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Purification and Properties of a β-Galactosidase With Potential Application as a Digestive Supplement

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Abstract

Functional-based screening of crude β-galactosidase activities from 42 yeast strains resulted in the selection of a single enzyme of potential interest as a digestive supplement. β-Galactosidase produced by *Kluyveromyces marxianus* DSM5418 was purified to homogeneity by a combination of gel filtration, ion-exchange, and hydroxylapatite chromatographies. The denatured (123 kDa) and native molecular masses (251 kDa) suggest that the enzyme is a homodimer. The optimum pH and temperature of the purified enzyme were 6.8 and 37°C, respectively. The unpurified β-galactosidase in particular displayed a high level of stability when exposed to simulated intestinal conditions in vitro for 4 h. Matrix-assisted laser desorption ionization mass sectrometry analysis revealed that the enzyme's trypsin-generated peptide mass fingerprint shares several peptide fragment hits with β-galactosidases from *Kluyveromyces lactis*. This confirms the enzyme's identity and indicates that significant sequence homology exists between these enzymes.

Index Entries: β-Galactosidase; *Kluyveromyces marxianus*; lactose intolerance; small intestinal conditions; peptide mass fingerprinting.

Introduction

Lactose is a disaccharide found in the milk of most mammals, usually at concentrations approaching 5% (w/v). It is hydrolyzed in vivo within the gastrointestinal tract by lactase phlorizin hydrolase (LPH or lactase), a membrane-bound enzyme of the small intestinal epithelial cells *(1)*. Intestinal lactase insufficiency results in lactose maldigestion and in some cases

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lactose intolerance. Digestive supplementation with exogenous lactase is the principal treatment for alleviating symptoms of lactose intolerance *(2)*. Numerous clinical studies with such lactase supplements have reported largely limited success in treating this condition *(3–5)*.

β-Galactosidases (EC 3.2.1.23) derived from various strains of *Kluyveromyces* and *Aspergilli,* have been utilized industrially for many years. Microbial β-galactosidases find application in the hydrolysis of lactose in whey and for the production of galactooligosaccharides *(6)*. Lactase digestive supplements also contain microbially derived β-galactosidases invariably obtained from the generally recognized as safe listed fungi, such as *Aspergillus niger* and *Aspergillus oryzae (3–7)*.

Studies in our laboratory suggested that these commercialized products are not ideally suited to lactose hydrolysis in vivo if their physicochemical characteristics are considered in the context of conditions encountered in the upper digestive tract *(8).* These supplemental lactases would be active mainly during the initial stages of gastric digestion but are significantly less suited to act in the small intestine owing to their low pH optima (pH 3.0–5.0, and displaying <30% residual activity at neutral pH values).

In this article, we describe the identification, purification, and characterization of a neutral lactase from *Kluyveromyces marxianus* that would likely be significantly more active in the small intestine than current commercial products.

Materials and Methods

Microbial Strains, Chemicals, and Equipment

Microbial strains were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) (Braunschweig, Germany). The chromatographic system, columns and media, polybuffer 74, isoelectric focusing (IEF) standards, and ampholyte 3-10 were obtained from Bio-Rad (Hercules, CA). An N-glycosidase F deglycosylation kit was obtained from Roche (Penzberg, Germany). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration molecular mass markers, along with all additional reagents, were obtained from Sigma-Aldrich (Dublin, Ireland).

Cultivation of Organism and Enzyme Production

Yeast strains were maintained on universal medium for yeasts (YM and YPD) *(9)*. Initial screening studies were undertaken according to the method of Nevalainen *(10)*. Enzyme was produced via submerged fermentation in a lactase-inducing medium (100 mL of medium in 500-mL shake flasks incubated at 37°C and 200 rpm for 48 h). The medium contained 10% (w/v) lactose, 0.3% (w/v) K_2HPO_4, 0.5% (w/v) yeast extract, and 0.3% (v/v) 5 *N* NH_4OH, pH 4.5, as described by Mahoney et al. *(11)*. Cellular

disruption was achieved by sonication (Soniprep 150 probe sonicator) of cell paste resuspended (10% [w/v]) in 0.1 *M* Tris-maleate buffer, pH 7.0, with 0.5 m*M* $MgSO_4$, 0.1 m*M* $MnCl_2$, 0.5 m*M* KCl, 1 m*M* dithiothreitol (DTT), and 1 m*M* phenylmethylsulfonyl fluoride (PMSF) *(12)*. Cellular debris was removed from the crude enzyme by centrifugation (12,000*g*, 4°C, 10 min).

