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Physicochemical Characteristics of Commercial Lactases Relevant to Their Application in the Alleviation of Lactose Intolerance

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Abstract

Selected microbial lactases are used to treat lactose intolerance. A series of experiments were carried out in vitro in order to determine the likely relative suitability of four major commercial lactase products used in this regard. The lactases displayed between 55 and 61% of maximum activity at 37°C and significant activity between pH 3.0 and 6.5. They retained between 0 and 65% of original activities after exposure to full simulated digestive tract conditions for 6 h. All four enzymes proved to be particularly acid sensitive and only two products were enteric coated.

The products demonstrated varying ability to hydrolyze lactose under simulated digestive tract conditions. The most effective product hydrolyzed 2.7 g lactose per capsule, suggesting that consumption of several capsules, as opposed to the usually recommended one or two, would be required to hydrolyze the entire 12 g lactose load characteristic of a dairy-based meal. All enzymes were substantially pure and displayed similar kinetic properties and molecular weights. None appeared ideally suited for use in the alleviation of lactose intolerance. The findings may in part explain the variability and often disappointing results previously reported for lactase-based clinical trials and will provide comparative baseline data against which candidate second-generation lactases may be assessed.

Index Entries: Lactase; β-galactosidase; lactose intolerance; gastrointestinal.

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Introduction

Lactose, or milk sugar, is a disaccharide found in the milk of most mammals. It is digested in vivo in the gastrointestinal tract by lactase phlorizin hydrolase (LPH), a membrane-bound enzyme of the small intestinal epithelial cells *(1)*. Intestinal lactase (β-galactosidase) insufficiency results in lactose maldigestion (inability to digest lactose but no symptoms experienced) and lactose intolerance (maldigestion with negative clinical symptoms). The incidence of lactase deficiency is reported to be of the order of 75% of the world's population, and is most prevalent amongst populations in Asia and the United States. LPH deficient populations have difficulty in consuming milk and other lactose-containing products in that its ingestion can result in abdominal pain, diarrhea, and flatulence *(2–4)*.

Digestive supplementation of lactase enzyme is the principal treatment for alleviating symptoms of lactose intolerance, other than the complete avoidance of lactose-containing foods *(5)*. The lactose content of milk may also be reduced/eliminated by its pre-treatment with lactase prior to consumption. This approach however has found limited application, as the resultant milk tastes sweet. There is significant demand for digestive supplements to alleviate lactose intolerance and many lactase-containing products are marketed as over the counter remedies. The majority of these digestive supplements are administered orally in tablet/capsule form.

Numerous clinical studies of lactase-based digestive supplements undertaken have reported mixed and often limited success in the alleviation of the symptoms of lactose malabsorption *(6–13)*. The principal enzymes exploited for use in such commercial supplements are obtained from GRAS listed fungi such as *Aspergillus niger* and *Aspergillus oryzae (5,14)*. *A. oryzae*-derived lactase has been preferred because of its higher residual activity at the natural pH of milk (pH 6.7) *(15,16)*. The relevance of this characteristic in enzyme replacement therapy is, however, questionable. The conditions experienced in the digestive tract are of obvious relevance in determining the suitability of a lactase enzyme for optimal hydrolysis of lactose in vivo *(7)*. Digestive proteases and gastrointestinal pH all influence enzymatic activity during transit through the gastrointestinal tract *(17)*. To date, no comparative study appears to have been published on the catalytic performance of commercial lactase digestive supplements when exposed to digestive tract influences. This is surprising given the variability in clinical trial results reported. In vitro simulation of digestive tract conditions is commonly employed to predict stability and efficiency of digestive enzymes for both human and animal use *(17–22)*. It is the purpose of this study to determine and compare physicochemical characteristics of four major commercial lactases relevant to their effective functioning in vivo.

