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Application Relevant Studies of Fungal β-galactosidases with Potential Application in the Alleviation of Lactose Intolerance

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Abstract Functional screening studies revealed that *Aspergillus carbonarius* ATCC6276 produced extracellular β-galactosidase activity potentially suited for use as a lactase digestive supplement in the treatment of lactose intolerance. The crude preparation contained two β-galactosidase activities, β-gal 1 and β-gal 2, which were separated by ion-exchange chromatography. Both enzymes were purified to homogeneity by a combination of gel filtration, ion-exchange, chromatofocusing and hydrophobic interaction chromatographies. β-gal 1 and β-gal 2 displayed differences in molecular mass (110 kDa versus 120 kDa as judged by SDS PAGE) and in a range of additional physicochemical properties. Km values of 83 and 309 mM, respectively, were recorded using lactose as substrate while temperature optima of 55°C versus 65°C were obtained. Unlike current commercialized supplemental lactases, both of the purified enzymes displayed significant stability when exposed to simulated gastric conditions, with β-gal 1in particular retaining 70% residual activity after exposure to pH 2.0 in the presence of pepsin for 2 h. Overall the results indicate that the β-galactosidases of *Aspergillus carbonarius* ATCC6276, either individually or in combination, may be suitable for use as a digestive supplement for the alleviation of lactose intolerance.

Keywords Lactase · β-galactosidase · *Aspergillus carbonarius* · Lactose intolerance

Introduction

Microbial β-galactosidases (β-D-galactoside-galactohydrolases, EC 3.2.1.23 or lactases) catalyze the hydrolysis of the disaccharide lactose, yielding its constituent monosaccharides glucose and galactose.

Fungal β-galactosidases are usually extracellular, typically possess pH optima in the range of pH 3–5, and have high temperature optima [1]. Bacterial and yeast β-galactosidases are usually intracellular, displaying pH optima in the neutral range [2, 3].

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β-Galactosidases, mainly derived from various strains of *Kluyveromyces* and *Aspergillus*, have found industrial application for many years. The use of fungal lactases predominates in many instances, largely due to their extracellular nature, high production levels, stability, and GRAS status. β-galactosidases from various strains of *Aspergillus niger* and *A. oryzae* are exploited commercially for the hydrolysis of lactose in whey, for the alleviation of lactose intolerance and for the production of galacto-oligosaccharides [1].

Lactose intolerance and some cases of infantile colic are lactose-triggered conditions. Their symptoms are often ameliorated by co-consumption of exogenous β-galactosidase enzyme supplements with milk/dairy-derived meals [4–8]. Lactase supplements, often marketed in tablet or capsule form, usually contain *Aspergilli*-derived β-galactosidases.

Studies in our laboratory suggest that these commercialized products are not ideally suited to lactose hydrolysis in vivo when their physicochemical characteristics are considered in the context of conditions encountered in the upper digestive tract [9].

Initial in-house screening of over 100 fungal strains in our laboratory suggested that crude lactase activity derived from *Aspergillus carbonarius* ATCC 6276 should prove more suitable than current commercial preparations in the gastrointestinal hydrolysis of dietary lactose, particularly in the stomach. The aim of this study was to purify this lactase activity and characterize it more fully in the context of potential application as a digestive supplement for the treatment of lactose maldigestion.

Materials and methods

Materials

Aspergillus carbonarius, strain ATCC 6276 was obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA. Over the counter lactase digestive supplements were obtained from: McNeil Consumer Products Co., 50 Commerce Dr., Trumbull, CT, USA (‘Lactaid’); Plainview LLC, West Long Branch, NJ, USA (‘Dairycare’); Gelda Scientific, 6320 Northwest Dr., Mississauga, ON, Canada (‘Lacteeze’) and The Natural Medicine Co., Burgage, Blessington, Co. Wicklow, Ireland (‘Lifeplan’). Chromatographic media, polybuffer 74, isoelectric focusing standards and ampholyte 3–10 were obtained from Bio-Rad, 2000 Alfred Nobel Dr., Hercules, CA 94547, USA.

