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Purification and characterization of a novel α-D-glucosidase from *Lactobacillus fermentum* with unique substrate specificity towards resistant starch

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Resistant starch is not digestible in the small intestine and is fermented by lactic acid bacteria in the large intestine into short chain fatty acids, such as acetate, propionate and butyrate, which result in several health benefits in analogy with dietary fibre components. The mode and mechanism of resistant starch degradation by lactic acid bacteria is still not understood. In the present study, we have purified α-D-glucosidase from *Lactobacillus fermentum* NCDC 156 by employing three sequential steps i.e. ultra filtration, DEAE-cellulose and Sephadex G-100 chromatographies. It was found to be a monomeric protein (~50 kDa). The optimum pH and temperature of this enzyme were found to be 5.5 and 37°C, respectively. Under optimised conditions with *p*-nitrophenyl-D-glucopyranoside as the substrate, the enzyme exhibited a K_m of 0.97 mM. Its activity was inhibited by Hg^{2+} and oxalic acid. N-terminal blocked purified enzyme was subjected to lysyl endopeptidase digestion and the resultant peptides were subjected to BLAST analysis to understand their homology with other α-D-glucosidases from lactobacillus species.

Key Words: α-D-glucosidase; *Lactobacillus fermentum*; N-terminal amino acid analysis; purification; resistant starch

Introduction

Food polysaccharides can be broadly classified as digestible (starch) and non-digestible dietary components (arabinoxylans, 1,3/1,4 β-D-glucans, glucomannans and cellulose). Many years of research and development have conclusively proved that starch, the main dietary polysaccharide, is not completely digested by the small intestinal enzymes and the resultant non-digestible part is designated as resistant starch (RS) (Roberfroid et al., 2010). RS is the retrograded amylose which cannot be digested by the action of salivary and pancreatic secretions. Broadly RS is classified as RS1, RS2, RS3 and RS4 (Walker et al., 2011). RS1 is physically unavailable starch and occurs in undamaged plant cells. RS2 exists as raw starch of some plant species such as potato. Gelatinized starch upon cooling forms retrograded starch which is termed as RS3 (Fuentes-Zaragoza et al., 2010). Cross-linking using chemicals such as phosphoryl chloride ($POCl_3$), sodium tripolyphosphate (STPP), epichlorohydrin or sodium trimetaphosphate (STMP) results in the modified starch termed RS4 (Walker et al., 2011). Microorganisms present in the large bowel ferment the undigested starch to produce short chain fatty acids such as acetate, propionate and butyrate along with isovalerate, isobutyrate and valerate (Kootte et al., 2012). Diet supplemented with RS significantly increases the population of lactic acid bacteria and reduces the harmful enterobacteria population (Rekiel et al., 2014). Colonic resistance against toxic substances in diet is improved with the increased levels of butyrate, which is the major short chain fatty acid produced from RS fermentation (Perera et al., 2010).

Since RS is retrograded amylose, its utilization by microorganisms may generally require the action of amylolytic enzymes; namely, α-amylase (E.C.3.2.1.1) that cleaves the substrate randomly, pullulanase (E.C.3.2.1.41) that cleaves α-1/6 dextrins into maltotriose and α-D-glucosidase (E.C.3.2.1.20), which hydrolyses the terminal α-1/4 linked residue from the non-reducing ends of starch, oligosaccharides and dextrins. So, the combination of the above three enzymes digests starch to glucose. There are many reports of the isolation and purification of α-amy-

lase from lactic acid bacteria grown on starch. Reports are available on RS ingestion by bifidobacteria in the colon of rats (Silvi et al., 1999) and pigs (Brown et al., 1997) which are degraded by extracellular amylase. In order to understand the mode of action of amylolytic enzymes on resistant starch, seven lactic acid bacteria (five lactobacillus species and two Bifidobacterium species) were screened in our preliminary studies which indicated high levels of α-D-glucosidase instead of alpha amylase. Out of the above seven microorganisms, *Lactobacillus fermentum* NCDC 156 was found to be the best with relatively higher amounts of α-D-glucosidase activity (unpublished data). Since there are no reports on the purification and characterization of α-D-glucosidase from any of the lactobacillus species which are grown on resistant starch, the present study was envisaged and in this present communication we are reporting the purification and characterization of α-D-glucosidase produced by *L. fermentum* NCDC156 grown on RS as the sole carbon source.

