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Determination of the Secondary Structure of *Kluyveromyces lactis* β-Galactosidase by Circular Dichroism and Its Structure−Activity Relationship as a Function of the pH

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The secondary structure of *Kluyveromyces lactis* β-galactosidase was determined by circular dichroism. It is mainly a β-type protein, having 22% β-turns, 14% parallel β-sheet, 25% antiparallel β-sheet, 34% unordered structure, and only 5% α-helix. The structure−activity relationship as a function of the pH was also studied. The pH conditions leading to the highest secondary structure content (100% ellipticity) of the enzyme was found at pH 7.0; at pH 6.5−7.0, the percent ellipticity decreased slightly, suggesting little structural change, but the activity decreased significantly, probably because of variations in critical residues. On the other hand, at pH's above 7.0, a more noticeable change in ellipticity was observed due to structural changes; the CD analysis showed a small increase in the helical content toward higher pH, whereas the maximum activity was found at pH 7.5, meaning that the changes produced in the secondary structure at this pH favored the interaction between the enzyme and the substrate.

KEYWORDS: Lactase; *Kluyveromyces lactis* β-galactosidase; secondary structure; circular dichroism.

INTRODUCTION

The yeast β-galactosidase or lactase (E.C. 3.2.1.23) is a widely used enzyme in the dairy industry for solving lactose-intolerance problems as well as technical troubles such as the disaccharide crystallization and its low sweetening capability. The three yeast species used as sources of neutral lactase are *Kluyveromyces lactis*, *Kluyveromyces marxianus*, and *Candida kefyr*, with the former being the most important from the commercial point of view (*1*). The three yeast species are philogenetically very closely related; therefore, the produced lactases have very similar characteristics (*2*). Despite the commercial importance of the yeast lactases, to the best of our knowledge, very little has been reported about its secondary structure and the relationship between its conformation and activity; moreover, some discrepancies concerning the number and molecular mass of subunits are found in the literature (*3*). Poch et al. (*4*) inferred a secondary structure for the β-galactosidase from *K. lactis* consisting of 11 β-strands and two α-helices based on its primary structure deduced from the LAC4 gene. In the current work, the secondary structure of the β-galactosidase from *Kluyveromyces lactis* was determined by circular dichroism, and the structure−activity relationship of the enzyme as a function of the pH was also studied.

MATERIALS AND METHODS

Enzyme and Chemicals. Crude extract of the lactase from *Kluyveromyces lactis* was purchased as a commercial preparation, Maxilact LX-5000 (Gist Brocades, Delft, The Netherlands). Orthonitrophenyl-β-D-galactopyranoside (ONPG) was obtained from Sigma Chemical Co. (St. Louis, MO). Other chemicals used were of analytical grade. Distilled deionized water was used throughout.

Purification. A Bio-Logic HR chromatography system was used. The enzyme was purified by gel filtration [Bio-Silect SEC 250-5 (300 × 7.6 mm) column]. One milliliter (0.666 mg/mL) of Maxilact LX-5000 was applied to the column equilibrated with 0.01 M potassium phosphate buffer, pH 7.0. Elution was carried out with the same buffer, and the fractions containing lactase activity were pooled, concentrated, and rechromatographed under the same conditions. Final purification was accomplished by means of anion-exchange chromatography. Solutions of approximately 0.09 mg/mL were injected into a UNO-Q12 column. Protein components were eluted from the column at a flow rate of 2 mL/min employing a linear gradient of 1 M KCl in 0.01 M potassium phosphate buffer, pH 7.0. All equipment was from Bio-Rad Laboratories (Hercules, CA).

Determination of Lactase Activity. Lactase activity was measured at pH 7.0 and 37 °C in a solution of 0.034 M orthonitrophenyl-β-D-

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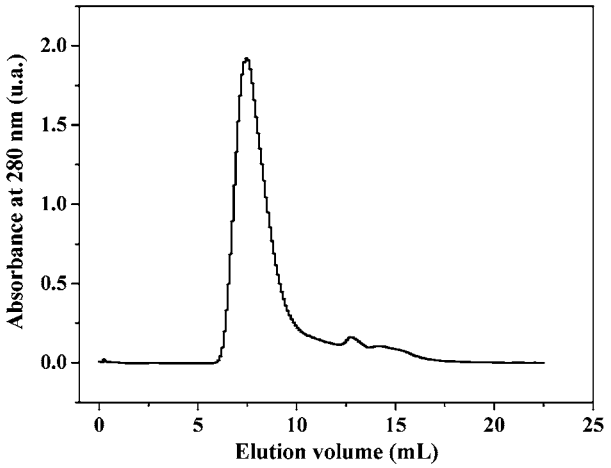

Figure 1. Gel filtration chromatography of β-galactosidase from *Kluyveromyces lactis* (Maxilact LX 5000). The sample was applied to a Bio-Silect SEC 250-5 (300 × 7.6 mm) column (Bio-Rad) and eluted with 0.01 M potassium phosphate buffer, pH 7.0.

