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Research Article

Purification, Characterization and Immobilization of an acid stable invertase from *Lagenaria siceraria* stem for the Production of Invert Syrup

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Abstract

A 67 kDa acid invertase (specific activity = 780 U/mg protein), appreciably present in Lagenaria siceraria stem (2,600 ± 200 U/100 g fresh wt.; 17,500 ± 300 U/100 g of lyophilized stem powder) was purified by $(NH_4)_2SO_4$ precipitation, ion exchange chromatography (DEAE Sephadex A50), size exclusion chromatography (Sephacryl S-300-HR) and HPLC. The enzyme was optimally active in the pH range of 4-5 (retaining 52 ± 1 % of its activity at pH 3) and stable in the pH range of 3-7 (retaining 37 ± 1% and 53 ± 1% of its activity at pH 2 and 8 respectively), up to 55°C, with a K_m of 5.84 mM (2 mg/ml) sucrose. Hg^{2+}> Ag^{+}>Cu^{2+}>Pb^{2+}>Cd^{2+} inhibited the enzyme activity. DTNB, iodoacetic acid, iodoacetamide, N-ethylmaleimide did not affect the activity suggesting the non-thiol nature of the enzyme. The enzyme hydrolyzed sucrose and raffinose, could slightly hydrolyze inulin, but was completely inactive upon maltose, levan, melezitose and trehalose. 8 U/ml of acid invertase almost completely hydrolyzed 10% (w/v) sucrose solution to invert syrup in 5 h at 50°C. Immobilization of the enzyme on oxidized bagasse (dialdehyde cellulose) increased its temperature optima (by 10°C) and thermo-stability (retaining 41 ± 1% and 30 ± 1 % of its activity at 70°C and 80°C respectively). Immobilized enzyme system efficiently produced invert syrup from sucrose, remaining 83 ± 1 % and 72 ± 1 % active after 15th and 25th cycles respectively.

Keywords: Lagenaria siceraria; Acid invertase; Sucrose; Invert syrup; Cellulose-dialdehyde

Introduction

Invertase (EC 3.2.1.26) or β-fructofuranosidase catalyses the hydrolysis of sucrose into an equimolar mixture of glucose and fructose known as invert syrup (non-crystallizable sugar syrup), which, because of its sweetness (is sweeter than sucrose due to high degree of sweetness of fructose), liquid form and hygroscopic properties is widely used in food (as fondant filling for chocolates, confectioneries, jams and candy making, artificial honey), beverage (in soft drinks) and pharmaceutical industries (Kulshrestha et al., 2013) [14]. The production of non-crystallizable sugar syrup from sucrose is one of the major applications of invertase enzyme. Alcoholic beverage manufacturers often add invert sugar (distillery grade) in the production of drinks like gin, beer and sparkling wines for flavoring. The most successful commercial invertase is derived from Baker's yeast.

Though phyto-enzymes in food industry offer new means to address energy savings and increase in efficiency and sustainability, yet they have not been seriously commercialized except that of malt amylase (from barley), papain and bromelain. Production of enzymes from microbial sources requires maintained upstream process technology, which is cost-effective, but no such technology is required for extracting plant enzymes. The plant source itself acts like a bioreactor producing the enzyme, which needs only to be harvested and extracted. Plant parts such as stems, producing appreciable amount of extracellular enzymes, are a cheap source, as these renewable parts can be harvested time to time (Mukherjee et al., 2012) [21].

Lagenaria siceraria (Molina) Standley (Family: Cucurbitaceae), commonly known as bottle gourd, is an annual herbaceous climbing plant, growing throughout the Indian subcontinent, and used since the ancient days both as vegetable food and traditional medicine

in the tropical and subtropical regions. The fruits, leaves, stem and seeds are edible and used by local people as folk medicines in the treatment of jaundice, diabetes, ulcer, piles, colitis, insanity, hypertension, congestive cardiac failure, and skin diseases (Prajapati et al., 2010) [25]. The plant is reported to possess immunomodulatory, hepatoprotective, cardioprotective, antioxidant, anti-stress and adaptogenic, antihyper lipidemic, analgesic, and anti-inflammatory properties (Ahmed et al., 2011) [2]. The plant shoot (stem and leaves) is known as 'lauki ka saag' in northern India and as 'lau saak' in eastern India and is very much used in culinary purpose. A wide range of chemical compounds including sterols, terpenoids, flavonoids, and saponins have been isolated from the species (Ahmed et al., 2017; Prajapati et al., 2010) [1, 25]. *Lagenaria siceraria* seeds are good sources of dietary proteins, crude fat and nutritionally important minerals (Ogunbusula, 2018) [23]. However, till date, no reports are available on the presence of macromolecules or enzymes from the plant stem. This is the first report of the presence of appreciable amount of any enzymatic activity (invertase) in the stems of *Lagenaria siceraria*.

It was recently reported by us that the medicinal herb*, Tinospora cordifolia* exhibits appreciable sucrase, maltase, and lactase activities (Mukherjee et al., 2012; Sengupta et al., 2013) [21, 26] which can be used as a remedy for disaccharide intolerance (Sengupta et al., 2013) [26]. The present study reports that the medicinal plant *Lagenaria siceraria* contains substantial amount of acid stable invertase activity (2600 ± 200 U /100 g fresh wt.; 17,500 ± 300 U/100 g of lyophilized stem powder).

Materials and methods

Chemicals

Sucrose, dinitrosalicylic acid (DNSA), bovine serum albumin (BSA, Fraction-V), DEAE-Sephadex (A50), bradford reagent, pepsin, trypsin (porcine pancreas), chymotrypsin (porcine pancreas), proteinase-K, maltose, raffinose, inulin (chicory root), melezitose, levan, trehalose were all purchased from Sigma chemicals, (St Loius, MO, USA). N-ethylmaleimide (NEM), p-chloromercuri benozoate (PCMB), iodoacetic-acid, iodoacetamide, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide-hydrochloride (EDAC) was purchased from Sigma Chemicals, (St Loius, MO, USA). 5, 5'-Dithiobis-2-nitrobenzoic acid (DTNB) was a product of SRL. Sephacryl S-300-HR was a product of Sigma chemicals. Electrophoresis and gel filtration standard marker protein kits were purchased from HiMedia, USA and Pharmacia Fine Chemicals, (Uppsala, Sweden) respectively. All other chemicals used were of chemically pure grade.