Materials and Methods

Materials

Over-the-counter lactase digestive supplements were obtained from McNeil Consumer Products Co., 50 Commerce Dr., Trumbull, CT ("Lactaid" 9000 FCC lactase units); Plainview LLC, West Long Branch, NJ ("Dairy-care," 3000 FCC units and *Lactobacillus acidophilus*); Gelda Scientific, 6320 Northwest Dr., Mississauga, ON, Canada ("Lacteeze," 4000 FCC units), and The Natural Medicine Co., Burgage, Blessington, Co. Wicklow, Ireland ("Lifeplan," 2000 FCC units). All other chemicals and reagents were of analytical grade and purchased from Sigma-Aldrich, Dublin, Ireland.

Extraction of Enzyme

Extraction of lactase from over-the-counter lactase digestive supplements was achieved by adding one tablet or capsule to 10.0 mL of 0.2 *M* sodium acetate buffer pH 5.5 followed by incubation in an orbital shaker (37°C, 200 rpm) for 20 min. Any insoluble material remaining was removed by centrifugation (10,000*g*, 10 min.).

Enzyme Assay

The lactase assay used was based on the method of Rasouli and Kulkarni *(23)* with some modifications. The assay system contained 0.8 mL of 5 m*M* ortho-nitrophenyl-β-D-galactopyranoside (ONPG) in 200 m*M* sodium acetate buffer pH 5.5 and 0.2 mL of suitably diluted enzyme. After co-incubation (15 min at 45°C), the assay was terminated by the addition of 1.0 mL of 0.1 *M* Na_2CO_3. One unit of lactase activity was defined as the amount of enzyme capable of releasing 1 µmol of *O*-Nitrophenol per minute under the defined assay conditions. All activity determinations were carried out in triplicate and with appropriate blanks.

Effect of Temperature and pH on Enzyme Activity

Temperature versus activity profiles were determined according to Nagy et al. *(24)*, by measuring lactase activity at 25–85°C. pH vs activity profiles were determined according to the method of Shaikh et al. *(25)*, by measuring lactase activity at pH 1.5–8.0, 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).

Stability of Lactases When Subjected to Simulated Gastrointestinal Conditions

The effect of simulated gastrointestinal tract conditions on lactase activity (both intact tablet/capsule and on enzyme extracted from the tablet/caplets) was determined using the modified methods of Xenos et al.

(17), Galia et al. *(26)*, and Ingels et al. *(27)*. Residual lactase activity (calculated relative to enzyme maintained in 200 m*M* sodium acetate buffer, pH 5.5) was determined after exposure to (a) simulated gastric digestion at pH 2.0 for periods ranging from 1 min to 2 h; (b) simulated upper intestinal digestion at pH 6.8 for 4 h; and (c) a combination of one following the other.

Simulation of gastric digestion entailed co-incubation (at 37°C and 150 rpm for time periods ranging from 1 min to 2 h) of either an intact tablet/capsule or 5.0 mL of suitably diluted (in distilled H_2O) enzyme extracted from the tablet/capsule, with 5.0 mL of simulated gastric fluid (SGF). SGF (pH 2.0) was prepared as per the United States Pharmacopoeia (USP) *(28)* and contained (per L) 3.2 g porcine pepsin, 7.0 mL HCl (6 *M*), and 2.0 g NaCl. A separate series of incubations were undertaken in which pepsin was omitted from the SGF ('SGF-PEP') 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.

Simulation of intestinal digestion entailed co-incubation (37°C, 150 rpm for 4 h) of either an intact tablet/capsule or 5.0 mL of suitably diluted (in distilled H_2O) enzyme extracted from the tablet/capsule, with 5.0 mL of simulated intestinal fluid (SIF). SIF (pH 6.8) was prepared as per the USP *(28)* and contained (per L) 10.0 g porcine pancreatin, 8.6 g monobasic potassium phosphate and 77 mL 0.2 *M* NaOH. Two separate series of incubations were also undertaken in which either pancreatin was omitted from the SIF ('SIF-PAN') or in which porcine bile salts (5 m*M*) were included (SIF+BS) but which were identical in all other respects. After incubation all samples were pH adjusted to 5.5 (using 0.5 *M* HCl) followed by activity assay.