Materials and Methods

Substrates and chemicals. DEAE-cellulose, ethylenediamine (EDTA), glucoamylase from *Aspergillus niger*, Sephadex G-100, *p*-nitrophenyl (PNP) substrates, termamyl from *Bacillus licheniformis* and other chemicals were obtained from Sigma Chemical (St. Louis, USA). Resistant starch was procured from HiMaize (Australia). Protein molecular weight markers were purchased from Genie, Bangalore, India. Microbiological media ingredients and culture media were procured from HiMedia, Mumbai, India. All chemicals and solvents used were of analytical grade.

Culture and maintenance. *Lactobacillus fermentum* NCDC156 was obtained from the National Collection of Dairy Cultures, NDRI, Karnal, India, and further maintained on lactobacillus MRS broth (HiMedia, Mumbai, India).

Enzyme activity assay. The α-D-glucosidase enzyme activity was determined using *p*-nitrophenyl-α-D-glucopyranoside (pNPG) as the substrate. A substrate solution (5 mM of 900 μl) in 0.05 M potassium citrate buffer (pH 5.0) was mixed with the crude extract (100 μl) and incubated at 37 $\pm$ 1°C for 20 min. Sodium carbonate (0.5 M of 100 μl) saturated solution was added to stop the reaction. The amount of para-nitro phenol (PNP) released was determined by measuring the absorbance spectrophotometrically at 410 nm. One unit of enzyme activity is equivalent to 1 μmol of PNP liberated from pNPG per min. Specific activity is defined in terms of units per mg protein (Farzadi et al., 2011).

Enzyme purification.
Culture conditions: *L. fermentum* NCDC156 was pre-cultured in MRS broth at 30°C for 18 h without shaking and used for inoculum preparation (conditions standardised with response surface methodology). *L. fermentum* NCDC156 inoculum (10^7 CFU/ml) was added to MRS broth medium (250 ml; dextrose was replaced with 2% (w/v) resistant starch) taken in a conical flask and incubated at 30°C for 48 h.

Purification: Cells were harvested from the culture medium by centrifugation (4,000 $\times$ g for 20 min at 4°C). Various chemical, enzymatic, and mechanical methods were performed in combination or alone for solubilisation of cell-bound enzyme. For disrupting the cells mechanically, ultra sonication (10 kHz) was performed for 15 min at 4°C followed by centrifugation (10,000 $\times$ g for 20 min at 4°C). Cells were suspended in Mcllvaine buffer added with 0.1% mercaptonol and sonicated (Farzadi at al., 2011). Centrifugation was carried out for all the above fractions and enzyme activities were measured in all the pellets and supernatants. Ultrasonication was carried out in ice for 3 min followed by centrifugation (3,000 $\times$ g, 20 min). The pellet was again subjected to the above procedure. The supernatant was used as a crude extract, and enzyme activity was measured and designated as membrane bound α-D-glucosidase activity.

Concentration and purification of the crude extract was carried out by ultra filtration using a 10 kDa MWCO ultra filtration membrane and the retained material was dialyzed against 20 mM Mcllvaine buffer (pH 5.5). A syringe filter (0.22 μm) was used to filter and the dialysate was loaded onto a DEAE-cellulose column which was prequilibrated with 20 mM Mcllvaine buffer (pH 5.5). To remove any unbound proteins, the column was washed with the equilibrating buffer. Fractionation of the bound proteins was carried out in the presence of a linear gradient of NaCl (0–0.5 M) in equilibrating buffer and checked for protein concentration as well as α-D-glucosidase activity. The fractions showing the α-D-glucosidase activity were pooled, dialyzed against 20 mM Mcllvaine buffer (pH 5.5) and concentrated in a Speed Vac Concentrator (Sigma). Further purification of the concentrated sample was carried out on Sephadex G-100 column (0.9 cm $\times$ 90 cm) previously equilibrated with 20 mM Mcllvaine buffer (pH 5.5) at a flow rate of 9.2 ml/h. Fractions containing α-D-glucosidase activity were pooled, dialyzed against 20 mM Mcllvaine buffer (pH 5.5) and concentrated. Using bovine serum albumin as the standard, the protein concentration in the fractions was checked by the Bradford method (Bradford, 1976). The homogeneity of the purified enzyme was determined by native polyacrylamide gel electrophoresis (native-PAGE) as described by Laemmli (1970).