Extraction of enzyme

Cleaned and washed *Lagenaria siceraria* stems (800 g) were chopped into 1-2 cm long pieces and were immediately dropped in 300 ml of buffer (0.1 M acetate buffer, pH 5.0) kept at 37°C. The mixture was blended for 5 min in a warring blender and the biomass was squeezed through a nylon cloth to collect the extract (1000 ml). The extract was centrifuged at 10,000 rpm at 4^0C and the clear supernatant obtained (970 ml) was used as the source of crude enzyme.

Determination of enzyme activity

Plant invertase activity was determined in 0.1M acetate buffer, pH 5.0 at 50°C using 2 % (w/v) sucrose as substrate. Reducing sugars formed were estimated by dinitro salicylic acid reagent (DNS) method (Sengupta et al., 2000). One unit of enzyme activity was taken as the amount of enzyme that could produce 1 µmole of reducing sugar/min under the above assay conditions (Mukherjee et al., 2010) [21].

Maltase and isomaltase, activities were determined using 0.5 % (w/v) each of maltose and isomaltose respectively, as substrates, and total glucose formed was estimated by glucose oxidase–peroxidase (GOD-POD) reagent method (Bergmeyer, 1983; Sengupta et al., 2013) [5, 27]. Protein was determined by coomassie brilliant blue protein assay reagent (Bradford, 1976) [7] using bovine serum albumin as standard and total carbohydrate was estimated by anthrone reagent (Ashwell, 1957) [4].

Determination of storage stability of Lagenaria siceraria as a source of invertase

Determination of the storage stability of the plant invertase was done in the following ways: (1) Fresh plant stem (400 g) were

chopped into pieces (10-12 cm long) and stored in an air tight container at 0-4°C. (2) 400 g of fresh plant stem was cut into small pieces (2-3 cm long) and the biomass lyophilized to dryness and finally blended to obtain dry powder (50 g) which was stored separately (25 g each) in air tight containers at room temperature and at 0-4°C. (3) 400 g of fresh plant stem was cut into small pieces, oven dried for 24 h at 40°C, ground into fine powder, and stored at room temperature in airtight containers; (4) 400 g of fresh plant stem were chopped and enzyme was extracted (480 ml) using 0.1 M acetate buffer, pH 5.0 as mentioned earlier. 240 ml of the extract was lyophilized to dryness and the dry powder stored in an air-tight container at 0-4°C; (5) Rest of the extract (240 ml) was stored in cold (0-4°C). Initial enzyme activities present in the stored plant materials were taken as 100 and residual enzyme activities were determined subsequently at fixed time intervals till 12 months of the study by withdrawing a fixed amount of the stored material.

Purification of the enzyme

The plant extract (800 ml) was concentrated to 200 ml by ultra filtration using PM-10KDa membrane (Sartorius, Germany) and further concentrated to 100 ml by lyophilization. The enzyme was precipitated by adding ammonium sulfate (90% saturation). The sample was then kept overnight at 4°C, centrifuged at 10,000 rpm for 10 min, the precipitate obtained was dissolved in 0.05 M acetate buffer, pH 5.0 (10 ml) and finally dialyzed against the same. The enzyme solution was further concentrated by lyophilization (10 ml) and was applied on to a DEAE-sephadex (A-50) column (3 cm X 25 cm) previously equilibrated with 0.05 M acetate buffer, pH 5. After passing 4 bed volumes of the same buffer, a 0-1 M NaCl gradient was used. The active fractions (2 ml each) were collected, pooled and lyophilized.

Concentrated enzyme solution (0.8 ml) containing 4 mg of protein was charged each time on a sephacryl S-300-HR column (1.5 cm X 70 cm), pre-equilibrated with 0.05 M acetate buffer, pH 5.0. The column was eluted with the same buffer and eluted fractions (1.5 ml each) were assayed for invertase activity and protein content. Active fractions were pooled, and lyophilized and further subjected to HPLC. A sample of 20 µl (0.100 mg protein) was applied to BioSep-SEC-S 2000 column (300 mm X 7.8mm), pre-equilibrated with 0.1M phosphate buffer, pH 6.8. The column was eluted with same buffer at a flow rate of 0.5 mL/min. The column was pre-calibrated with pure molecular weight marker proteins (Pharmacia). Purified protein sample (40 µg) was subjected to denaturing SDS–PAGE carried out at pH 8.3 according to the method of Laemmli, (1970) using 12.5 % gel and 1% (w/v) SDS in the LKB Midget unit (10 cm x 10 cm x 1.5 mm slab). Protein molecular weight markers (Broad range; HiMEDIA) also were run parallel in SDS–PAGE. The gels were also subjected to Periodic Acid-Schiff (PAS) stain.

Determination of pH and thermal stability

The pH optimum of *Lagenaria* invertase was determined in the pH range of 3.0 to 9.0. The buffer systems used were 0.1M glycine-HCl (pH 3.0), 0.1M acetate buffer (pH 4.0 and 5.0), 0.1M phosphate buffer (pH 6.0 and 7.0), 0.1 M Tris-HCl (pH 8.0), 0.1M glycine-HCl (pH 9.0). The reaction mixtures (2 ml) containing 4.0 U of invertase was incubated with 2 % (w/v) sucrose at 50°C for 15 min in buffer of different pH values. The enzyme activity was determined as described earlier.

The pH stability of enzyme was determined by incubating a mixture containing 10 U/ml of plant invertase in different pH buffers (pH 2.0 – pH 9.0) at 37°C for 3 h. Residual enzyme activity was determined after dilution of the enzyme in optimum pH buffer (0.1M acetate buffer, pH 5.0).

The optimum temperature for plant invertase was determined in the temperature range of 20-90°C. In the reaction mixture (2 ml), 5 U of enzyme was incubated with 2% (w/v) sucrose in 0.1M acetate buffer, pH 5.0 at different temperatures for 15 min. Temperature stability was determined in the range of 20-90°C. The incubation mixtures containing 12 U/ml of invertase in 0.1M acetate buffer, pH 5.0 were pre-incubated at different temperatures for 60 min. Fixed aliquots were withdrawn at different time intervals, and residual enzyme activities determined.