N-glycosidase F deglycosylation kit was obtained from Roche applied science, Nonnenwald 2, 82372 Penzberg, Germany. SDS-PAGE and gel filtration molecular mass markers, the trinider glucose detection kit and all additional reagents were obtained from Sigma-Aldrich, Dublin, Ireland.

Organism Cultivation and Enzyme Production

Aspergillus carbonarius, strain ATCC 6276 was maintained on solid potato dextrose agar plates, stored at 4°C. Induction of β-galactosidase production was achieved via submerged fermentation using wheat bran medium [10], inoculated with a 1.0 ml suspension containing 10^7 spores ml^{-1} and incubation for 144 h at 30°C in an orbital shaker at 200 rpm. Harvesting of extracellular crude β-galactosidase entailed filtration through Whatman no. 1 filter paper with subsequent centrifugation of the filtrate (12,000×g, 20 min at 4°C).

Enzyme Assay

β-galactosidase activity assay was based upon the method of Rasouli and Kulkarni [11], using as substrate 5 mM *O*-nitrophenyl-β-D-galactopyranoside (ONPG) in 200 mM sodium acetate buffer, pH 5.5. One unit of β-galactosidase activity was defined as the amount of enzyme capable of releasing 1 μmol of *O*-nitrophenol per minute under the defined assay conditions.

Enzyme Purification

Crude extracellular β-galactosidase was concentrated (13-fold) by ultrafiltration using a 30 kDa ultrafilter membrane and the retentate was clarified by centrifugation (12,000×g, 20 min at 4°C). Concentrated enzyme was loaded onto sephacryl S200 HR gel filtration column pre-equilibrated with 10 mM sodium phosphate buffer pH 6.0 (flow rate, 1.0 ml/min). Pooled activity was subsequently loaded onto a DEAE-sepharose CL 6B column, pre-equilibrated with 10 mM sodium phosphate buffer pH 6.0 and bound enzyme was eluted over an ascending linear salt gradient (0–0.25 M NaCl) in 10 mM sodium phosphate buffer pH 6.0 (flow rate; 1.0 ml/min). Pooled activity fractions were concentrated and buffer exchanged into 25 mM piperazine-HCl buffer pH 5.5 by ultrafiltration (10 kDa cut-off membrane) and loaded onto a PBE 94 chromatofocusing column pre-equilibrated with 25 mM piperazine-HCl pH 5.5. Fractions were eluted over a descending pH gradient 5.5–4.0, using polybuffer 74-HCl pH 4.0. The pooled activity was exchanged into 10 mM sodium phosphate buffer pH 6.0 containing 3.0 M ammonium sulphate by ultrafiltration and was loaded onto phenyl-agarose column pre-equilibrated with the same buffer. Bound enzyme was eluted over a linear descending ammonium sulphate gradient (3.0–1.0 M). All chromatography was performed at 4°C using a biologic LP chromatography system (Bio-Rad). Protein concentration was determined by the method of Bradford [12], using bovine serum albumin as the standard.

Electrophoretic Analysis and Molecular Mass Determination

SDS-PAGE was carried out using an 8% gel and a vertical electrophoretic system as described by Laemmli [13]. Non-denaturing electrophoresis was carried out with the omission of SDS from the gel running and loading buffers, and the sample was not pre-treated under denaturing conditions. Protein bands were visualised by staining with coomassie brilliant blue R-250. β-galactosidase activity staining was carried out by incubating the native gel with X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) solution [0.02% (*w*/*v*) in 0.2 M sodium acetate buffer pH 5.5] substrate solution for 30 min at 45°C and 100 rpm. Native molecular mass was determined using a sephacryl S300 HR gel filtration column.