Enzyme Assay

β-Galactosidase activity assay was based on the method of Mahoney and Whitaker *(13)*. Ortho-nitrophenyl-β-d-galactopyranoside (ONPG) substrate was dissolved to a final concentration of 5 m*M* in 200 m*M* potassium phosphate buffer, pH 6.8. One unit of β-galactosidase activity was defined as the amount of enzyme capable of releasing 1 μmol of *O*-nitrophenol/min under the defined assay conditions.

Enzyme Purification

Ion-exchange chromatography was carried out using a 1 × 10 cm econo-column, packed with DEAE-Sepharose CL 6B (5-mL bed volume). The column was preequilibrated with 100 m*M* Tris-maleate buffer, pH 7.0, containing 0.5 m*M* $MgSO_4$, 0.1 m*M* $MnCl_2$, 0.5 m*M* KCl, 1 m*M* DTT, and 1 m*M* PMSF (running buffer). After extract application, the bound β-galactosidase was eluted from the column using running buffer with an ascending linear salt gradient (0–250 m*M* NaCl). The partially purified enzyme was subsequently applied to a 2 × 35 cm Econo-column packed with Sephacryl S200 HR (100-mL bed volume) and equilibrated with running buffer. The final chromatographic step was hydroxylapatite chromatography. A 1 × 10 cm Econo-column was packed with macro-prep ceramic type 1 hydroxylapatite medium (5-mL bed volume). The column was preequilibrated with running buffer (10 m*M* potassium-phosphate buffer, pH 6.8, with 0.5 m*M* $MgSO_4$, 1 m*M* DTT, and 1 m*M* PMSF). Bound protein was eluted by an ascending running buffer concentration gradient from 10 to 400 m*M* potassium phosphate. All chromatography was performed at 4°C using a biologic LP chromatography system. Protein concentration was determined by the method of Bradford *(14)*, using bovine serum albumin as the standard.

Electrophoretic Analysis and Determination of Molecular Mass

SDS-PAGE was carried out using an 8% gel and a vertical electrophoretic system as described by Laemmli *(15)*. Protein bands were visualized by staining with Coomassie brilliant blue R-250. Native molecular mass was determined using a Sephacryl S300 HR gel filtration column with a bed volume of 200 mL.

Determination of Isoelectric Point and Glycosylation Analysis

Polyacrylamide gel IEF was carried out according to the method described by Bio-Rad *(16)*. Glycosylation analysis (N-linked) was carried out using an N-glycosidase F deglycosylation kit in accordance with the manufacturer's instructions.

Effect of Temperature and pH on Enzyme Activity

Temperature vs activity profiles (20–55°C) were determined according to the method of Nagy et al. *(17)*. pH vs activity profiles (3.5–10.0) were determined according to the method of Shaikh et al. *(18)* using the following buffers: 0.2 *M* citric acid, pH 3.5–4.5; 0.2 *M* sodium acetate–acetic acid, pH 5.0–5.5; 0.2 *M* disodium hydrogen phosphate–sodium dihydrogen phosphate, pH 6.0–8.0; 0.2 *M* potassium chloride–boric acid, pH 8.5–10.0.