Effect of different chemical modifiers on enzyme activity

In different incubation mixtures (1 ml), 20 U of purified invertase was pre-incubated at 37°C with iodoacetic acid (50 mM), iodoacetamide (50 mM), PCMB (1mM), DTNB (5 mM), water soluble carbodiimide (50 mM) and PMSF (2mM) at their respective recommended pH buffers (Mukherjee et al., 2010) [21] Inactivation kinetics was monitored till 3 h of incubation.

Substrate specificity and kinetics of sucrose and raffionse hydrolysis

The activity of purified invertase on various substrates (sucrose, maltose, raffinose, melezitose, trehalose, inulin, levan, p-nitrophenyl-a-D-glucopyranoside, isomaltose) was determined in 0.1 M-acetate buffer, pH 5.0 at 50°C. The kinetics of sucrose and raffinose hydrolysis was studied in terms of liberation of reducing groups. Reaction mixtures (20 ml) containing 1% - 10% (w/v) substrate (sucrose and raffinose) in 0.1 M-acetate buffer, pH 5.0 and 20 - 200 U of purified invertase were incubated at 50°C for 5 h. Fixed aliquots were withdrawn at different time intervals and the amount of reducing sugar was estimated as mentioned earlier. Glucose was estimated by GOD POD reagent method as mentioned in 2.1. Lineweaver–Burk plot for the determination of K_m value was prepared by measuring the enzyme activity for increasing substrate (sucrose) concentration followed by regression analysis.

Effect of various metal ions on purified amylase

Incubation mixtures (1 ml) containing 5 U of enzyme in 0.1M acetate buffer, pH 5.0 were pre-incubated separately for 10 min in presence of different metal ions (Hg^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Cd^{2+}, Ba^{2+}, Cu^{2+}, Ag^{+}, Pb^{2+} and Mn^{2+}) of 1mM and 2 mM concentration at 37°C. Reaction was initiated with the addition of 1ml of 2% (w/v) sucrose and the incubation continued for 15 min at 50°C. The residual enzyme activities were subsequently determined.

Effect of proteolytic enzymes on enzyme stability

Reaction mixtures (1 ml), containing 20 U of purified invertase was incubated separately with different proteolytic enzymes, *viz.* pepsin [150 U/ml], trypsin [200 U/ml], chymotrypsin [132 U/ml], papain [100 U/ml] and proteinase K [40 U/ml] in their respective buffers (0.1 M glycine-HCl buffer, pH 3 for pepsin; 0.1M phosphate buffer, pH 7.0 for trypsin, chymotrypsin and proteinase K; 0.1 M acetate buffer, pH 5.5 for papain) for 120 min at 37°C. Suitable 'control' sets were maintained where the same amount of purified enzyme was incubated in presence of the above mentioned buffers in the absence of proteolytic enzymes. Fixed aliquots were withdrawn from each of the incubation mixtures and residual enzyme activity was subsequently determined.

Immobilization of Lagenaria sisceraria invertase on bagasse dialdehyde cellulose

Processing of bagasse for enzyme immobilization

Bagasse collected from the local market was purified according to the method of Varavinit *et al.,* (2002) [34]. 500 g of bagasse was boiled thrice in distilled water (0.5 L each time) for 1 h followed by drying the fibers in a hot air oven (60°C). Dried fibers were cut into small pieces (0.1 - 0.3 cm long). 70 g of dry bagasse fibers (per batch), was boiled alternately in 1.5 L of 1.25 % (v/v) sulphuric acid and 1.5 L of 1.25 % (w/v) sodium hydroxide for 1 hour each time, followed by thorough washing with distilled water and finally with 500 ml of 95% (v/v) ethanol. The biomass was then dried in hot air oven at 70 °C for 3 h to obtain purified bagasse.

Oxidation of bagasse

Purified bagasse was oxidized according to the method of Varavinit et al., (2002) [34]. 50 g of purified bagasse obtained from the above step was soaked in 1 L of 0.03 M periodic acid. The pH of the solution was adjusted to 3.0 (using dilute sulphuric acid), and kept in a water bath at 90°C for 15 h with constant stirring. The bagasse was then filtered, washed thrice with distilled water (3 X 1000 mL) and finally dried and stored in air tight containers. The amount of reducing groups (aldehyde) released from cellulose by the periodate oxidation was estimated by DNSA reagent, using 20 mg of purified bagasse (dry weight), either oxidized or un-oxidized (control).

Immobilization of Lagenaria invertase

400 ml of crude concentrated enzyme solution (60,000 ± 2,000 Units) in 0.1M acetate buffer, pH 5.0 was coupled with 25 g cellulose-dialdehyde (oxidized bagasse) in five steps. Initially, 5 g of dried oxidized-bagasse was immersed in the above mentioned enzyme solution (400 ml) and incubated at 45°C for 30 min with constant shaking. The treated bagasse was then vacuum filtered, and the filtrate containing un-immobilized enzyme (residual enzyme) was collected to be used in the next step. Vacuum filtered treated bagasse was washed twice in distilled water, press-dried on a blotting paper and finally stored at 4°C in 0.1M acetate buffer, pH 5.0. This process was repeated five times till the entire bagasse biomass (25 g) was utilized; immersing 5 g of dry oxidized bagasse each time in the drained un-immobilized enzyme solution recovered from the previous step. Immobilized invertase activity was determined and the activity yield was expressed by the following equation (Varavinit et al., 2002).

$$\text{Activity yield (\%)} = 100 \times \frac{\text{Activity of the Immobilized enzyme}}{A - B} \quad \text{(Eq.1)}$$

Where, A is the activity of free enzyme added, and B is the total activity of unused enzyme (un-immobilized enzyme at the end of each step + enzyme washed out with water).

Assay of immobilized enzyme activity

Immobilized invertase activity was determined by adding 40 mg of bagasse-immobilized enzyme (dry weight) into 20 mL of 4% (w/v) sucrose solution in 0.1M acetate buffer, pH 5.0 at 50°C with constant shaking for 15 min. The reaction was stopped by removing the bagasse from solution. Enzyme activity was expressed in terms of µ mole of reducing sugar released /min as determined by the DNSA method.