Effect of Temperature and pH on Enzyme Activity

Temperature versus activity profiles (25–75°C) was determined according to Nagy et al. [14]. pH versus activity profiles (1.5–8.5) were determined according to the method of Shaikh et al. [15], using the following buffers: 0.2 M HCl–KCl (pH 1.5–2.5), 0.2 M glycine–HCl (pH 2.5–3.5), 0.2 M sodium acetate-acetic acid (pH 4.5–5.5), 0.2 M disodium hydrogen phosphate–sodium dihydrogen phosphate (pH 6.0–7.5), 0.2 M Boric acid–NaOH (pH 8.0).

Enzyme Stability When Subjected to Simulated Gastric Conditions

Simulation of gastric digestion using modified methods of Xenos et al. [16] and Ingels et al. [17] entailed co-incubation (up to 2 h, 37°C, 150 rpm) of 5.0 ml of suitably diluted (in dH_2O) enzyme with 5.0 ml of simulated gastric fluid (SGF). SGF (pH 2.0) was prepared as per the United States Pharmacopoeia [18] and contained (per liter) 3.2 g porcine pepsin, 7.0 ml conc. HCl and 2.0 g NaCl. A separate series of incubations were undertaken in which pepsin was omitted (SGF-NPEP) but which were identical to the above in all other respects. After incubation, all samples were pH adjusted to 5.5 using 0.5 M NaOH followed by activity assay.

Determination of Kinetic Properties

Kinetic properties were determined with respect to varying concentrations of chromogenic substrates ortho-nitrophenyl-β-D-galactopyranoside (ONPG, 0.1–15 mM), para-nitrophenyl-β-D-galactopyranoside (PNPG, 0.5–16 mM) and lactose (10–900 mM) using the modified method of Chakraborti et al. [19]. Enzymatic hydrolysis of lactose was estimated by determining glucose levels produced using a trinider glucose detection kit as per manufacturers' (Sigma-Aldrich) instructions. Lineweaver–Burk plots and initial velocity against substrate concentration plots were constructed.

Isoelectric Point Determination and Glycosylation Analysis

Polyacrylamide gel isoelectric focussing (IEF) was carried out as per manufacturer's instructions. Glycosylation analysis (N-linked) was carried out using an N-glycosidase F deglycosylation kit in accordance with the manufacturer's instructions.

Peptide Mass Fingerprinting and MS/MS Analysis

Gel slices of the deglycosylated protein bands were excised from a coomassie stained SDS-PAGE gel and supplied to Pinnacle Laboratory at the University of Newcastle Upon Tyne, UK. Pepsin digestion and MS analysis (using an Applied Biosystems Voyager DESTR mass spectrometer) were carried out as described by Gonnet et al. [20] with subsequent submission of MS data to the matrix database for protein identification (www.matrixscience.com [21]).

Results and Discussion

Purification, Electrophoretic Analysis and Molecular Mass Determination

Two β-galactosidase activities were resolved and purified to homogeneity (Table 1). Separation of the two activity peaks was achieved by the ion exchange step (Fig. 1). A final purification factor of 6 and 3 was achieved for β-gal 1 and β-gal 2, respectively, values similar to those obtained for β-galactosidase isoforms purified from *Aspergillus nidulans* [22].

The percentage yield of both enzymes (5 and 1% for β-gal 1 and β-gal 2, respectively) is relatively low, reflecting the fact that only fractions from the centre of chromatographic activity peaks were pooled to ensure purification to homogeneity. While the chromatofo-

Table 1 Purification table for β-galactosidases (β-gal 1 and β-gal 2) from A. carbonarius ATCC 6276.