Zymogram analysis and molecular weight determination. Zymogram analysis was conducted on native-PAGE polyacrylamide gel according to Bachmann and McCarthy (1991). Upon electrophoresis, the gel was rinsed with one volume of 50 mM Tris/HCl buffer and sandwiched to a 1% (w/v) agarose gel containing 10 mM pNPG, which is supported on a gel bound, sealed with cling and incubated at 37°C for 1 h. Enzyme activity was identified as a yellow band resulting from the release of PNP by α-D-glucosidase (data not shown).

Running gel (12%) of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weight of the purified α-D-glucosidase. The standard molecular weight markers (Genie) used were phophorylase b (97.0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (29.0 kDa) and aprotinin (6.5 kDa).

Table 1. Purification of α-D-glucosidase from *Lactobacillus fermentum* NCDC156.

	Total protein (mg)	Total activity (U)	Specific activity (U/mg of protein)	Yield (%)	Fold purification
Crude enzyme	12.80	24.8	1.9	100	1
Ultrafiltration	9.78	20.3	2.07	81.9	1.08
DEAE-cellulose	0.19	3.8	14.7	15.3	7.75
Sephadex G-100	0.01	0.4	40	1.61	21

Effects of temperature, pH, inhibitors and different metal ions on α-D-glucosidase. pNPG was used as a substrate to determine the optimum pH of purified α-D-glucosidase in different pHs of 0.05 M sodium acetate buffer (pH 4 to 6), phosphate buffer (pH 6 to 7.5) and Tris-HCl buffer (pH 7.5 to 9). pH stability was determined by incubating the purified α-D-glucosidase in the above-mentioned buffers for 30 min at $37 \pm 1°C$ and checking for the enzyme activity. The relative activity was plotted against different pHs taking the unincubated activity (at pH 5.5) as 100%.

For thermal stability measurements, enzyme was incubated in 50 mM Mcllvaine buffer (pH 5.5) without substrate for 30 min. At the end of 30 min, substrate was added and the residue activity was determined. The unincubated enzyme activity was added and the residue activity determined. The unincubated enzyme activity was taken as 100% and a relative activity at different test temperatures was plotted.
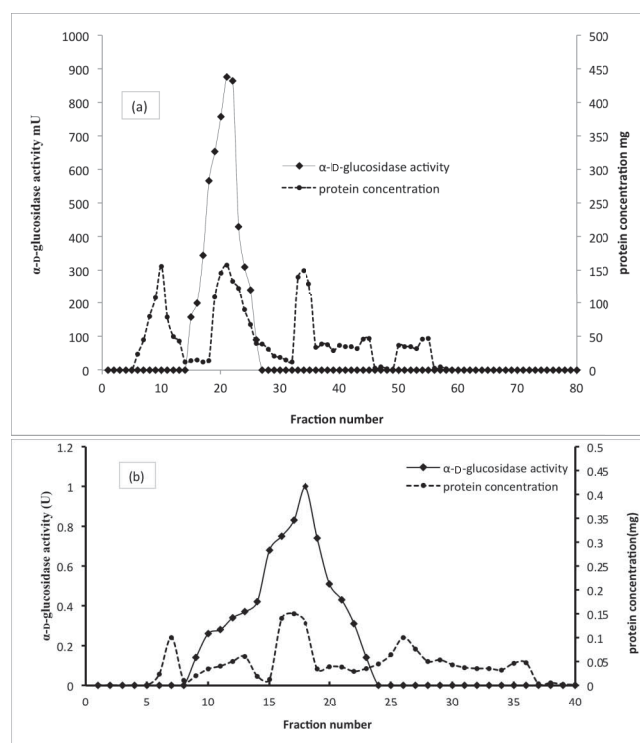
Under standard assay conditions the effect of various metals ions ($HgCl_2$, $FeCl_3$, $CaCl_2$, $PbCl_2$, $NiCl_2$, $MgCl_2$, KCl and $MnCl_2$) on α-D-glucosidase activity was determined in a 5 mM concentration and compared with the control (without added metal ions). The control was taken as 100%. The effect of $HgCl_2$ on enzyme activity was studied in the concentration range of 0.5–5.0 mM.