Determination of the Efficacy of Commercial Lactases in Hydrolyzing Lactose Under Simulated Gastrointestinal Conditions

One intact tablet/capsule was co-incubated with 20 mL of SGF and 23 mL of a 53% (w/v) lactose solution at 37°C and 100 rpm for 120 min. The solution pH was then adjusted to 6.8 with 0.2 *M* NaOH immediately followed by the addition of 20 mL of SIF+BS, with further incubation at 37°C and 100 rpm for 240 min. Subsequently, solutions were placed on crushed ice and the pH adjusted to more than 9.0 with 20 mL of 0.1 *M* Na_2CO_3. The levels of glucose produced were determined in triplicate using a glucose trinider detection kit (Sigma Diagnostics) as per manufacturer's instructions.

Electrophoretic Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using an 8% gel and a vertical electrophoretic system *(29)*. Non-denaturing electrophoresis was undertaken in the same manner but with omission of SDS from the gel running and loading buffers,

and the sample was not pre-treated under denaturing conditions. Protein staining was carried out using Coomassie R-250. Activity staining was carried out by incubating the native gel with ONPG substrate solution for 30 min at 45°C and 100 rpm.

Native Molecular Mass Determination

Native enzyme molecular mass was determined by gel filtration chromatography, using a 150 mL Sephacryl S200 column and 100 m*M* sodium acetate-acetic acid buffer pH 5.5 as running buffer. Protein standards (10 mg/mL) used were albumin (bovine serum, MW 66,000), alcohol dehydrogenase (yeast, MW 150,000), β-amylase (sweet potato, MW 200,000), carbonic anhydrase (bovine erythrocytes, MW 29,000), and cytochrome *c* (horse heart, MW 12,400).

Determination of Kinetic Properties

Kinetic properties were determined with respect to lactose under optimal assay conditions of pH 4.5 and 55°C. A lactose concentration range of 50–600 m*M* was used. Enzymatic hydrolysis of lactose was estimated (in triplicate) by determining glucose levels released using a trinider detection kit (Sigma Diagnostics) as per manufacturer's instructions. Lineweaver–Burk plots and initial velocity against substrate concentration plots were constructed and kinetic constants K_m (Michaelis constant) and V_{max} (Maximum velocity) were estimated.

Results and Discussion

Effect of Temperature and pH on Enzyme Activity

All four preparations display the same optimal temperature for activity (55°C) and have very similar activity-vs-temperature profiles (Fig. 1). The percentage relative activity of the digestive enzymes at physiological temperature (37°C) was found to range from 55 to 61% of the maximum activity. Product information provided with the digestive supplements stated that all the preparations were derived from *Aspergilli* (products 1, 2, and 4 contained enzyme derived from *A. oryzae* whereas product 3 enzyme was derived from an "*Aspergillus* sp."). These data hint that all four enzymes may be produced by closely related fungal strains. The temperature optimum (55°C) is in agreement with that obtained for a lactase characterized from a strain of *A. oryzae* by Park et al. *(30)*. The activity at physiological temperature reported by Park et al. *(30)* and Tanaka et al. *(31)* for lactases isolated from *A. oryzae* strains were in the region of 75% of optimal activity.

All four lactase supplements displayed very substantially similar pH profiles (Fig. 2), reinforcing the possibility that all four enzymes are sourced form similar strains. Significant levels of activity were recorded between pH 3.0 and 6.5. They all exhibited an optimum pH of 3.5–4.5. These pH profiles/optima are also similar to those reported in the literature for lactases produced by other strains of *Aspergilli (30,31)*.