Enzyme Stability When Subjected to Simulated Intestinal Conditions

Simulation of small intestinal digestion using modified methods of Xenos et al. *(19)* and Ingels et al. *(20)* entailed coincubation (4 h, 37°C, 150 rpm) of 5 mL of suitably diluted (in dH_2O) enzyme with 5 mL of simulated intestinal fluid (SIF). SIF (pH 6.8) was prepared as per the *United States Pharmacopoeia (21)* and contained 6.8 g/L of monobasic potassium phosphate, 77 mL of 0.2 *N* NaOH, 10 g/L of porcine pancreatin mix, and dH_2O to 1 L. The pH of SIF was adjusted to 6.8 with 0.2 *M* HCl/NaOH. A separate series of incubations was undertaken in which pancreatin was omitted (SIF–NP) but in which all the other aforementioned conditions were identical. A second series of separate experiments was undertaken in which the SIF was supplemented with bile salts to a final concentration of 5 m*M* (SIF + BS). After incubation the pH of all samples was adjusted to 5.5 using 0.5 *M* NaOH and then activity assay was conducted.

Determination of Kinetic Properties

Kinetic properties were determined with respect to varying concentrations of chromogenic substrates ONPG (0.1–15 m*M*), para-nitrophenyl-β-D-galactopyranoside (PNPG) (0.5–16 m*M*), and lactose (10–600 m*M*) using the modified method of Chakraborti et al. *(22)*. Enzymatic hydrolysis of lactose was estimated by determining glucose levels produced using a trinider glucose detection kit according to the manufacturer's instructions. Kinetic constants were determined from Lineweaver-Burk plots and initial velocity against substrate concentration plots. The effect of potential inhibitors (galactose, glucose, sucrose, and isopropyl-β-D-thiogalactoside (IPTG) was investigated by enzyme assay in the presence of varying concentrations (5–50 m*M*) of the inhibitor.

Peptide Mass Fingerprinting

Gel slices of the protein bands were excised from a Coomassie-stained SDS-PAGE gel and supplied to Pinnacle Laboratory at the University of Newcastle Upon Tyne, UK. Trypsin digestion and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis (using an Applied Biosystems Voyager DESTR mass spectrometer) were carried out as described by Gonnet et al. *(23)* with subsequent submission of MALDI-MS data to the MASCOT database for protein identification *(24)*.

Results and Discussion

Initial Screening Studies

Initial functional screening studies focused on yeast-derived lactases, because these tend to display neutral pH optima, likely rendering them more suited to small intestinal application than the acid-active fungal lactases. All but 4 of the 42 yeast strains grew on solid medium containing lactose as the sole carbon source. However, only 21 strains produced detectable β-galactosidase activity when grown by inducing submerged fermentation. Functional screening entailed determination of application-relevant physicochemical characteristics of the unpurified preparations. These included the effect of temperature, pH, and simulated small intestinal conditions on enzyme stability and activity as well as 6-wk storage stability at 4°C and room temperature (data not shown). β-Galactosidase produced by *K. marxianus DSM5418* was found to be the sole enzyme displaying a combination of physicochemical characteristics potentially suited to application as a small intestine–targeted digestive aid. This enzyme was therefore chosen for purification and characterization in an application-relevant context. This strain (previously known as *Kluyveromyces fragilis*) had previously been identified as a high lactase producer *(11,25)* and was partially purified (purification factor of 15 with 9 bands evident on SDS-PAGE gels) *(12)*. The strain has also been investigated for use as baker's yeast *(26)* and for the production of single-cell protein, pectinase, and inulinase *(25,26)*.

Purification and Determination of Molecular Weight

The purification scheme employed yielded a purification factor of 115 with a percent yield of 53% (Table 1). This compares favorably with previously reported purification data for several *Kluyveromyces*-sourced β-galactosidases (e.g., see refs. *27* and *28*). The purified enzyme migrated as a single band on an SDS-PAGE gel, confirming purity (Fig. 1). The enzyme also retained good stability during purification. Instability/significant activity loss has been reported for several such yeast β-galactosidases during their purification (e.g., see ref. *29*). The estimated molecular mass of the purified enzyme using native (gel filtration) and denaturing (SDS-PAGE) analysis was 251 and 123 kDa, respectively, indicating that the active enzyme form is that of a homodimer. Mahoney and Whitaker *(12)* and Uwajima et al. *(27)* reported similar native molecular masses of 201 and 203 kDa for β-galactosidases from closely related strains. Similar native molecular masses have been reported for β-galactosidases from *Lactobacillus helveticus* (257 kDa) *(30)*, *Rhizomucor* sp. (250 kDa) *(18)*, and *Lactobacillus delbrueckii (31)*. The dimeric structure of β-galactosidases from *K. marxianus* strains has previously been reported *(12,32)*.