Kinetics of sucrose hydrolysis by the immobilized invertase was determined similarly using 5-10% (w/v) sucrose solution (40 ml). Aliquots withdrawn at different time intervals were assayed for reducing sugars and glucose liberated during the incubation period (5 h). Results were expressed in terms of mg reducing sugar liberated with time (min).

The enzyme-immobilized bagasse system was used for 25 successive cycles (each cycle of 5 h duration) of sucrose hydrolysis (10% w/v sucrose in 0.1 M acetate buffer, pH 5.0) and the efficiency of the immobilized-enzyme system was determined after every cycle of operation.

Determination of the thermostability of the immobilized enzyme

Temperature optimum of the immobilized enzyme system was determined (in the range of 20°C - 90°C) by adding 0.1 g of enzyme immobilized bagasse in 20 ml of 2% (w/v) sucrose solution in 0.1 M acetate buffer, pH 5.0. Thermo-stability was determined in the range of 20°C - 90°C. The incubation mixtures containing immobilized enzyme (0.16 g in 5 ml of 0.1M acetate buffer, pH 5.0) were pre incubated at different temperature for 1 h. The incubation mixtures were quickly equilibrated to 50°C, followed by addition of 5 mL of 4% (w/v) sucrose solution. Residual enzyme activities were determined as mentioned earlier.

Statistical analysis

Triplicate sets of experiments were conducted (for purification, kinetics of sucrose hydrolysis, determination of various physico-chemical properties, determination of yield and efficacy of enzyme immobilization etc) and their mean values calculated. Standard deviation was also determined and the results were expressed either as the average (mean) value or as mean ± SD.

Results and discussion

Yield and storage stability

Invertases occur in many plant tissues and play an important role in sucrose metabolism, translocation and storage (Walker et al., 1997) [36]. Two types of invertases are recognized on the basis of their pH optima. (a) Neutral or alkaline invertase (location- cytosol; non-glycosylated) (Stommel and Simon, 1990; Chen and Black, 1992) [28, 8]. (b) Acid invertase - located in the apoplast and vacuole and is glycosylated (Wagner and Wiemken, 1987) [35]. Much progress has been made in understanding of the protein structures of both alkaline (Chen and Black, 1992) and acid invertases (Sturm and Chrispeels, 1990; Unger et al., 1994) [17, 32]. Though invertase activity has been reported in plants, like potato tuber (Bracho and Whitaker, 1990), [6] carrot (Lee and Sturm, 1996), [17] mung bean (Lee et al., 1998), [16] apple fruit (Pan et al., 2005), [24] cherry fruit (Krishnan and Pueppke, 1990), [23] chicory root (Van Den Ende and Van Laere, 1993.), [33] *Tinospora cordifolia* stem (Mukherjee et al., 2012; Sengupta et al., 2013) etc., [22, 27] yet, except in *Tinospora cordifolia* (4500±500 U/ 100 g fresh stem) the presence of appreciable amount of enzyme has not been reported till date.

Lagenaria siceraria stem gave an appreciable yield of acid stable invertase. Fresh plant stem contained 2600 ± 200 Units of invertase /100 g (when extracted with 0.1 M acetate buffer, pH 5.0 at 37°C). Lyophilzed stem powder yielded 17,500 ± 300 U/100 g, while lyophilized aqueous extract yielded as high as 85,000 ± 1,000 U/100 g powder. Oven-dried and powdered stem pieces gave a low yield (9,500 ± 300 U/100 g).

Lagenaria siceraria is a well-known vegetable food plant used both in culinary purpose and herbal medicine over thousands of years throughout the Indian subcontinent. In the process for the preparation of commercial enzyme grades from the plant stem, the enzyme extraction protocol was optimized. The optimum extraction buffer was found to be 0.1M acetate buffer, pH 5.0. The raw material i.e., fresh plant stem was found to lose activity quickly on storage either in cold or at room temperature (Fig. 1) but that was excellently prevented by prior drying in cold (lyophilization) of the chopped stem pieces followed by preservation at cold (0-4°C) or at room temperature, retaining 94 ± 2 % of its original activity till 12 months of the study (Fig. 1). Similar result was observed in the case of aqueous stem extract lyophilized and stored at 0-4°C (Fig.1). On the other hand, enzyme extracted from fresh plant (aqueous extract) and stored at 0-4°C remained considerably stable, retaining 91 ± 2 % and 75 ± 2 % of its activity till 6months and 12 months respectively (Fig 1). Oven dried plant stem pieces, powdered and stored at cold, retained almost about 63± 2 % of its activity (till 12 months). It must be mentioned here that fresh stem pieces of *Tinospora cordifolia*, whether stored in cold or at room temperature, was also found to lose its enzyme activity quickly (Mukherjee et al., 2012) [22].

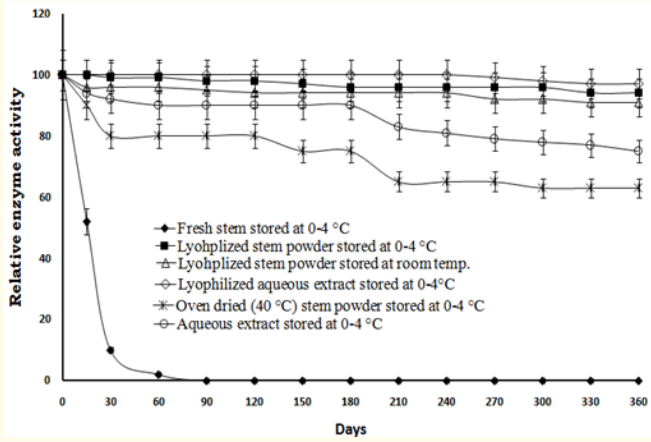

Figure 1: Storage stability of raw material. The plant stem and the extracted enzyme were stored in different ways (the details of which are mentioned in the text). Initial enzyme activities of the stored materials were taken as 100 and relative enzyme activities were determined subsequently at fixed time intervals till 12 months of the study by withdrawing a fixed amount of the stored material. The data represents the mean ± SD value of the triplicate sets of experiments.