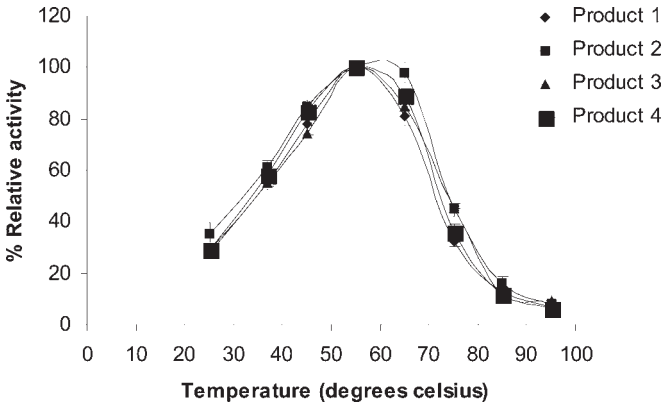

Fig. 1. Temperature vs activity profiles of the commercial lactase preparations (data expressed as a percentage of maximum activity). Each value represents mean ± SD (n = 3).

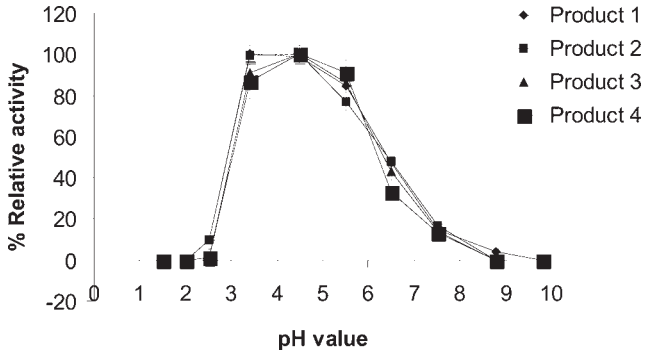

Fig. 2. pH vs activity profiles of the commercial lactase preparations (data expressed as a percentage of maximum activity). Each value represents mean ± SD (n = 3).

The pH profiles suggest that the enzymes, if released in the stomach, would be active only during the initial stages of gastric digestion. Immediately after initial ingestion of a typical meal, gastric pH is usually between 5.0 and 6.0 *(7,32)*. However, this falls progressively so that 30 min post ingestion the gastric pH is between 2.5 and 3.5 and falls further to values of 1.5–2.2 *(32,33)*. On the basis of pH all four enzymes would be catalytically active in the small intestine, although they would display less than 40% of maximum activity at mid duodenal pH (6.8) *(27,33,34)*.

Stability of Lactases When Subjected to Simulated Gastrointestinal Conditions

All four lactases, after extraction from the tablet/capsule format, lost total activity upon incubation under SGF conditions for 1 min (Table 1). Similar results were obtained when the extracted enzymes were incubated

Table 1
Effect of In Vitro Simulated Gastrointestinal Tract Conditions on Lactase Extracted From Digestive Supplements

Incubation time	Treatment	Relative activity ± SD (%) Product 1	Product 2	Product 3	Product 4
1 min	SGF[a]	0	0	0	0
	SGF-PEP[b]	0	0	0	0
4 h	SIF[c]	84 ± 3	94 ± 1	96 ± 2	93 ± 2
	SIF-PAN[d]	86 ± 1	110 ± 8	102 ± 2	101 ± 4
	SIF + BS[e]	85 ± 8	97 ± 1	95	91

Values expressed as a percentage of original activity (activity of a sample maintained at pH 5.5 and not exposed to any simulated digestive fluids). Each value represents mean ± SD ($n = 3$).

[a]Simulated gastric fluid; [b]simulated gastric fluid without pepsin; [c]simulated intestinal fluid; [d]simulated intestinal fluid without pancreatin; [e]simulated intestinal fluid with bile extract.

in SGF devoid of pepsin, indicating that activity loss was as a result of low pH.

In contrast, all four extracted enzymes were relatively stable when incubated under conditions simulating the small intestine, retaining between 84 and 96% of residual activity after a 4-h incubation period (Table 1). Omission of pancreatin resulted in a modest (but statistically significant, $p < 0.05$) increase in observed residual activity in the case of products 2, 3, and 4, indicating that these lactases were likely subject to partial proteolysis by the pancreatin proteolytic fraction. Inclusion of bile salts in SIF made very little difference to residual activity levels, suggesting that the commercial lactases were unaffected by the presence of these detergent molecules.