The influence of inhibitors such as oxalic acid, citric acid, boric acid, EDTA, and *p*-chloromercuribenzoate (PCMB) on α-D-glucosidase activity was determined in the concentration range of 1–10 mM. Pre-incubation of the respective inhibitor with the purified enzyme was carried out for 1 h at 4°C, followed by the standard enzyme assay as described above. The control was taken as 100%.

Determination of kinetic parameters and specificity of α-D-glucosidase. Various concentrations of pNPG (ranging from 0.2 to 1 mM) were incubated with the purified enzyme under optimum assay conditions to determine the kinetic parameters. K_m and V_{max} were estimated from a Lineweaver-Burk plot by determining the initial velocities.

p-nitrophenyl derivatives are used to determine the substrate specificity of α-D-glucosidase by measuring the rate of PNP liberated during hydrolysis from 2 mM of the substrates in 50 mM Mcllvaine buffer (pH 5.5) at $37 \pm 1°C$ for 10 min and detected spectrophotometrically at 410 nm.

Amino acid composition. The purified α-D-glucosidase enzyme was hydrolyzed *in vacuo* at 110°C in constant boiling HCl for 24 h using the Pico-Tag workstation. Amino acid analysis was performed by pre-column derivatization using phenyl isothiocyanate. The phenyl

Fig. 1. Elution profiles of α-D-glucosidase from *L. fermentum* NCDC156 on (a) DEAE-Cellulose exchange (b) Sephadex G-100.

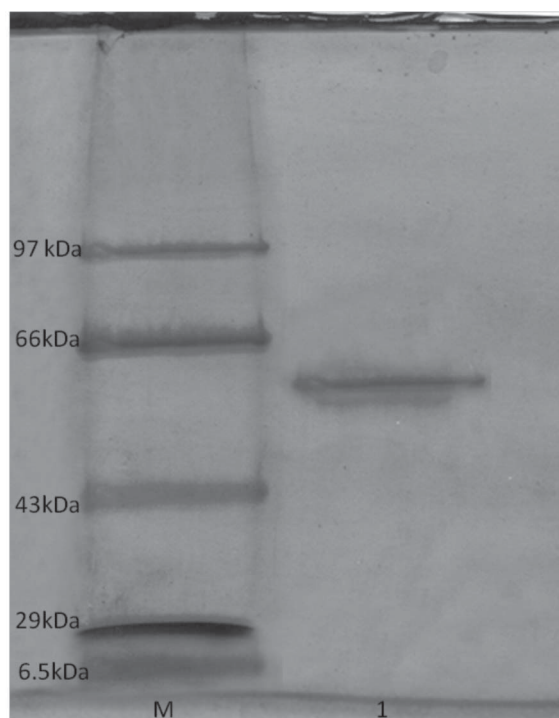thiocarbamoyl amino acids were analyzed by reverse phase HPLC (Bidlingmeyer et al., 1984).

N-terminal sequence analysis. Purified α-D-glucosidase enzyme was electroblotted onto PVDF membranes (Immobilon[TM], 0.45 mm, Millipore, Bedford, MA, USA) following the manufacturer's instructions. Ponceau 3R was used to stain the protein band. Internal sequences were determined by digesting the protein band using lysyl endopeptidase (Iwamatsu and Yoshida-Kubomura, 1996). Amino acid sequences were analyzed by an automated protein sequencer (Shimadzu PPSQ-10).