Purification Step	Total vol. (ml)	Total Protein (mg)	Total activity (U)	Protein (mg/ml)	Act. (U/ml)	Sp. act. (U/mg)	Percent yield	Purification factor
Crude	390	193.83	356	0.497	0.913	1.84	100	1
UF	30	129.48	338	4.316	11.283	2.61	95	1.42
GF	75	72.53	260	0.967	3.472	3.58	73	1.95
Ion ex.								
β-gal 1	135	6.705	124.8	0.050	0.924	18.60	35	10.12
β-gal 2	157	2.090	21.2	0.013	0.135	10.14	6	5.51
CF & UF								
β-gal 1	123	4.866	37	0.040	0.300	7.60	10	4.13
β-gal 2	131	1.345	7.6	0.010	0.058	5.65	2	3.07
HIC								
β-gal 1	164	1.711	19.1	0.010	0.116	11.16	5	6.07
β-gal 2	105	0.686	3.80	0.007	0.036	5.54	1	3.01

UF, ultrafiltration; *GF*, gel filtration; *CF*, chromatofocusing; *HIC*, hydrophobic interaction chromatography; *Ion ex.*, ion exchange; *Act*, activity; *Sp. Act.*, specific activity.

cusing step is noteworthy in that it appears to reduce the purification factor (Table 1), SDS PAGE analysis confirmed the necessity of the step to remove a specific contaminating band.

Purified β-gal 1 and β-gal 2 each migrated as single bands on both native and SDS PAGE gels with SDS PAGE yielding estimated subunit molecular weights of 110 and 120 kDa, respectively (Fig. 2). Conformation that native gel protein bands possessed β-galactosidase activity was obtained by zymogram analysis (data not shown). Native molecular masses of enzymes 1 and 2, as estimated by gel filtration, were 139 and 152 kDa. This suggests both enzymes are monomeric. The discrepancy between electrophoretic- and chromatographic-based estimates is relatively significant but consistent. Overall, subunit molecular mass values obtained and the monomeric nature of the enzymes broadly resemble those obtained for β-galactosidases purified from other *Aspergillus* strains [23]. Although the ability of *Aspergillus carbonarius* to produce β-galactosidase activity had been reported [24], the occurrence and purification of molecular isoforms and application-relevant studies has not previously been documented.

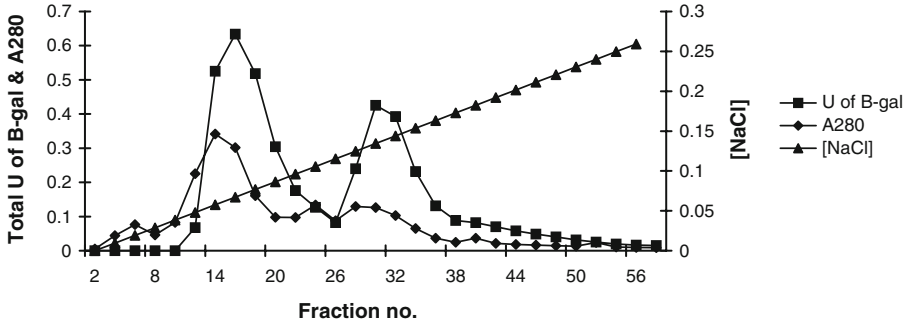

Fig. 1 Separation of the 2 β-galactosidase activities on a DEAE sepharose CL 4B ion exchange column (fraction volume, 3.0 ml, flow rate, 1.0 ml/min)

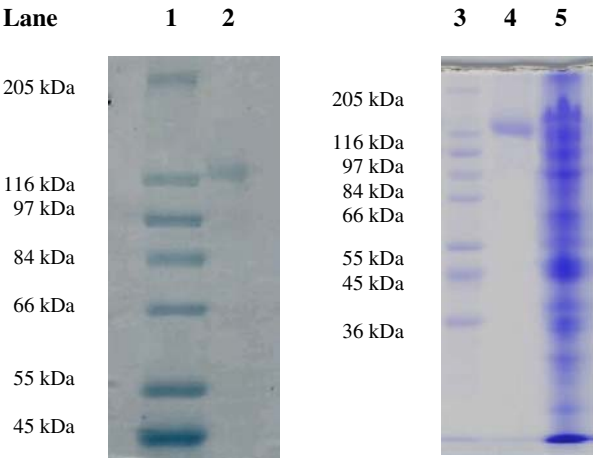

Fig. 2 SDS-PAGE of purified β-galactosidases. *Lane 1* and *3*: molecular mass markers, mass indicated alongside. *Lane 2*: purified β-gal 1. *Lane 4*: purified β-gal 2. *Lane 5*: Crude β-galactosidase preparation