Purification

The enzyme solution (aqueous extract) was initially concentrated by ultra filtration and lyophilization, without causing any significant loss of activity and recovering about 95% of the total enzyme activity (Table 1). Protein recovered by 90% ammonium sulphate saturation had a slight higher specific activity and loss of enzyme activity was about 20% (Table 1). The subsequent ion exchange chromatography on DEAE-sepahdex (A-50) column increased the specific activity to almost 5 times (Table 1). Gel filtration chromatography of the enzyme preparation on Sephacryl S-300-HR column indicated presence of a non-sucrase protein contamination eluted separately in lower molecular weight range (Fig. 2), thus increasing the specific activity of the enzyme. The purified enzyme was recovered to the extent of 44.5% of the original activity with a specific activity of 684.6 U/mg of protein (Table 1). HPLC of the active fraction on BioSep-SEC-S 2000 column showed a single peak and the native molecular weight was found to be 67 KDa (Fig. 3). The enzyme was homogenous in Native PAGE and gave a single band in SDS PAGE (Fig. 4). Both the bands gave positive response to PAS staining, suggesting it to be a glycoprotein. It must be mentioned that purified acid invertase from potato tuber was found to be a glycoprotein with 10.9% carbohydrate content (Bracho and Whitaker, 1990) [6] and *Kluyveromyces fragilis* invertase was also found to be a glycoprotein (66% carbohydrate, by wt.) (Workman and Day, 1983) [37]. *Lagenaria siceraria* invertase showed a molecular weight of 67 KDa in SDS PAGE (Fig. 4), similar to that of mung bean invertase (Lee et al., 1998), [16] while invertase from mango fruit (Li et al., 2017) and neutral invertase from carrot (Lee and Sturm, 1996) [17] displayed a molecular weight of 45 KDa and 57 KDa on SDS PAGE respectively.

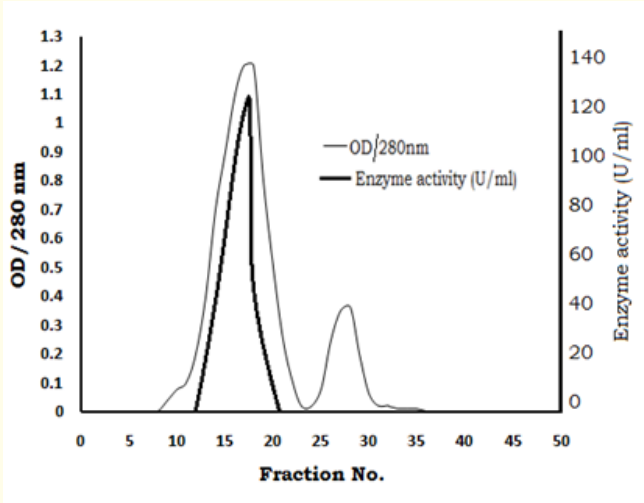

Figure 2: Purification of *Lagenaria siceraria* invertase by size exclusion chromatography. Concentrated enzyme solution (0.8 ml) containing 4 mg of protein was charged each time on a Sephacryl S-300-HR column (1.5 cm X 70 cm), pre-equilibrated with 0.05 M acetate buffer, pH 5.0. The column was eluted with the same buffer and eluted fractions (1.5 ml each) were assayed for invertase activity and protein content.

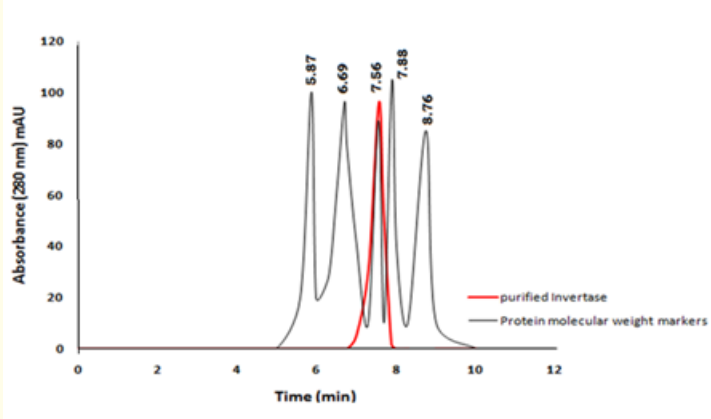

Figure 3: Purification of *Lagenaria siceraria* invertase by HPLC. 20 µl (0.100 mg protein) of concentrated purified protein (obtained from Sephacryl S-300 column) was applied to BioSep-SEC-S 2000 column (300 mm X 7.8mm), pre-equilibrated with 0.1M phosphate buffer, pH 6.8. The column was eluted with same buffer at a flow rate of 0.5 ml/min. The column was pre-calibrated with molecular weight marker proteins [Glutamate dehydrogenase (290 KDa) – 5.87 min; IgG (150 KDa) - 6.69 min; Enolase (67 KDa) – 7.56 min; Albumin (44 KDa) – 7.88 min; Myoglobin (17KDa)- 8.76 min].

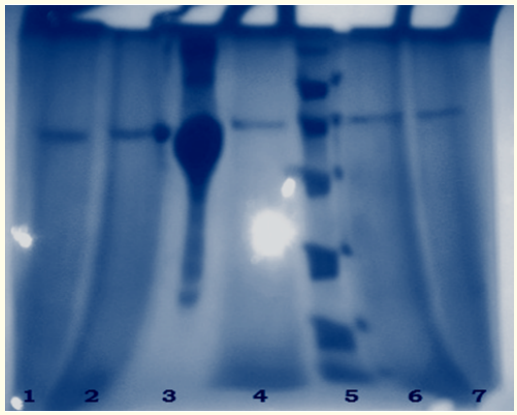

Figure 4: SDS PAGE of *Lagenaria* invertase. Purified protein sample (40 µg) was subjected to denaturing SDS–PAGE carried out at pH 8.3 according to the method of Laemmli, (1970) using 12.5 % gel and 1% (w/v) SDS in the LKB Midget unit (10 cm x 10 cm x 1.5 mm slab). Protein molecular weight markers (broad range) also were run parallel in SDS–PAGE. Lane **1, 2, 4, 6** and **7** – Purified invertase; Lane **5**-Molecular weight marker proteins; Lane **3**- crude enzyme (Ammonium suplhate precipitated enzyme).