Determination of Isoelectric Point and Glycosylation Analysis

The isoelectric point of the purified enzyme was determined to be 5.1, which is broadly similar to values reported for additional *Kluyveromyces-*

Table 1
Purification for β-Galactosidase from *K. marxianus* DSM5418

	Total volume (mL)	Total protein (mg)	Total activity	Protein (mg/mL)	Activity (U/mL)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude	30	41.7	286.8	1.39	9.56	6.87	100	1.00
Ion exchange	88.5	3.097	238.7	0.035	2.6971	77.06	83	11.22
Gel filtration + ultrafiltration	46	1.058	222.3	0.023	4.833	210.13	77.5	30.59
Hydroxylapatite	120	0.1913	151.28	0.0016	1.2649	790.56	53	115.10

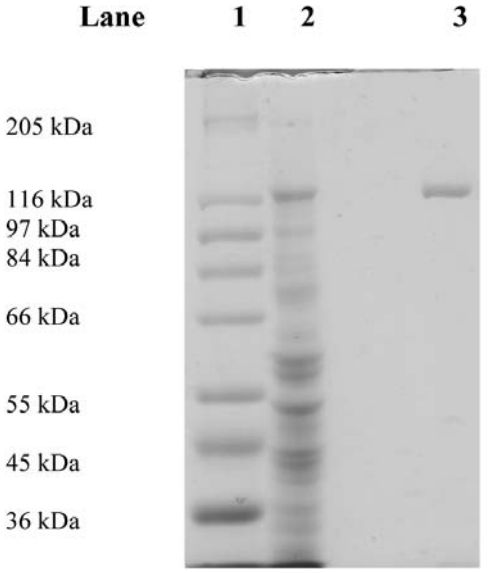

Fig. 1. SDS-PAGE of purified β-galactosidase from *K. marxianus* DSM5418. Lane 1, molecular mass markers; lane 2, crude intracellular enzyme; lane 3, purified β-galactosidase.

derived β-galactosidases *(27)*. Lack of glycosylation was suggested by failure of the protein bands on electrophoretic gels to stain with carbohydrate stain (data not shown). Treatment of the purified enzyme with N-glycosidase F also failed to reveal the presence of N-linked oligosaccharide chains, a finding in keeping with the protein's intracellular location.

pH and Temperature vs Activity Profiles

The pH vs activity profile of both the crude and purified enzyme was similar to that previously reported for *Kluyveromyces*-derived β-galactosidases *(13,27,33)*. The profile (pH optimum of 6.8, with 50% or greater residual activity between pH 5.6 and 7.8) suggests that it would be suitable for lactose hydrolysis in the small intestine. The pH of chyme typically ranges from 5.4 to 7.8, with a mean pH of 6.8 by the midduodenum *(34)*.

The temperature vs activity profile (temperature optimum of 37°C, with 50% or greater residual activity from 20 to 45°C) was similar to that derived from other *Kluyveromyces* strains such as *K. fragilis* and *K. lactis (7,32)* and indicates that the enzyme displays maximum activity at physiologic temperatures.

The facts that both crude and purified enzyme preparations display essentially identical pH vs activity and temperature vs activity profiles, and that no peak splitting is observed during chromatographic purification, strongly suggest that the yeast produces a single intracellular β-galactosidase.