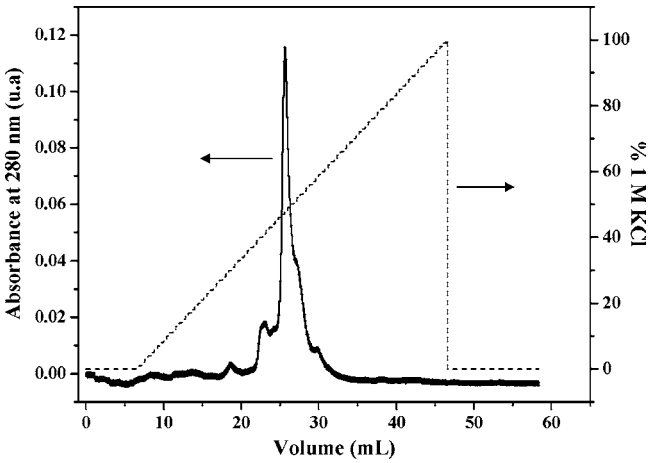

Figure 2. Purification of β-galactosidase from *Kluyveromyces lactis* (Maxilact LX 5000) by anion-exchange resolution chromatography. Solutions of gel filtration rechromatography with enzymatic activity were injected into a UNO-Q12 (Bio-Rad) column. Proteins components were eluted from the column at flow rate of 2 mL/min employing a linear gradient of 1 M KCl in 0.01 M potassium phosphate buffer, pH 7.0.

galactoside (ONPG, Sigma Chemical Co., St. Louis, MO) in 0.01 M potassium phosphate buffer as substrate, and enzyme activity was measured spectrophotometrically at 412 nm by means of a Shimadzu UV 160A spectrophotometer (Shimadzu Corporation, Kyoto, Japan) based on the release of orthonitrophenol (ONP). The effect of the pH on the lactase activity was determined in the range of 5.5−8.5.

Protein Concentration. During the different stages of purification, the total protein concentration was determined by Bradford's method (*5*).

Polyacrylamide Gel Electrophoresis. Enzyme purity was determined by native polyacrylamide gel electrophoresis (PAGE) (T = 7.5%), and the number of subunits in the pure enzyme was determined by denaturing SDS−PAGE. Electophoresis runs were performed in a mini-PROTEAN II cell, with a PowerPac-300 power supply using a constant voltage of 200 V, and gels were stained with Coomassie brilliant blue (0.25%) in a mixture of water, methanol, and acetic acid (50/40/10%) and/or Sypro orange protein stain; quantification was done by means of an image analyzer (Gel-Doc 1000 with Molecular Analyst software). All reagents and equipment were from Bio-Rad Laboratories (Hercules, CA).

Circular Dichroism (CD). CD spectra were recorded in a Jasco J-715 spectropolarimeter (Jasco, Tokyo, Japan) (*6*). Measurements in the far-ultraviolet region (195−240 nm) were performed on protein solutions (0.089 mg/mL) employing a 0.5-cm-path-length cell at 25 °C. Samples used in the CD studies were first dialyzed against deionized water (final pH 7.0). The mean residue ellipticity, $[\theta]_{mrw}$, was calculated using a mean residue molecular mass of 110 Da (*7*). To study the effect of pH on the conformation of the lactase from *K. lactis*, the pH was adjusted with either 1 M HCl or 1 M NaOH maintaining the same protein concentration in all of the samples, and the CD spectra were recorded under the same conditions running three scans for each sample. To estimate the content of secondary structure, the CD data were analyzed by the CDSSRT method (*8*−*10*) available in DICHROWEB (*11*, *12*).

RESULTS AND DISCUSSION

Purification. Figure 1 shows the elution profile obtained by gel filtration of the commercial lactase extract (Maxilact LX 5000). The fractions with enzymatic activity were pooled and rechromatographed under the same conditions. The rechromatographed protein peak (RCPP) had a specific activity of 1709 units/mg. This active preparation was then subjected to anion-exchage chromatography in the UNO-Q12 column. The purity of the enzyme was assessed by native PAGE, showing a single band (data not shown).