Incubation of the intact tablets/capsules under simulated gastric conditions resulted in the progressive and total inactivation of lactases present in products 1 and 3, whereas products 2 and 4 retained full/near full activity (Table 2). Product literature indicated that products 2 and 4 are enteric coated and these capsules did retain integrity during SGF incubation, protecting the enzymes therein from inactivation. Products 1 and 3 are apparently non-enteric coated and disintegrate over time in the SGF, exposing the enzymes to the denaturing influence of low pH. All four intact products disintegrated when incubated with SIF and retained full activity after a 4-h incubation period (Table 2). Resistance of other extracellular fungal enzymes to proteolysis by pancreatin has also been previously observed *(35)*. Predictably, only products 2 and 4 retained significant activity when incubated under full-simulated digestive tract conditions (2-h incubation with SGF immediately followed by a 4-h incubation with SIF, which is representative of the typical period of exposure to these conditions in vivo *[33,36]*).

Table 2
Effect of In Vitro Simulated Gastrointestinal Tract Conditions on Tablet/Capsule Forms of Digestive Supplements

Incubation time	Treatment	Relative activity ± SD (%) Product 1	Product 2	Product 3	Product 4
1 min	SGF	111 ± 3	111 ± 3	93 ± 2	97 ± 2
	SGF-PEP	104 ± 1	104 ± 1	94 ± 1	99 ± 2
30 min	SGF	29 ± 1	100 ± 1	1	95
	SGF-PEP	27	100	0	97
2 h	SGF	6	100	0	90
	SGF-PEP	2	100	0	90
4 h	SIF	105 ± 5	95 ± 1	95 ± 4	105 ± 4
	SIF-PAN	105 ± 1	97 ± 1	107 ± 3	112 ± 4
	SIF + BS	95 ± 1	133 ± 6	106 ± 10	144 ± 8
6 h	Complete GI tract (SGF + SIF + BS)	1 ± 1	63 ± 9	0	65 ± 17

Values expressed as a percentage of original activity (activity of a sample maintained at pH 5.5 and not exposed to any simulated digestive fluids). Each value represents mean ± SD (n = 3).

The results clearly demonstrate the necessity of enteric coating to prevent enzyme inactivation under low pH gastric conditions. It is important, however, to note that the simulated gastric data presented represent the worst case scenario, in that it represents the low pH (2.0) state. The fasted state was chosen as the most convenient and desirable time for administration of lactase digestive supplements is prior to or simultaneously to ingestion of a lactose load *(3,7,11)*, and that the option of administration prior to consumption of lactose load would afford the individual greater flexibility. However, if administered concurrently/directly after a lactose load, and if released in the stomach, the commercial lactases would likely display some activity initially, until the pH approaches/reaches 2.0.

Determination of the Efficacy of Commercial Lactases in Hydrolyzing Lactose Under Simulated Gastrointestinal Conditions

Predictably, only products 2 and 4 hydrolyzed any significant quantities of lactose upon subjecting the intact four commercial tables/capsules to simulated total digestive tract conditions in the presence of a 12 g lactose load (approximately the amount of lactose contained in a 200 mL glass of milk). The difference in hydrolysis levels achieved by these two products was not statistically significant ($p > 0.05$) (Fig. 3). Even in these cases, only a fraction of the available lactose was hydrolyzed (Fig. 3). Based on mean values, product 4 would achieve the greatest degree of lactose breakdown (2.7 g lactose/intact capsule), thereby requiring 4.4 tablet equivalents to completely hydrolyze the 12 g lactose load. Products 1 and 3,