Statistical analysis. All the experiments are performed in triplicate and the values are represented as mean values ±standard deviation (SD).
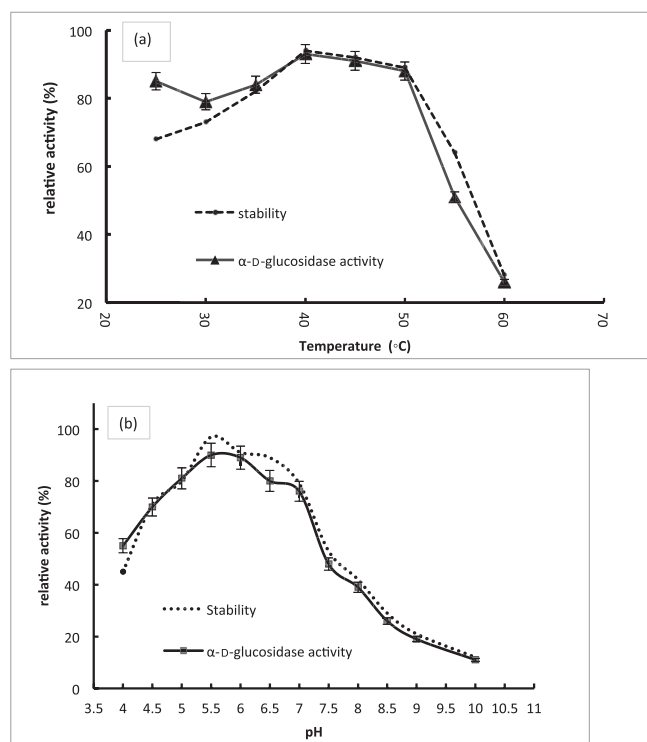
Results

Purification

In the current study, α-D-glucosidase from *L. fermentum* NCDC156 grown on RS was purified and characterized. The activity of the enzyme was found to be higher (66 U mL^{-1}) in the dialyzed cell-free extract prepared by
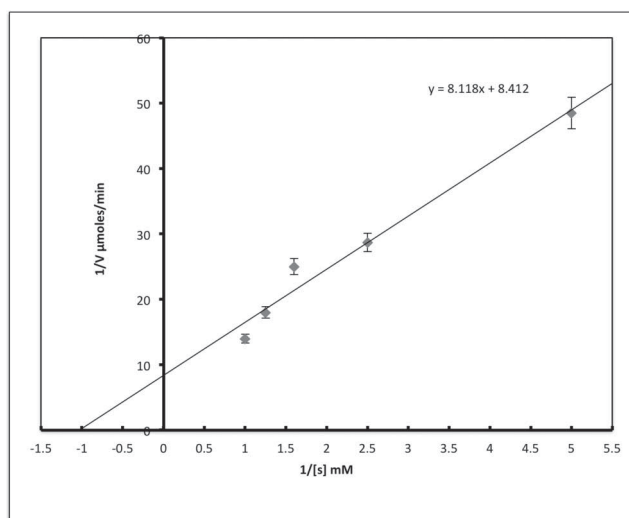
Fig. 2. SDS-PAGE (12%) of the fractions obtained during the purification.

Lane M, marker proteins with relative molecular masses indicated on the right; Lane 1, purified α-D-glucosidase.

Fig. 3. Effects of different temperatures (a) and pH (b) α-D-glucosidase activity and stability.

Data are given as means ±SD, $n = 3$.

Fig. 4. Lineweaver-Burk plot for K_m and V_{max} values of the α-D-glucosidase in the presence of different concentrations of pNP-α-D-glucopyranoside.

Data are given as means ±SD, $n = 3$.

Table 2. Effect of organic compounds on the activity of purified α-D-glucosidase from *Lactobacillus fermentum* NCDC156.

Organic compound	α-D-glucosidase activity (% activity)
Control	100 ± 0^a
L-Cysteine (1 mM)	124 ± 1^a
L-Histidine (10 mM)	123 ± 1^a
Tris (100 mM)	132 ± 1^b
Tris (10 mM)	121 ± 1^a
EDTA (50 Mm)	112 ± 1^c
EDTA (10 mM)	103 ± 1^d

Data is given as means ±SD, $n = 3$; values not sharing common alphabets within the row are significantly different ($P < 0.05$).