Figure 2 shows the elution pattern from the anion-exchange chromatography of the RCPP. Only one of the separated components showed lactase activity.

Electrophoresis. Denaturing gel electrophoresis (SDS−PAGE) demonstrated that the pure enzyme was formed by two monomers of 125.4 and 95.3 kDa (**Figure 3**). According to Becerra et al. (*3*), the *K. lactis* lactase can be found as a dimer of approximately 240 kDa formed by two monomers of 124 kDa; although this molecular mass is very close to the value we found (220.7 kDa), there is a difference in the size of one of the monomers. It is noticeable in the literature (*3*) that there are discrepancies concerning the number and molecular masses of the subunits of *K. lactis* lactase. Some authors attribute these differences to the glycosilation degree, which varies with the assay conditions (*13*, *14*); this could at least partially account for the discrepancy in molecular mass of the monomers.

Circular Dichroism (CD). The CD spectrum in the far-UV region of lactase from *K. lactis* at pH 7.0 and 25 °C is shown in **Figure 4**. The spectral region studied, from 190 to 240 nm, corresponds to the zone in which the CD spectrum of a protein is a reflection of its secondary structure content (*15*). The main observed spectral characteristic of lactase CD curve is the presence of one broad negative band centered at 210−213 nm; according to Manavalan and Johnson (*16*), this characteristic is consistent with backbone-folding pattern of the type of β-pleated-sheet-rich structure. The content of secondary structure (**Table 1**) obtained from the analysis of the CD spectrum supports this proposal.

The CDSSRT method (*8*−*10*) was used to estimate the content of secondary structure in the lactase because it gave the best fitting. The CD curve calculated by the CDSSRT method is shown as points in **Figure 4**. Results from this analysis gave an estimated curve that reproduces the experimental points with a normalized root mean square deviation (nrmsd) fitting of 0.104. **Table 1** shows the relative contributions of secondary structure to the CD curve of *K. lactis* lactase at pH 7.0 (native conformation), 5.5, and 8.5. In the native conformation, pH 7.0, the antiparallel β-sheet appears as the main type of regular conformation (25%). α-Helices make only a minimum contribution to the secondary structure of this enzyme (5%).

