (30 ml/h) percentage reduction of RFOs was higher (94%) but at higher flow rate (150 ml/h) operational difficult was observed. Optimum flow rate is one of the important parameter for effective operation in a fluidized bed reactor. The polyvinyl alcohol immobilized α-galactosidase beads

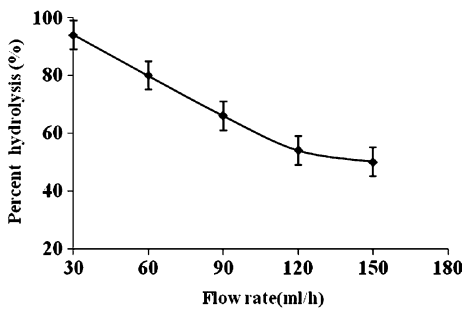

Fig. 1 Effect of flow rate on hydrolysis of RFOs in chickpea milk

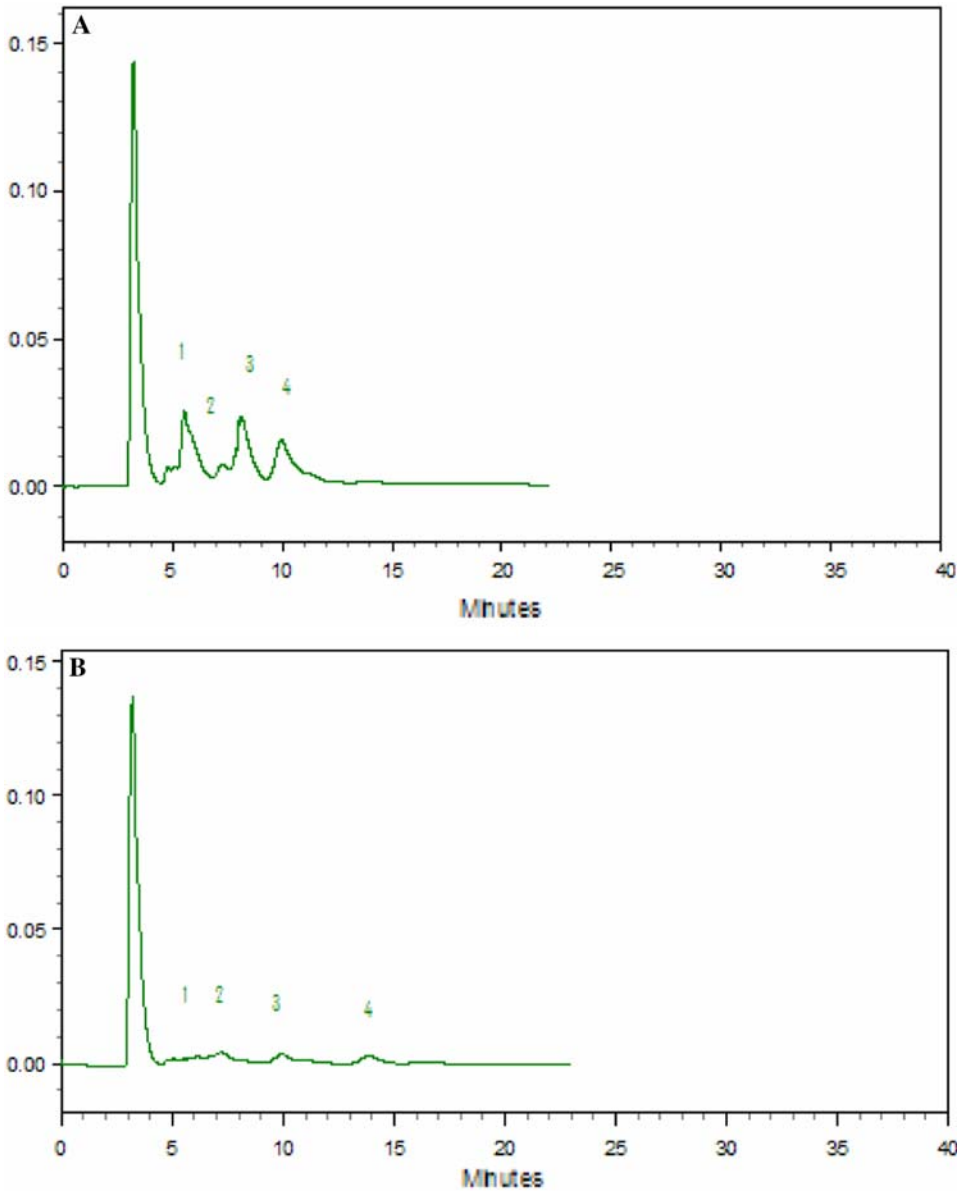

Fig. 2 HPLC separation of oligosaccharides from chickpea milk. **a** Raw chickpea milk: *1* sucrose, *2* raffinose, *3*, Stachyose, *4* verbascose. **b** PVA-immobilized α-galactosidase treated chickpea milk: *1* sucrose, *2* raffinose, *3* stachyose, *4* verbascose

were used, eight repeated uses in fluidized bed reactor without considerable decrease in its percentage hydrolysis (data not shown). Sucrose, raffinose, stachyose and verbascose were separated by HPLC and their retention time was compared with standards. The retention time of raw chickpea milk (Fig. 2a) and the enzymatic hydrolysis of HPLC pattern (Fig. 2b) confirmed that there is considerable decrease in RFOs content in chickpea milk.

Conclusions

The removal of RFOs present in chickpea milk showed 96% after 12 h incubation with α-galactosidase from *A. oryzae*. The organism *A. oryzae* is regarded as safe to use in food processing. The immobilization of α-galactosidase and its subsequent use is comparatively safe, simple, and cheap with durable enzyme activity; well suited for immobilization of α-galactosidase and its utilization in fluidized bed reactor for removal of RFOs in chickpea milk. The results obtained from our studies are of considerable interest for industrial purposes.

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