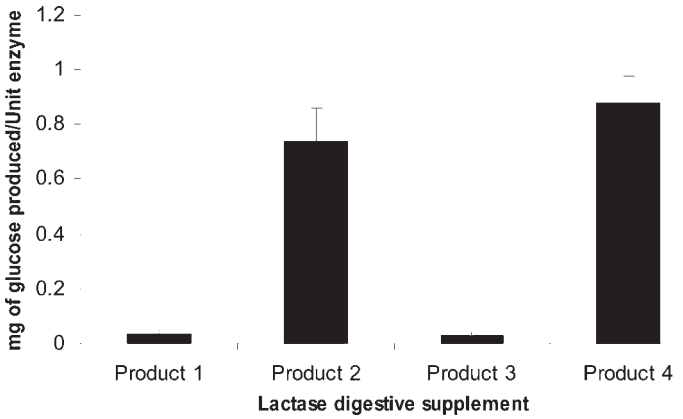

Fig. 3. Glucose levels (expressed as mg glucose produced per unit of activity), as produced by commercial lactase preparations under in vitro simulated gastrointestinal tract conditions. Error bars represent SD about the mean (n = 6).

Table 3
Summary of the Kinetic Properties of Digestive Supplement Enzymes on Lactose (K_{cat} = Catalytic Constant)

	K_m (mM)	V_{max} (μmol/min/mg)	K_{cat} (s^{-1})	K_{cat}/K_m (M/s)
Product 1	110	34,440	70,093	6.4×10^5
Product 2	260	34,636	65,864	2.5×10^5
Product 3	160	34,250	65,304	4.0×10^5
Product 4	120	25,789	50,947	4.2×10^5

if taken simultaneously/immediately after a lactose containing meal, would likely promote a significantly greater degree of lactose hydrolysis than that reported (Table 3), for the reasons outlined in the previous section. Overall, therefore, the data suggest that consumption of several tablets/capsules (as opposed to the usually recommended one to two tablets) would be required to hydrolyze most/all the lactose load characteristic of a dairy-based meal.

Electrophoretic and Chromatographic Analysis

The purity and the denatured molecular masses of the lactases formulated in the four digestive supplements were determined by SDS-PAGE (Fig. 4A). The SDS gels reveal the products, particularly products 1–3 to be very substantially pure. Although this may reflect chromatographic purification post fermentation, it could also reflect the fact that, through careful strain development and optimization of fermentation, lactase represents the sole protein produced in significant quantities. The high degree of purity is also reflected in the native gel-banding patterns (Fig. 4B). The diffuse nature of these bands is characteristic of many extracellular fungal enzymes and may be as a result of heterogeneity of glycosylation. Confirmation that