Table 3. Amino acid composition of α-D-glucosidase enzyme purified from *L. fermentum* NCDC156 grown on RS.

Amino acids	gm/100 gm of protein	Standard deviation
Asp	11.13	0.46
Glu	8.82	0.24
Ser	10.31	0.44
Gly	4.74	0.01
His	1.40	0.47
Arg	4.68	0.21
Thr	10.92	0.75
Ala	7.47	0.47
Pro	3.94	0.23
Tyr	6.36	0.06
Val	6.19	0.03
Met	1.34	0.63
Cys	1.13	0.45
Ile	4.07	0.21
Leu	8.04	0.25
Phe	6.58	0.01
Lys	2.88	0.34

ultrasonication of cell pellets in lysis solution (membrane bound α-D-glucosidase) as compared with the culture supernatant (1.1 U mL^{-1}, α-D-glucosidase and intracellu-

lar α-D-glucosidase of 4.2 U mL^{-1}). Membrane-bound α-D-glucosidase was subjected to a three-step purification and the results are summarized in Table 1. The crude en-

Fragment 1

D8IHI3	D8IHI3_LACFC	225GDVEPVVANGHRMHEFLQEMHQAVMAKHDLVTVGETPGATTDDAKKYANLEQTELN 280
U2II93	U2II93_LACFE	225GDVEPVVANGHRMHEFLQEMHQAVMAKHDLVTVGETPGATTDDAKKYANLEQTELN 280
T0SCJ7	T0SCJ7_LACFE	225GDVEPVVANGHRMHEFLQEMHQAVMAKHDLVTVGETPGATTDDAKKYANLEQTELN 280
V4XG41	V4XG41_LACFE	225GDVEPVVANGHRMHEFLQEMHQAVMAKHDLVTVGETPGATTDDAKKYANLEQTELN 280

Fragment 2

D8IHI3	D8IHI3_LACFC	225GDVEPVVANGHRMHEFLQEMHQAVMAKHDLVTVGETPGATTDDAKKYANLEQTELN 280
U2II93	U2II93_LACFE	225GDVEPVVANGHRMHEFLQEMHQAVMAKHDLVTVGETPGATTDDAKKYANLEQTELN 280
T0SCJ7	T0SCJ7_LACFE	225GDVEPVVANGHRMHEFLQEMHQAVMAKHDLVTVGETPGATTDDAKKYANLEQTELN 280
V4XG41	V4XG41_LACFE	225GDVEPVVANGHRMHEFLQEMHQAVMAKHDLVTVGETPGATTDDAKKYANLEQTELN 280

Fig. 5. Alignment of (1) N-terminal (α-D-glucosidase from *L. fermentum* NCDC156), and (2) internal sequences of Alphaglucosidase from *Lactobacillus fermentum* strain CECT 5716 (NCBI, D8IHI3); Oligo-1,6-glucosidase from *Lactobacillus fermentum* MTCC 8711 (NCBI, T0SCJ7); ABC transporter, substrate-binding protein *Rhodococcus equi* ATCC 33707 (NCBI, E9SXI1).

The amino-terminal sequence of purified α-D-glucosidases (from *L. fermentum* NCDC156 grown on resistant starch fragments generated by lysyl endopeptidase digestion were determined, as described in Section "Materials and Methods". The amino acid residue numbers of α-glucosidases and other enzymes are indicated on both sides of the corresponding sequences.

zyme was concentrated by ultra filtration, followed by successive steps of purification using anion exchange and gel filtration (Figs. 1a and 1b) to obtain a pure enzyme with a yield and purification of 1.61% and 21-fold, respectively (Table 1).

Enzyme properties

Native PAGE and Zymogram (data not shown) analysis was carried out to confirm the identity of the purified α-D-glucosidase (Fig. 2). The molecular weight corresponding to the band was estimated to be ~50 kDa on the basis of its relative mobility. The single band on the gel revealed that the purified enzyme was monomeric.