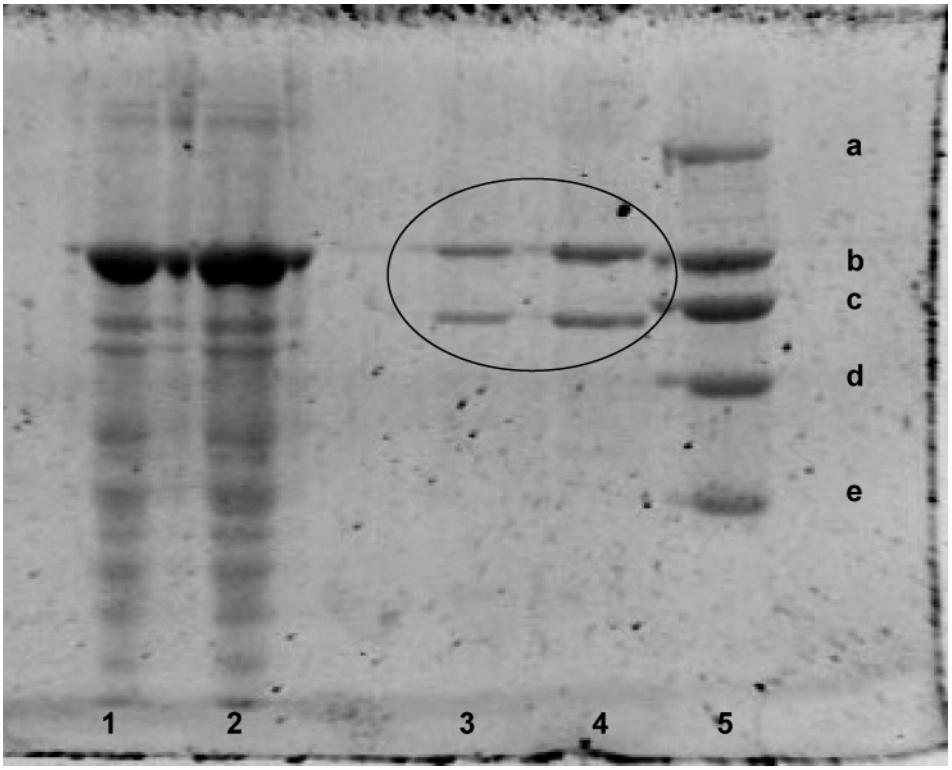

Figure 3. SDS–PAGE of the purified fraction of *K. lactis* lactase. Lanes 1 and 2, Maxilact LX 5000. Lanes 3 and 4, Purified fraction of *Kluyveromyces lactis* β-galactosidase, showing circled two monomers of the enzyme with molecular mass of 125.4 and 95.3 kDa, respectively. Lane 5, Molecular mass standards (Bio-Rad): (a) Myosin, 200 kDa; (b) *E. coli* β-galactosidase, 116.3 kDa; (c) phosphorylase b, 97.4 kDa; (d) serum albumin, 66.2 kDa; and (e) ovoalbumin, 45 kDa.

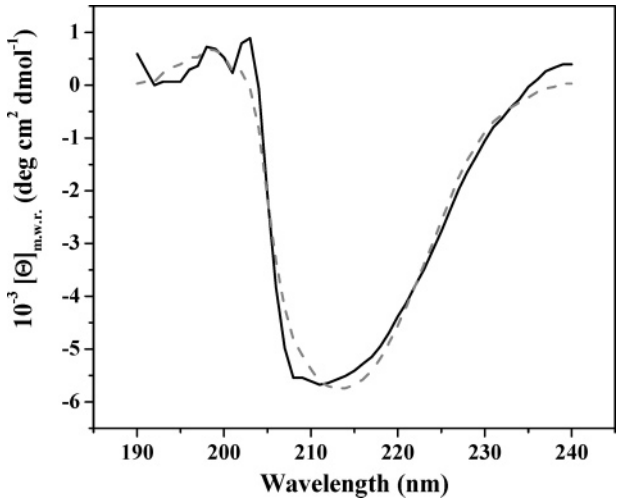

Figure 4. CD spectrum of β-galactosidase from *Kluyveromyces lactis* (Maxilact LX 5000) at pH 7.0 and 25 °C. Experimental curve (—), calculated curve (CDSSRT method) (– – –).

Poch et al. (*4*) inferred the secondary structure of *K. lactis* β-galactosidase from its primary structure based on the sequence of the LAC4 gene. They concluded that it contains 11 β-strands and two α-helices. Our analysis showed a higher proportion of β-structure with respect to α-helices (which was about 5%), similar to the structure predicted by Poch et al. (*4*). Furthermore, using the same primary structure as reported by Poch et al. (*4*), we predicted the secondary structure composition using the software available on the Internet at http://www.embl-heidelberg.de/predictprotein/submit_def.html, also resulting in a higher proportion of β-structure (37.1%) and a lower content of α-helices (9.7%), which is in agreement with our experimental data.

Table 1. Contribution of Secondary Structures (%) to CD Curves of β-galactosidase from *Kluyveromyces lactis* at Different pH's Calculated by the CDSSRT Algorithm

		β-sheet					
pH	α-helix	antiparallel	parallel	β-turns	unordered	total	nrmsd[a]
5.5	5	23	14	23	34	99	0.081
7.0	5	25	14	22	34	100	0.104
8.5	8	25	14	22	30	99	0.090

[a] Relative error calculated by the CDSSRT algorithm.

Additionally, when the LINCOMB algorithm (*17*) was used, it also reflected that *K. lactis* lactase was a mainly β-type protein (38.27%) and that there was a high contribution of non-peptide groups (aromatic residues and disulfide bonds) (31.70%) to the secondary structure of lactase. Mahoney (*18*) reported that *K. marxianus* (*fragilis*) β-galactosidase, which is very similar to the *K. lactis* lactase (*2*), contains six reactive sulfhydryl groups and 12−14 disulfide bonds; according to our results, this high number of residues could account for this observation.

Effect of pH on the Conformation and Activity of Lactase. The effect of pH on the conformation of *K. lactis* lactase was studied by CD. In the pH region 5.5−8.5, the spectral features of the enzyme remained approximately constant, with variations only in magnitude (**Figure 5**). The secondary structure contents of lactase at pH 5.5 and 8.5 were similar to that of the native conformation (pH 7.0); nevertheless, at pH 8.5, a small increase in the α-helical content (8%) was observed with respect to that of the native conformation (5%) (**Table 1**). At pH's below 5.5 or above 8.5, protein aggregation was observed, indicating that gross conformational modifications took place under these