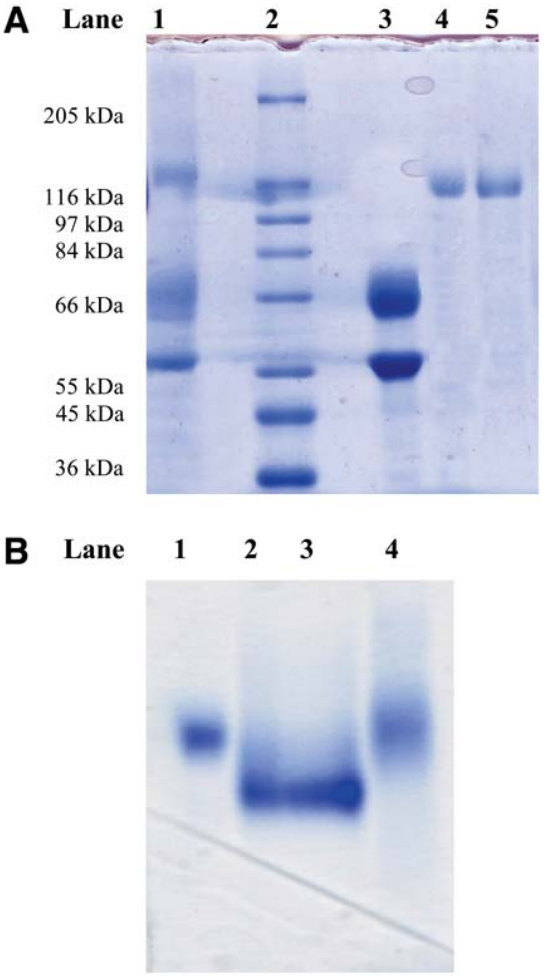

Fig. 4. **(A)** SDS-PAGE gel of the commercial lactase preparations. Lane 1: product 4, lane 2: molecular mass standards, lane 3: product 1, lane 4: product 2, lane 5: product 3. **(B)** Native PAGE gel of the commercial lactase preparations. Lane 1: product 1, lane 2: product 2, lane 3: product 3, and lane 4: product 4. All gels were stained using Coomassie blue R250.

the protein bands observed in the native PAGE gel possessed lactase activity was obtained by zymogram (data not shown).

A combination of electrophoretic and gel filtration analysis reveal product 1 enzyme to be a heterodimer, with the two subunits displaying masses of 68 and 57 kDa. The combined molecular mass of the subunits is 125 kDa, which is similar to that obtained when confirming the native molecular mass (124 kDa) by gel filtration.

Products 2 and 3 enzymes are monomeric, with molecular masses estimated at 115 kDa by both SDS-PAGE and gel filtration. SDS-PAGE analysis of product 4 reveals three main bands (57, 68, and 122 kDa), whereas gel filtration analysis reveals a native molecular weight of 124 kDa.

Determination of Kinetic Properties

The enzyme followed Michaelis-Menten kinetics with modest product inhibition by galactose. This concurs with the widely accepted model for fungal lactases. Product 1 and 4 enzymes display similar K_m values (110 and 120 m*M*, respectively; Table 3). The Product 2 and 3 enzymes displayed higher K_m values of 260 and 160 m*M*. Such values are in broad agreement with those previously reported for *Aspergillus*-derived lactases. The enzyme from *A. oryzae* studied by Park et al. *(30)* displayed a K_m for lactose of 50 m*M*. Lactase isolated from an *A. niger* strain by Widmer and Leuba *(37)* displayed a K_m for lactose of 85–125 m*M*.

Similar V_{max} values were recorded for products 1–3, whereas that of product 4 was significantly lower. These values are significantly higher than those reported for *A. nidulans* (0.4 μmol/min/mg of protein) *(38)*, *A. niger* (51.2 U/mg) *(39)*, *A. oryzae* (121.9 U/mg) *(31,* and *A. niger* by Widmer and Leuba *(37)* (104 U/mg). There are few reported values of the K_{cat}/K_m ratio obtained for other lactases in the literature. The values recorded for the *Pseudoalteromonas* enzyme (47.5 M^{-1} s^{-1}) by Turkiewicz et al. *(35)* and *Arthrobacter* sp. enzyme (218 mM^{-1} s^{-1}) characterized by Karasova-Lipovova et al. *(40)* are much lower than those reported here. The kinetic properties displayed by the four commercial enzymes may well have been significantly optimized by developmental programs.

Conclusions

Despite publication of numerous clinical trials, no comparative assessment of the stability and activity of commercial lactase supplements, when subjected to simulated gastrointestinal tract conditions, could be found in the published research literature. In addition to providing a possible molecular insight into the often disappointing and variable results reported by clinical trials, the findings provide baseline data against which candidate second-generation lactases may be initially assessed. Enzyme companies such as Solvay enzymes and Novozymes are currently revisiting the digestive enzyme market, with a view to generating next generation products *(41)*. The data suggest that none of the current commercial preparations fully meet criteria likely characteristic of an ideal supplemental lactase. It illustrates the importance of enteric coating to protect current supplemental enzymes from the effects of low pH gastric state and suggests that higher dosage levels than those currently recommended may be required to achieve complete/near complete hydrolysis of the lactose load characteristic of a typical dairy-based meal.

Acknowledgments

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