Effect of Temperature and pH on Enzyme Activity

β-gal 1 and β-gal 2 retained 31 and 56% residual activity, respectively, at physiological temperature (Fig. 3). The relatively high temperature optima of the enzymes (B-gal 1in particular) could also render them of broader industrial interest for application in oligosaccharide production or for lactose hydrolysis during the milk pasteurization process. Both enzymes were active over a wide range of pH values characteristic of the stomach (Fig. 4). Gastric pH usually falls between 5.0–6.0 immediately after ingestion of a meal, but decreases progressively to approximately 2.0 due to stimulation of gastric acid secretion. Both enzymes would therefore likely be effective in hydrolyzing lactose *in vivo* during the gastric phase of digestion. On the basis of these parameters alone, there is little to distinguish the commercial enzymes [9] and β-gal 1 in the context of application as a digestive aid. β-gal 2 however retains activity over the full pH range likely encountered in the stomach, displaying almost 80% maximal activity at pH 2.0. Neither β-gal 1 (Fig. 4) nor the commercial enzymes [9] display significant activity at pH values significantly below 3.0. The modest dip in activity of both enzymes at pH 4.5 is unlikely to have any significant application-relevant consequences. Such twin-peak pH versus activity profiles have been reported for a range of additional extracellular microbial enzymes, including beta glucanases and phytases [25, 26].

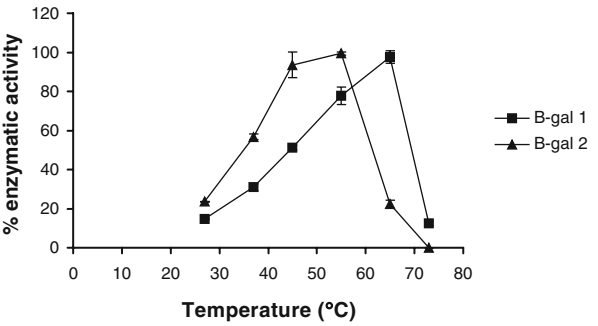

Fig. 3 Activity versus temperature profiles of the purified β-galactosidases. Enzyme activity is plotted as a % value relative to the activity displayed at the enzyme's optimum temperature. 100% activity equates to 0.5 U/ml absolute activity. Error bars indicate the standard deviation of the measured data values from the mean, $n=3$

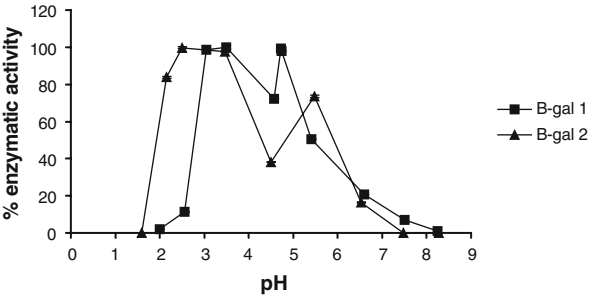

Fig. 4 Activity versus pH profiles of purified β-galactosidases. Enzyme activity is plotted as a % value relative to the activity displayed at the enzyme's optimum pH. 100% activity equates to 0.5 U/ml absolute activity. Error bars indicate the standard deviation of the measured data values from the mean, $n=3$