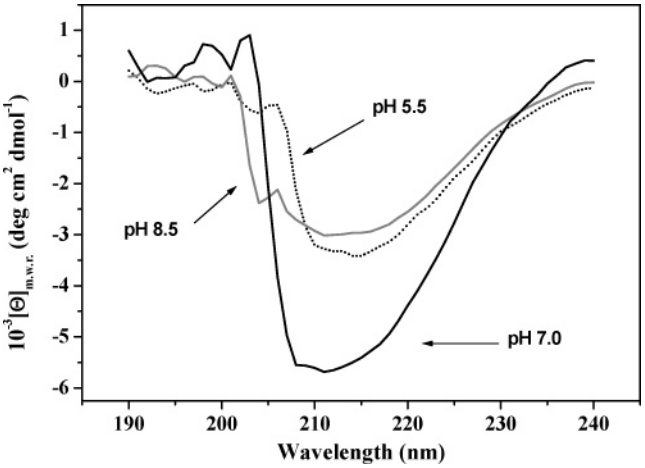

Figure 5. CD spectra of β-galactosidase from *Kluyveromyces lactis* (Maxilact LX 5000) at pH 5.5, 7.0, and 8.5.

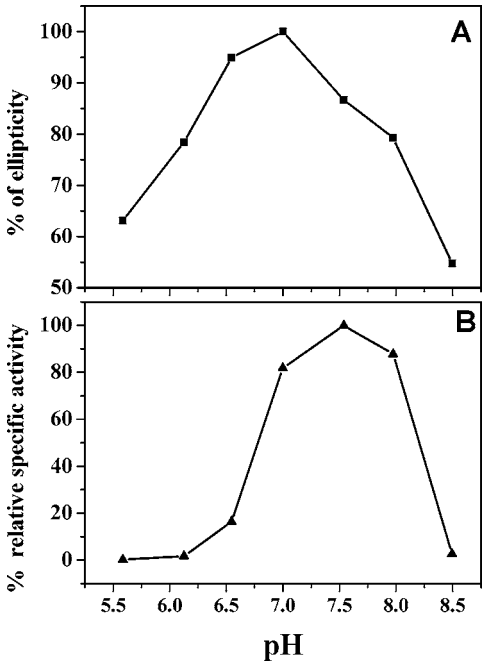

Figure 6. Effect of pH on the ellipticity at 215 nm (A, ■) and enzymatic activity (B, ▲) of β-galactosidase from *Kluyveromyces lactis* (Maxilact LX 5000). The ellipticity values are expressed as percentages of the value at pH 7.0 (100%).

conditions. A similar behavior was also found (at both acidic and alkaline pH conditions) for β-lactamase by Goto and Fink (*19*), who reported changes of the same order of magnitude as those obtained by us.

Figure 6A shows the changes in ellipticity of lactase at 215 nm as a function of pH; it can be seen that the highest content of secondary structure was found at pH 7.0 (100% ellipticity). At pH values between 6.5 and 7.0, the percentage of ellipticity decreased slightly, suggesting that little structural change took place. On the other hand, at pH's above 7.0, a larger change in ellipticity was observed that was due to structural changes, in agreement with the CD analysis, which showed a small increase in the helical content toward higher pH.

Figure 6B shows the pH−activity curve in which it can be observed that the maximum lactase activity was found at pH 7.5, which does not correspond to the maximum ellipticity. When comparing the results of the two curves, it was observed that, at pH 6.5, the activity decreased significantly, whereas the ellipticity varied only slightly, due to slight structural arrangements of the protein, which could not be detected by CD. This suggests that, at this pH, the diminution of the activity is due to local changes, such as the charges of some residues. On the other hand, when the pH rose from 7.0 to 7.5, the activity increased to its maximum, corresponding to the increase in the helical content of the secondary structure; this could indicate that the maximum enzyme activity does not correspond to the native conformation and that this structural shift favors the interaction between the enzyme and the substrate. At pH 8.5, the activity was lost, which could be explained by the fact that, at this pH, lactase aggregation was observed.

In conclusion, according to its CD spectrum, *K. lactis* β-galactosidase is mainly a β-type protein; the antiparallel β-sheet appears as the main type of regular conformation (25%). The LINCOMB analysis also showed that there is a high contribution of non-peptide residues, reflecting its high content of disulfide bonds.

The CD spectra showed that pH 7.0 leads to the highest secondary structure content, whereas the maximum activity was displayed at pH 7.5. The behavior of the activity in relation to the pH varies in two different ways: when moving to the acid side, the activity decreases because of local changes in charged residues that could not be detected by CD, whereas when moving to the alkaline side, structural changes such as the increase in the helical content seem to favor the enzyme−substrate interaction, resulting in an increase of activity.

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