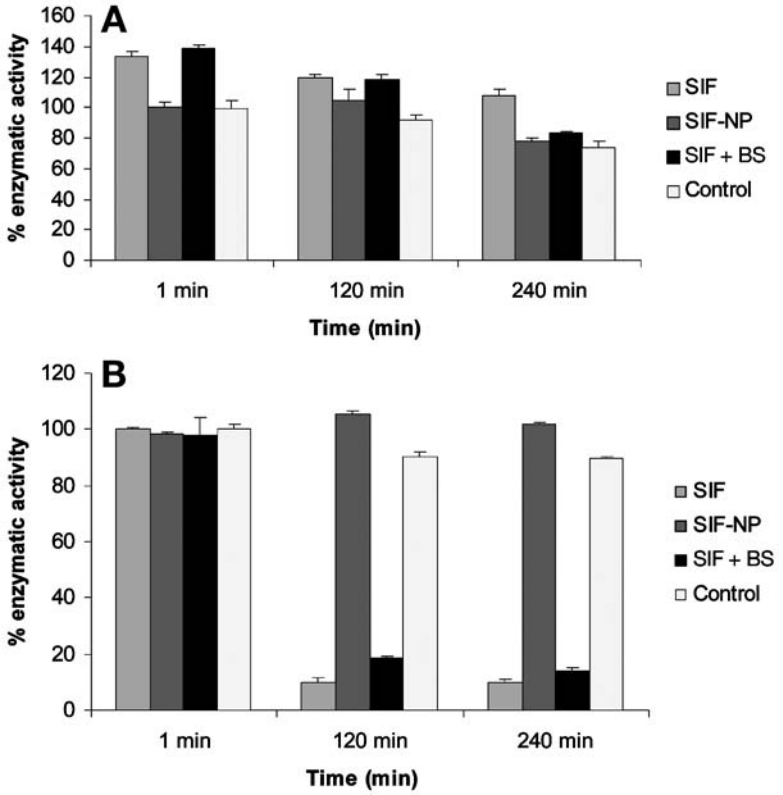

Fig. 2. Effect of in vitro simulated intestinal conditions on (A) crude and (B) purified intracellular β-galactosidase. SIF, enzyme incubated with simulated intestinal fluid; SIF–NP, enzyme incubated with SIF with omission of pancreatin; SIF + BS, enzyme incubated with SIF containing bile extract; control, enzyme incubated in 0.2 *M* sodium phosphate buffer, pH 7.0. The percentage of enzymatic activity remaining for the enzyme after subjection to various treatments was calculated as a percentage of the control incubated for 1 min. Error bars indicate the SD of the measured data values from the mean (n = 6).

Effect of Simulated Small Intestinal Conditions on Enzyme Activity

Incubation of the crude enzyme with SIF resulted in substantial enzyme activation relative to the control, an effect negated by the omission of pancreatin (Fig. 2A). Activation could be owing to a partial proteolytic cleavage of the β-galactosidase yielding a more active enzyme form, a phenomenon previously demonstrated in the context of several other enzymes *(35–37)*. Other possibilities could include pancreatin-mediated destruction of a lactase inhibitor present in the unpurified enzyme preparation or potentially the release of sulfhydryl groups believed to activate yeast lactases *(38)*. The addition of bile salts at physiologic concentrations to SIF had little effect at earlier incubation time points, whereas after 240 min a significant reduction in activity relative to SIF alone was observed. Over time the surfactant

action of the bile salts may be leading to partial enzyme inactivation. Overall, however, the crude β-galactosidase displays excellent stability when incubated over 4 h under simulated small intestinal conditions.

Incubation of the purified enzyme under simulated intestinal conditions, however, led to a far more significant degree of inactivation (Fig. 2B). Modest activation (about 10%) was evident relative to control only when enzyme was incubated in SIF devoid of pancreatin and at longer incubation time points, an effect for which there is no obvious explanation. SIF (with or without bile salts) resulted in significant activity loss over time (about 12% of residual activity was left after 240 min). Omission of pancreatin negated this effect, indicating that activity loss was owing to proteolytic degradation. The pure enzyme is likely susceptible to more extensive hydrolysis than the crude preparation, owing to the removal of alternative proteolytic targets (i.e., contaminating proteins) by purification.