Sample	*Total Enzyme Activity (U)*	*Total Protein (mg)*	*Specific Activity (U/mg protein)*	*Fold Purification*	*% Recovery*
Crude Extract (1000 ml) [obtained from 850 g fresh stem]	20,000	440	45.5	1	100
Concentrated (ultrafiltration and lyophilization) extract (100 ml)	19,000	390	48.7	1.07	95
Ammonium Sulfate recipitation (90 % saturation) (15 ml)	16,000	310	51.6	1.13	80
DEAE-Sephadex (A-50) (5 mL)	10,000	45	222.2	4.88	50
Sephacryl S-300 (1 ml)	8900	13	684.6	15.04	44.5
HPLC (BioSep-SEC-S 2000)	7800	10	780	17.14	39

Table 1: Steps of purification of invertase from *Lagenaria siceraria*. Experimental details have been mentioned in the text. The data given are the average (mean) value of the triplicate similar sets of experiments carried out.

Physicochemical properties

Purified invertase from *Lagenaria* was found to be optimally active in the pH range of 4.0 – 5.0 (Fig. 5), similar to that of *Tinospora codifolia* sucrase (Sengupta et al., 2012), acid invertase from potato tubers (Bracho and Whitaker, 1990), invertase from chicory roots (Van Den Ende and Van Laere, 1993) [33] and cherry fruit invertase (Krishnan and Pueppke, 1990) [13]. *Lagenaria* invertase retained 52 ± 1% of its optimal activity at pH 3 (Fig. 5). Activity of the enzyme increased with the increase in temperature till 55°C and was optimally active between 50-60 ^{0}C and stable upto 55°C. The enzyme activity fell sharply beyond 60°C.

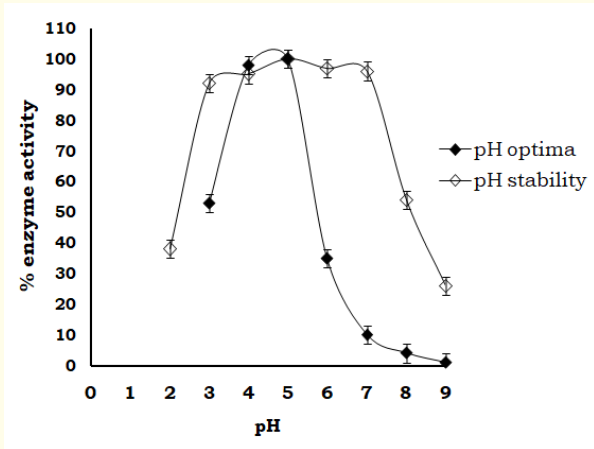

Figure 5: pH optima and stability of *Lagenaria siceraria* sucrase. pH optima and stability of the enzyme were determined in the pH range of 2-9. The buffers used and the experimental details are mentioned in the text. The data represents the mean ± SD value of the triplicate sets of experiments carried out.

Purified invertase from *Lagenaria* displayed remarkable pH stability, retaining 92± 2% and 94 ± 1% of its activity at pH 3 and 7 respectively, after 3 h of incubation (Fig. 5). The enzyme could retain and 37 ± 1% and 53± 1% of its activity at pH 2 and 8 respectively.

It was found that the proteolytic enzymes (trypsin, chymotrypsin, proteinase K) at their optimal pH lowered the activity of *Lagenaria siceraria* invertase; the invertase to protease ratio being 1:10 (for trypsin), 1:6.6 (for chymotrypsin) and 1:2 (for proteinase K). Invertase activity was completely lost in presence of chymotrypsin and proteinase K while tryspin caused a loss of about 67± 1% of activity. Incubation in presence of papain and pepsin, however, did not cause any loss of activity.

Lagenaria siceraria invertase was highly sensitive to 1mM PCMB. More than 90% of enzyme activity was lost within 20 min of incubation. On the other hand, DTNB, iodoacetamide, iodoacetic acid, NEM did not inhibit the invertase activity suggesting it to be a non thiol enzyme. Several plant enzymes like papain, *T.cordifolia* amylase, bromelain and ficin were found to be thiol enzyme. Water soluble carbodimide (EDAC) and PMSF had no significant effect on *Lagenaria* invertase activity.

Lagenaria siceraria invertase, similar to that of mung bean sucrase (Lee et al., 1998), [16] showed highest enzyme activity with sucrose as substrate while raffinose was hydrolyzed at a lower rate. The enzyme was slightly active upon inulin but failed to hydrolyze maltose, levan, treahalose and melezitose. Purified invertase displayed a K_m of 2.0 mg/ml (5.84 mM) and V_{max} of 0.98 µmole/min with sucrose as substrate. 10 U/ml of invertase completely hydrolyzed 10 % (w/v) sucrose solution in 3 h while the same was achieved in 5 h by 8 U/ml of enzyme (Fig. 6). 5 U/ml of enzyme could hydrolyze 73±1 % of sucrose solution (10 % w/v) in 5 h while 2 U/ml of enzyme hydrolyzed 47 ±1 % of the same in 5 h (Fig. 6).

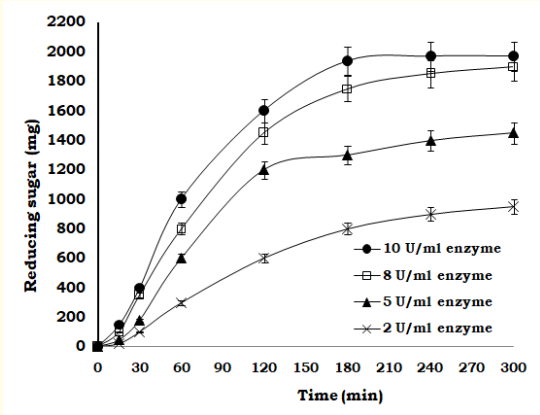

Figure 6: Kinetics of sucrose hydrolysis by *Lagenaria siceraria* invertase. Reaction mixtures (20 ml) containing 10 % (w/v) sucrose in 0.1 M-acetate buffer, pH 5.0 and 40 - 200 U of purified invertase were incubated at 50 ^{0}C for 5 h. Fixed aliquots were withdrawn at different time intervals and the amount of reducing sugar was estimated as mentioned earlier. The data represents the mean ±SD value of the triplicate sets of experiments carried out.