Stability When Subjected to Simulated Gastric Conditions

Purified β-gal 1 retained high levels of activity (70%) when subjected to simulated gastric conditions (pH 2.0 in the presence of pepsin) for 2 h (Fig. 5). Activity loss can be attributed almost entirely to low pH, likely causing enzyme denaturation although further analysis by, e.g. circular dichroism or X-ray diffraction would be required to confirm this. β-gal 2 retained less activity (20%) after the full 2 h incubation, with activity loss largely attributable to low pH, although pepsin had a statistically significant additive effect ($p<$ 0.01). In marked contrast we have already reported [9] and verified as part of this study, that the lactase active ingredient present in the 4 major commercialized digestive supplements are all completely inactivated when incubated under identical conditions for time periods as short as 1 min.

This data represent a worst-case scenario, as co-ingestion of a meal would increase the initial gastric pH encountered to well above fasted state levels for 30 min or more. Both commercial and test enzymes would therefore likely be active during the initial phase of

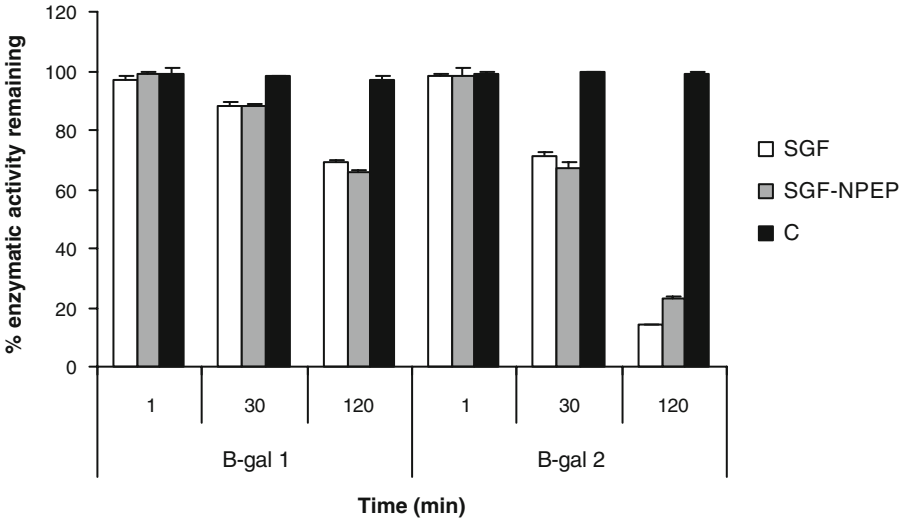

Fig. 5 The effect of in vitro simulated fasted gastric conditions on purified enzymes. SGF: enzyme incubated with simulated gastric fluid. SGF-NPEP: enzyme incubated, with SGF devoid of pepsin. *C*, control (enzyme incubated in 200 mM sodium acetate buffer pH 5.5). Enzyme activity is plotted as a % value relative to the activity displayed by the control incubated under the indicated conditions for 1 min. 100% equated to 0.5 U/ml. Error bars indicate the standard deviation of the measured data values from the mean, $n=3$

gastric digestion. However, the test enzymes alone would likely retain any activity after this initial phase, potentially providing significantly extended contact time between active enzyme and substrate. Food typically remains in the stomach for up to 2 h [27].

The commercial enzymes could be protected from gastric inactivation by enteric coating, and two of the four commercial enzymes tested are enteric coated, ensuring their release only in the small intestine. However, pH versus activity profiles of the commercial enzymes [9] suggests that these enzymes could retain only between 10–20% of maximum activity within the small intestine.

Kinetic Properties, Isoelectric Point and Glycosylation Analysis

Kinetic constants (K_m and V_{max}) determined at the optimum pH and temperature of the enzymes, are presented in Table 2. β-gal 1 displayed a pI of 4.5 while that of β-gal 2 was 4.1. Electrophoretic analysis of the N-glycosidase F treated enzymes indicated that both enzymes contain two N-linked glycan chains. The total reduction in the molecular mass of the enzymes after full N-deglycosylation was similar (20 and 18%).