From an application perspective, inactivation of the pure enzyme is unlikely to be of relevance. Supplemental digestive enzymes contain crude enzyme preparations and the supplemental enzyme would encounter digestive tract conditions in vivo in the presence of yet additional milk/other food proteins, which would afford it protection from extensive proteolytic degradation. From this perspective, the effect of simulated small intestinal influences on the crude enzyme preparation would be more physiologically relevant. While *Kluyveromyces*-derived lactases have been studied in the context of various industrial applications, to our knowledge this is the first time such an enzyme has been subjected to simulated digestive conditions.

Kinetic Studies

The kinetic constants presented in Table 2 show that the enzyme had greatest affinity for lactose (K_m = 4 m*M*). The enzyme was catalytically most efficient with PNPG as substrate. Santos et al. *(39)* reported a similar K_m value for soluble *K. fragilis* (marxianus) β-galactosidase (4.6 m*M*). The enzyme was most efficient in catalyzing the hydrolysis of PNPG with ONPG and lactose in that order. However, the kinetic constants of the enzyme in hydrolyzing lactose compare favorably with those reported for other microbial β-galactosidases *(13,40)*.

The enzyme followed Michaelis-Menten kinetics with modest product inhibition, a characteristic of many yeast β-galactosidases *(41,42)*. Table 3 presents the effects of native reaction products as well as some structural analogs on the purified enzyme. Most notable from an application perspective is the strong inhibition by galactose at low concentrations, an effect relieved at higher concentrations. This observation has been reported previously in the context of other lactases *(43)*. It is difficult to predict the application relevance of such inhibition. Lactose is present in bovine milk at high concentrations (260 m*M*), but the hydrolysis products would be quickly removed from the intestinal lumen via absorption.

Table 2
Summary of Kinetic Properties of Purified β-Galactosidase from *K. marxianus* DSM5418

	K_m (m*M*)	V_{max} (μmol/min/mg)	K_{cat} (s^{-1})	K_{cat}/K_m (*M*/s)
ONPG	4.7	1781	8559	1.9×10^6
PNPG	7.1	5.7×10^5	2.7×10^6	3.9×10^8
Lactose	4.0	1300	6300	1.58×10^6

Table 3
Determination of Effect of Potential Inhibitors on Enzymatic Activity of Purified β-Galactosidase from *K. marxianus* DSM5418[a]

Inhibitor	Relative activity (%)
Galactose	
5 m*M*	93 ± 7
25 m*M*	81 ± 6
50 m*M*	66
Glucose	
5 m*M*	86 ± 6
25 m*M*	96 ± 9
50 m*M*	105 ± 14
IPTG	
5 m*M*	91 ± 2
25 m*M*	85 ± 6
50 m*M*	84 ± 9
Sucrose	
5 m*M*	114 ± 1
25 m*M*	99 ± 6
50 m*M*	105 ± 11

[a]The percentage of relative activity was calculated as a percentage of the control assay value. The values displayed are the mean of triplicate experiments with the SD.

Peptide Mass Fingerprinting

Figure 3 presents the MALDI-MS chromatogram obtained from analysis of tryptic peptides of pure β-galactosidase from *K. marxianus* DSM5418. The peptide mass data were used to query protein sequence databases using the MASCOT tool. The masses of 25 tryptic peptide fragments matched the masses of theoretical tryptic peptides cleaved from NCBInr database deposited sequences of β-galactosidases derived from *K. marxianus* var *lactis* (score 2102) and *K. marxianus* var *marxianus* (score 2100) (NCBI