Effect of pH and temperature

The optimum temperature of the enzyme was found to be 37°C, and was stable up to 50°C for 35 min but rapidly lost its activity above 50°C. The temperature and stability curves of the purified enzyme are shown in Fig. 3. The optimal activity of the enzyme was found at pH 5.5 and the stability was in the range of 5.5–7.5. The enzyme was stable at pH 5.5 for 48 h, however thereafter it lost its activity. The activity of the purified enzyme decreased above pH 7.0 and was completely lost beyond pH 9.0.

Kinetic studies

A Lineweaver-Burk plot exhibited a linear correlation between the concentration of *p*-nitrophenyl α-D-glucopyranoside (10 ± 240 mol) and the amount of *p*-nitrophenol released. Kinetic studies in the existing analysis showed apparent K_m and V_{max} values of 0.97 mmol/L and 0.11 mol/min for purified α-D-glucosidase (Fig. 4). The Arrhenius plot was linear over the range of 35–60°C and the activation energy (Ea) was found to be 32 kJ mol^{-1} (data not shown).

Effect of cations and other chemicals

The influence of various metals ions on α-D-glucosidase activity was determined. Alphaglucosidase activity was enhanced by Ca^{2+} by 39%. Alphaglucosidase activity was inhibited by Hg^{2+}, Pb^{2+} by 96% and 58% respectively. There was no significant effect on α-D-glucosidase activity by other metal ions (Table 2). EDTA did not show any significant effect on enzyme activity. In the present study, at 10 mM concentration α-D-glucosidase activity was inhibited by PCMB (84%). At a similar concentration, there was complete inhibition of the enzyme by oxalic acid.

Amino acid composition

The amino acid composition of α-D-glucosidase is presented in Table 3. Polar amino acids, such as aspartate (11.13%), threonine (10.92%) and serine (10.31%), are present in greater concentrations than the nonpolar amino acids such as glycine (4.74%) and isoleucine (4.07%). A low concentration of cysteine (1.13%) was found.

N-terminal amino acid sequencing

Purified resistant starch-degrading enzyme (α-D-glucosidase) was analyzed with respect to an N-terminal amino acid sequence. Even at 100 pmol of α-D-glucosidase, an amino acid sequence could not be obtained, which indicated the blockage of an N-terminal residue of α-D-glucosidase. Hence, this enzyme was treated with lysyl endopeptidase digestion resulting in two peptides (Fig. 5a). BLAST analysis was performed using the amino acid sequence obtained from the two fragments in order to identify a possible homology with the enzymes from other sources. In the BLASTP analysis, two fragments which were designated as F1 (internal sequence **ATTDDA**) and F2 (internal sequence **KAANLE**) exhibited about 92.3% and 90% similarity/identity, respectively. F1 has a high

degree of homology to the corresponding regions of α-D-glucosidase (amino acid identity: 92.3%) from *Lactobacillus fermentum* (strain CECT 5716), *Lactobacillus fermentum* (strain NBRC 3956/LMG 18251), and *Lactobacillus mucosae* LM1. F2 was highly homologous (90% amino acid identity) with the corresponding region α-D-glucosidase from *Lactobacillus fermentum* (strain CECT 5716), and *Lactobacillus fermentum* (strain NBRC 3956/LMG 18251 (Fig. 5b). All the above-mentioned glucosidases belong to the glycosyl hydrolase family 13 (GH13) (19), and F1 and F2 and can therefore be designated as putative α-glucosidases belonging to this GH13 family.

Discussion

RS acts as an important substrate for the growth of beneficial bacteria in the large intestine. It degrades slowly by the action of enzymes secreted by lactic acid bacteria, thereby acting as a carbon source to the colonic microflora. There is a lacuna regarding information about the enzymes involved in the digestion of RS in humans and its effect on the colonic microbiota. In this work, we describe for the first time the purification of α-glucosidase from *L. fermentum* grown on resistant starch as the substrate. The enzyme showed similarities with other starch-degrading enzymes isolated from fungi and plant sources. Enzyme purification was carried out by three sequential steps involving ultra filtration, ion exchange and gel permeation chromatographies. The apparent molecular weight of α-D-glucosidase of *L. fermentum* NCDC156 in the present study is found to be 50 kDa, which is in agreement with most of the reported microbial amylolytic enzymes (Souza, 2010), but, however, is slightly higher than that reported from *Lactobacillus amylovorus* (47 kDa) (Xian et al., 2015).