Excellent pH and thermal stability makes *Lagenaria siceraria* invertase very much acceptable commercially for the production of invert syrup. Though invert syrup can be prepared from acid hydrolysis of sucrose, yet enzymatic hydrolysis is always preferred because it does not lead to the formation of undesirable flavoring agents and colored impurities - a common disadvantage of the acid hydrolysis process (Linda and Stanley P, 2007) [19]. It is to be mentioned in parts of the southern states of the USA, the juice of sweet sorghum (which higher in invert sugar than cane juice) is heated, clarified by skimming, and concentrated into a syrup (invert syrup)- a light-brown-colored syrup with a distinctive, pungent odor and flavor, in addition to its sweetness and molasses-like flavor. (Clarke, 2003) [9].

As the source plant is edible and completely non-toxic, *Lagenaria* invertase may also be used as an effective remedy (oral enzyme

therapy) for the treatment of sucrose intolerance. Disaccharide intolerance is an inherited disaccharidase deficiency that leads to the inability to digest certain carbohydrates due to a lack of one or more intestinal disaccharidases (e.g., lactase, maltase, isomaltase and sucrase). Symptoms include diarrhea, abdominal distention and flatulence. Management of the disorder by external enzymes supplementation has not yet been much attempted (due to unavailability of commercial disaccharidase package) except by sacrosidase (yeast invertase) as reported by Treem et al., (1993); Treem et al., (1999) [30, 31]. However, the yeast invertase preparation (in liquid form) was reported to be unstable (showing approximately 46% and 37% residual activity at in 4^{th} and 5^{th} h respectively) at room temperature (Treem et al., 1993) [30]. *Lagenaria siceraria* sucrase is stable at a temperature range of 0 - 55^0C. Moreover, to be used as a therapy for sucrose intolerance or for other commercial purposes, *Lagenaria* invertase need not be purified; it can be used as a crude concentrated powder or liquid (as the plant is completely non-toxic).

Lagenaria siceraria invertase was found to be highly sensitive to Hg^{2+} which completely inhibited the enzyme activity at 1mM concentration. Other heavy metals like Ag^{+} (1mM) inhibited 82±1 % of enzyme activity while Cu^{2+} (1mM) inhibited 51±1 % of enzyme activity. Cd^{2+} and Pb^{2+} (1mM) inhibited 23±1 % and 32 ±1 % of enzyme activity respectively while Fe^{3+}, Mg^{2+}, Zn^{2+} (at 1mM and 2mM concentration) had no significant effect on enzyme activity. However, Ca^{2+} and Mn^{2+} at 1mM concentration slightly increased the enzyme activity. Ag^{+} (2mM) and Cu^{2+} (2mM) completely inhibited the inhibited the enzyme activity.

Immobilization: yield and efficacy

Using free or soluble enzyme is a wasteful method as the enzyme cannot be recovered at the end of the reaction. Immobilization facilitates the separation of enzyme from the hydrolysate and therefore decreases the labor and overhead costs (Mohamad et al., 2015) [20]. Immobilization increases the process yield per unit of enzyme and its repetitive utilization, gives protection and stabilization to the enzyme by preventing inactivation from various physical and chemical denaturing agents, and also enhances the enzyme properties (Cong et al., 1995; Garcia-Galan et al., 2011) [10, 11]. Enzymes can be immobilized to a multitude of different methods like entrapment within cross linked polyacrylamide gel, covalent binding to the surface of sepharose gel beads induced by cyanogen bromide (CNBr), entrapment within calcium alginate beads etc (Aksoy et al., 1998) [3].

According to the bagasse oxidation protocol (Fig. 7) used, 0.52 ± 0.04 m moles of reducing groups (glucose equivalent) / g of purified bagasse was produced. The binding of the soluble enzyme on the dialdehyde-cellulose matrix (Fig. 7), increased with the increase in the amount of matrix added but the strength of the immobilized enzyme (units/g bagasse) decreased significantly when optimum enzyme was fixed at a high amount of bagasse. Table 2 shows the five repetitive cycles carried out for the complete immobilization of acid invertase (400 mL concentrated enzyme extract) on dialdehyde-cellulose (25 g). The % activity yield of the immobilization of acid stable invertase, calculated (after 5 cycles) using equation (2) was found to be 53 ± 2 % (Table 2). Total loss of enzyme activity (by washing) was about 8 % (Table 2).

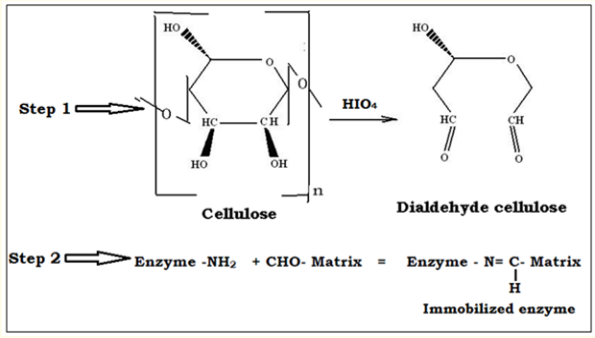

Figure 7: Scheme of enzyme immobilization.

	Initial total enzyme activity (Units)	*Enzyme immobilized (Units)*	*Post-immobilization treatment*	*Enzyme activity lost during washing (Units)*	*Residual enzyme activity (Units)*
Step 1 5 g dry oxidized-bagasse was immersed in concentrated enzyme solution (400 ml)	60,000 ± 2,000	10,000 ± 500	Treated bagasse was vacuum filtered, washed twice in distilled water, press-dried on a blotting paper and stored at 4^0C in 0.1M acetate buffer, pH 5.0	1,000 ± 100	49,000 ± 500
Step 2 (Repetition of step 1)	49,000 ± 500	6,000 ± 800		1,200 ± 150	42,000 ± 500
Step 3 (Repetition of step 2)	42,000 ± 500	7000 ± 450		1,000 ± 250	34,000± 500
Step 4 (Repetition of step 3)	34,000± 500	5,000 ± 550		1100 ± 200	28,000 ± 500
Step 5 (Repetition of step 4)	28,000 ± 500	4,000± 500		1000 ± 200	23,000 ± 1500
Total yield		***32,000 ± 2500***	Total 25 g of enzyme-immobilized bagasse was stored in 0.1M acetate buffer, pH 5.0 at 4^0C.	***5,300 ± 850***	***23,000 ± 1500***

Table 2: Immobilization of *Lagenaria siceraria* invertase on dialdehyde bagasse.
The table shows the five repetitive cycles carried out for the complete immobilization of acid invertase (400 mL concentrated enzyme extract) on dialdehyde-cellulose (25 g). Residual enzyme activity of each cycle was utilized for the next cycle. The data represent the average value (mean) ±SD of the triplicate similar sets of experiments carried out.