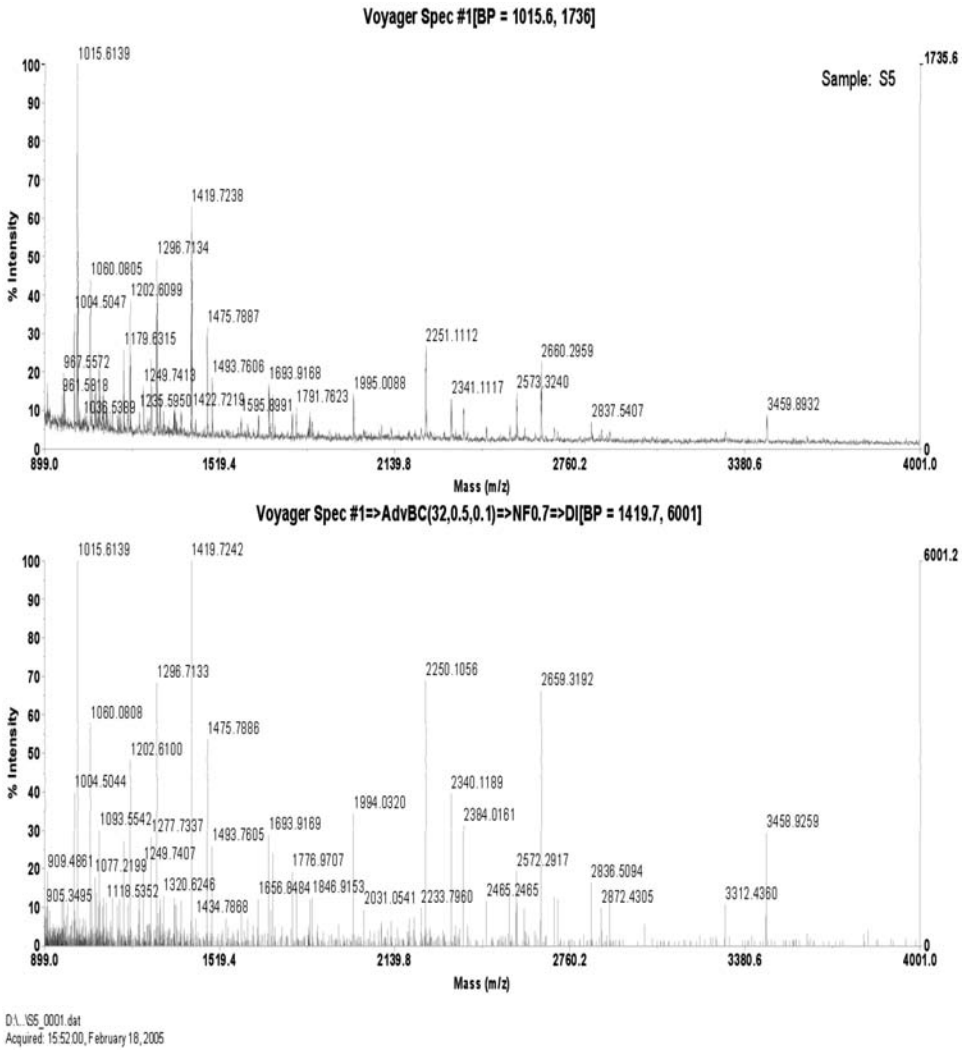

Fig. 3. MALDI-MS chromatogram of purified enzyme after cleavage with trypsin.

accession no. P00723 and Q6QTF4). The mass spectroscopic data therefore confirm the enzyme's identity and indicate that significant sequence homology exists with the deposited *Kluyveromyces* enzymes.

Conclusion

Although a number of *Kluyveromyces*-derived β-galactosidases have been well characterized in the literature, their stability under simulated intestinal conditions/potential application as a digestive supplement has not been considered. Purified β-galactosidase from *K. marxianus* DSM5418 displayed physicochemical properties broadly similar to those of previ-

ously characterized yeast β-galactosidases. The stability of the unpurified enzyme when exposed to simulated small intestinal conditions suggests excellent suitability as a small intestine–targeted supplemental lactase, unlike the major current commercial supplemental preparations. Transit time through small intestine is also twice that of stomach, facilitating longer duration of product action. The enzyme preparation would require enteric coating in order to protect it from stomach acid, but this is inexpensive and technically straightforward to achieve. The enzyme preparation could be administered instead of current preparations, or concurrently with them, in order to afford continuous lactose hydrolysis through both stomach and small intestine. Industrial-scale production would be aided by the fact that *K. marxianus* DSM5418 is a high lactase producer, and substantial work on fermentation optimization has already been undertaken *(44–46)*. However, its efficacy as a digestive supplement can ultimately be assessed only via clinical trials.

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