The optimum temperature of this α-D-glucosidase was found to be 37°C, which is similar to those reported from *Bifidobacterium breves* (Ryan et al., 2006). The enzyme was stable up to 50°C for 35 min and rapidly lost its activity above 50°C. This is in general agreement with the temperature optima (36–50°C) of starch-degrading enzymes (Li et al., 2010). The enzyme was stable at pH 5.5 for 48 hours, and thereafter it lost its activity. Similar results were reported from other lactic acid bacteria (Ryan et al., 2006). In general, most of the starch-degrading enzymes in fungi are stable over an optimal pH range of 4–11 (Michelin et al., 2010). Amino acids such as aspartic acid, histidine, arginine, and tyrosine, are present in the active site of members of starch-degrading enzymes and are responsible for exhibiting a similar type of pH profiles (Boraston et al., 2004; Van der Maarel et al., 2002; Van Hijum et al., 2006).

The K_m of the purified enzyme in the present study with respect to pNPG was 0.97 mmol/L, which was in tune with an earlier report of α-amylase-from *Penicillium camemberti* showing 0.92 mmol/L (Nouadri et al., 2010). In the present study, the substrate specificity of the alphaglucosidase was tested with other substrates, soluble starch, amylopectin and maltooligosachharides (DP 2-7). The purified enzyme showed a high activity against pNP-α-D-glucopyranoside but didn't have any activity on the other pNP derivatives (*p*-nitrophenyl-α-D-xylopyranoside; *p*-nitrophenyl α-D-arabinofuranoside; *p*-nitrophenyl α-D-galacto pyranoside; *p*-nitrophenyl α-D-mannopyranoside), soluble starch and amylopectin. Purified alphaglucosidase treated maltooligosachharides reaction products were determined by thin-layer chromatography with respect to time. The terminal glucose residue was released, indicating the enzyme to be exo-glucosidase in nature (data not shown). With an increase in the time duration, transglucosidase activity of the purified enzyme was observed. The alphaglucosidase activity was affected in the presence of heavy metals, such as Hg^{2+} and Pb^{2+}, and this result was in agreement with the available reports (Mfombep and Senwo, 2012). The Hg^{2+} ion acts as a strong inhibitor of enzymes isolated from different sources and this suggests the occurrence of thiol groups at the catalytic site of the enzyme (Lasrado and Gudipati, 2013).

Among divalent metal ions, only calcium increased the alphaglucosidase activity. Ca^{2+} is reported to form co-ordinate bonds with active site amino acids, such as aspartic acid and glutamic acid, which, in turn, is responsible for the positive effect of Ca^{2+} on enzyme activity (Nirmala and Muralikrishna, 2003). EDTA did not show any significant inhibitory activity which clearly indicates the absence of metal ions in the catalytic site. A similar result was reported for α-D-glucosidase, purified from *Lactobacillus acidophilus* (Saeed and Salam, 2013). Amino acid composition exhibited a lower concentration of cysteine which is in contrast with the available reports from other sources (Pal et al., 2010).

Though RS is gaining attention related to its health benefits, its degradation by the enzymes of probiotic bacteria such as Lactobacilli and Bifidogenic is lacking. The structural information of the purified enzyme is warranted to clone it in bacterial species. Hence, the N-terminal amino acid sequence was carried out. Since the N-terminal end of the purified enzyme is blocked, digestion with lysyl endopeptidase was carried out which resulted in two peptides. These resultant peptides were analysed by BLAST analysis which indicated more than 90% homology with the reported alpha glucosidases. On the first iteration, the closest hits were obtained with different strains of Lactobacillus. The increasing number of known nucleotide sequences of genes encoding α-D-glucosidase suggests the role of certain conserved amino acid residues in catalysis. Four conservative regions in primary structures of α-D-glucosidase have been identified from yeast, bacilli and insects (Zhou et al., 2015). Further studies pertaining to the cloning of this α-D-glucosidase is under progress.

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