Kinetic analysis indicates that the enzymes have a higher affinity for the synthetic substrates, which is possibly due to the greater hydrophobicity of the aglycon moieties [28]. β-gal 1 displays a higher K_m value for ONPG than that recorded for other *Aspergilli*. K_m values with ONPG ranging from 0.72 to 2.4 mM have been reported for β-galactosidases isolated from *Aspergilli* [23, 29]. The β-gal 1 K_m for lactose (82.68 mM) is higher than that recorded for an enzyme from an *Aspergillus oryzae* strain but is similar to that recorded for other *Aspergilli* including *A. nidulans* (71 mM), and various strains of *A. niger* (85–125 mM) [30].

β-gal 2 displays a lesser affinity for lactose (K_m of 309 mM), highlighting the differences between the two enzymes. β-Galactosidases exhibiting similar K_m values for ONPG include the *Bacillus megatarium* and *Lactobacillus helveticus* enzyme [1]. The V_{max} for β-gal 1 on lactose is similar to that reported for *A. oryzae* by Tanaka et al., [31] (121.9 $U.mg^{-1}$) and *A. niger* by Widmer and Leuba [30] (104 $U.mg^{-1}$).

Recorded pI values fall within the range typical for previously characterized fungal β-galactosidases and the total carbohydrate content is similar to that reported for an *A. niger* β-galactosidase isoform [1, 32].

Peptide Mass Fingerprinting and MS/MS Analysis

Peptide mass data from deglycosylated, trypsin-digested, purified β-gal 1 and β-gal 2 obtained directly from SDS PAGE gel bands did not closely match any mass data derived from protein sequences in the NCBInr database by the Mascot tool. The peptide mass

Table 2 Kinetic properties of the purified enzymes.

	Substrate	K_m (mM)	V_{max} (μmol.min^1.mg-^{1})	K_{cat} (s^1)	K_{cat}/K_m ($M.s^{-1}$)
β-gal 1	ONPG	2.63	1,200	440	1.67×10^5
	PNPG	1.60	200,000	74,000	4.62×10^7
	Lactose	82.68	146	55	6.62×10^2
β-gal 2	ONPG	0.563	75	96	1.71×10^5
	PNPG	1.165	11,249	15,754	1.35×10^7
	Lactose	308.79	9.3	13	4.2×10^1

fingerprints obtained for the enzymes were distinct but did share three identical peptide mass values (1,060, 1,275 and 1,296 Da). MS/MS analysis was carried out on the shared peptides and ion data from the 1,275 Da shared peptide returned a hit in the NCBInr database. The peptide sequence was identified as GPLNEGGLYAER. Interrogation of the database showed this sequence to be conserved amongst several fungal, particularly *Aspergillus* derived β-galactosidases [21].

Differences in molecular weight and other properties, including mass spectrometry based analysis, although very strongly suggestive, do not prove conclusively that β-gal 1 and β-gal 2 enzymes are different gene products as opposed to, e.g., differentially glycosylated forms of the same gene product. Direct cloning and sequencing experiments would be the most convenient way to unambiguously resolve that issue.

General Conclusion

Commercial lactase digestive supplements have been on the market for many years and invariably contain 'off the shelf' lactase enzymes not specifically developed for this purpose. The often sub-optimal responses recorded in clinical studies is likely due, in part at least, to this fact.

Unlike the commercialized enzymes, both purified β-galactosidases (particularly β-gal 1) proved stable when incubated under simulated gastric conditions. β-gal 2 (in contrast to beta-gal-1) also displayed very significant activity levels at pH 2.0. In this context the two enzymes complement one another and a crude preparation of both could well prove of interest as stomach-active supplemental lactases. Definitive proof of their suitability, either individually or in combination however would require direct clinical trials.

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