Immobilization of enzyme on bagasse had significant beneficial effects on the catalytic activities the enzyme. Immobilized invertase was found to be optimally active at 70^0C instead at 60^0C (optimum for soluble enzyme) and displayed 20 ± 2 % more activity than soluble enzyme at 80^0C (Fig. 8). Thermal stability of the enzyme was also improved by the process of immobilization (Fig. 8). Soluble invertase, which was sharply inactivated beyond 55^0C, excellently remained active (after immobilization) beyond this temperature retaining 41 ± 1 % and 30 ± 1 % of its activity at 70^0C and 80^0C respectively (Fig. 8).

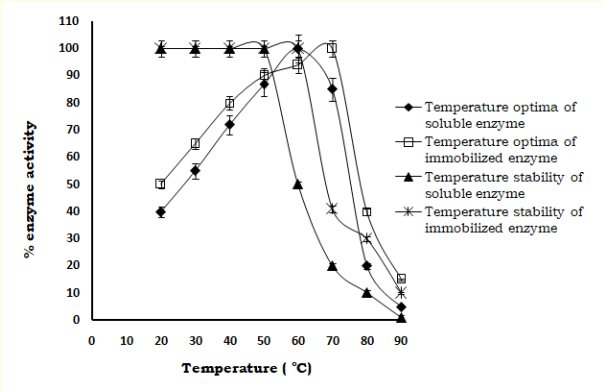

Figure 8: Thermo-stability of the immobilized enzyme system. Temperature optimum and thermo-stability of the immobilized enzyme system was determined in the range of 20 – 90 ^{0}C. Experimental details have been mentioned in the text. The data represent the average value (mean) with ± SD of the triplicate sets of experiments carried out.

The kinetics of sucrose hydrolysis (5- 10 % w/v) by the immobilized enzyme system showed that it was in no way less efficient than soluble enzyme preparation (figure not shown). The immobilized enzyme system completely hydrolyzed 10 % (w/v) sucrose solution in 5 h. The immobilized enzyme system could efficiently run for 25 successive cycles (each cycle of 5 h duration), producing invert syrup from sucrose, remaining 83 ± 1 % and 72 ± 1 % active after 15th and 25th cycles respectively (Fig. 9). It must be mentioned that the use of immobilized invertase is important for continuous hydrolysis of sucrose as the resulting shifts in the pH can prevent the formation of oligosaccharides by the transferase activity associated with the soluble enzyme (Kotwal and Shankar, 2009).

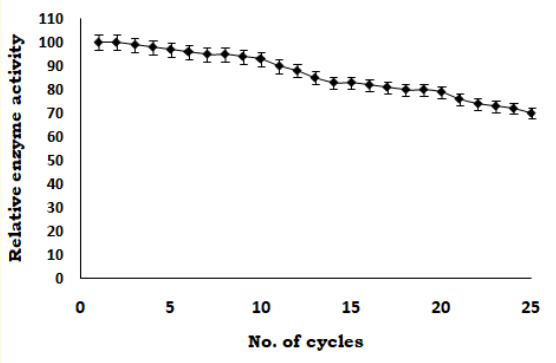

Figure 9: Efficiency of the immobilized enzyme system. The enzyme immobilized-dialdehyde-cellulose (bagasse) system was used for 25 successive cycles (each cycle of 5 h duration) of sucrose (10 % w/v) hydrolysis and the efficiency of the immobilized enzyme system was determined after every cycle of operation. Experimental details have been mentioned in the text. The data represent the average value (mean) + SD of the triplicate sets of experiments carried out.

Bagasse, as a cellulosic matrix selected for the enzyme immobilization, has some advantages: (i) low cost, (ii) easy availability, (iii) good fiber strength, (iv) purity of the matrix and (v) hydrophilicity. In the cellulose oxidation process, it was observed that excess generation of aldehyde group by periodate oxidation affected the mechanical strength of the fibers, making it brittle. Therefore, the oxidation process was balanced to generate reasonable amount of aldehyde groups without weakening the matrix strength. Cellulose dialdehyde is a nonspecific activated matrix which could bind proteins in general, by Schiffs base reaction with free amino groups of protein. In an enzyme immobilization system, a high activity yield is always recommended. Too low activity yield indicates a useless immobilization process. If 20 % activity yield is obtained, it indicates that 5 cycles of the immobilized enzyme system be operated to be equivalent to the use of soluble enzyme. In this case, the activity yield was quite high (53 $\pm$ 2 %), which indicates that just 2 cycles of operation of the immobilized enzyme system is worth better (considering the thermo-stability) than using the soluble enzyme.

Conclusion

Appreciable yield, excellent acid stability, ability to efficiently hydrolyze sucrose into invert syrup and the source plant being edible and completely non-toxic, renders *Lagenaria siceraria* invertase a commercial acceptance to be used in food industry. The enzymes are stable in the duodenal pH (pH 5-7) and are obtained from a safe medicinal plant and therefore the enzyme need not be purified; crude concentrated extracts of the plant can prove to be a useful and safe remedy for sucrose intolerance.

Highlights

- The stem of *Lagenaria siceraria* (bottle gourd) contains substantial amount of acid stable invertase activity.
- 2600 $\pm$ 200 units of invertase present /100 g fresh stem.
- The enzyme is stable in the pH range of 3-7 (with 37$\pm$1% of its activity at pH 2).
- Enzyme was purified (67 kDa; specific activity=780 U/mg protein; K_m=2mg/ml sucrose).
- Enzyme completely hydrolyzed 10% (w/v) sucrose solution to invert syrup in 5 h at 50^0C.
- Enzyme was immobilized on oxidized bagasse (dialdehyde- cellulose).

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