JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2008, 56, 5859–5863 5859

Role of Lysine ε-Amino Groups of β-Lactoglobulin on Its Activating Effect of *Kluyveromyces lactis* β-Galactosidase

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Native β-lactoglobulin binds and increases the activity of *Kluyveromyces lactis* β-galactosidase. Construction of a three-dimensional (3D) model of β-lactoglobulin showed that lysine residues 15, 47, 69, and 138 are the most exposed ones, thus the ones more likely to interact with β-galactosidase. Molecular docking estimated the interaction energies of amino acid residues with either lactose or succinic anhydride, showing that Lys^{138} is the most likely to react with both. Affinity chromatography demonstrated that succinylated β-lactoglobulin diminished its ability to bind to the enzyme. Furthermore, when activity was measured in the presence of succinylated β-lactoglobulin, its activating effect was lost. Since succinylation specifically blocks Lys ε-amino groups, their loss very likely causes the disappearance of the activating effect. Results show that the activating effect of β-lactoglobulin on β-galactosidase activity is due to the interaction between both proteins and that this interaction is very likely to occur through the Lys ε-amino groups of β-lactoglobulin.

KEYWORDS: β-Lactoglobulin; *Kluyveromyces lactis* β-galactosidase; Lys ε-amino groups; molecular docking

INTRODUCTION

Kluyveromyces lactis β-galactosidase (β-gal) is by far the most important commercial lactase used in the dairy industry. Several reports have established that the presence of some proteins in the reaction medium may affect the activity of β-gal; speculations raise the possibility that the proteins present a masking effect of inhibiting metal ions in the reaction medium (*1, 2*). Jiménez-Guzmán et al. (*3*) studied the effect of whey proteins in β-gal activity and demonstrated that the activity of *Kluyveromyces lactis* β-gal increased when it was measured in the presence of either β-lactoglobulin (β-lg) or bovine serum albumin; this finding is particularly interesting since these proteins are available in milk and whey, which are the natural reaction media for this enzyme in dairy processing. Affinity chromatography assays demonstrated that β-gal bound specifically to β-lg, resulting in enzyme activation. Moreover, heating pure β-lg further increased β-gal activity, but heating it in the presence of lactose diminished its activating effect. Other reports have established that heating β-lg in the presence of lactose resulted in a reaction between the protein and the sugar (lactosylation) (*4*), which diminished the binding capacity of the protein to the enzyme and therefore its activating effect (*3*). Jiménez-Guzmán et al. (*3*) concluded that β-lg raises β-gal activity through two different mechanisms, one of which depends on the release of sulfhydryl groups by heat treatment from the denatured protein (*5*) and the other as a result of the ability of the native protein to bind the enzyme (*3*).

Lysine ε-amino are some of the most exposed and reactive groups in β-lg, with Lys^{47} and Lys^{138} being the most exposed ones (*6*). It has also been reported that lactosylation of β-lg occurs through the amino groups of lysine, specifically Lys^{47} (*4, 7, 8*). Since it has been reported that lactosylation causes a diminution of the interactions between β-lg and β-gal and of its activating effect (*3*), it is very likely that the same region of the molecule of β-lg may be involved in the binding between β-lg and β-gal; thus, this interaction might be through β-lg's Lys ε-amino groups. The aim of this work was to determine the role of the Lys ε-amino groups of β-lg on its activating effect of *K. lactis* β-gal.

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10.1021/jf703785p CCC: $40.75 © 2008 American Chemical Society
Published on Web 06/27/2008

MATERIALS AND METHODS

Three-Dimensional Modeling and Molecular Docking. A three-dimensional (3D) model was built with PyMOL software (DeLano Scientific LLC, 2007) using the NMR coordinates of bovine β-lactoglobulin in solution obtained at pH 7.1 in deuterium and found in the Protein Data Bank (PDB) database (PDB ID: 1CJ5) (*9*).

Molecular docking was then performed to locate the binding site and estimate the binding energies between the different amino acid residues of β-lg and lactose or succinic anhydride using AutoDock 3.05 software (The Scripps Research Institute, 2006). Results were obtained simulating a temperature of 25 °C and were clustered taking into account a range of rmsd of 5 Å. The 3D models for lactose and succinic anhydride were drawn at the Dundee PRODRG2 Server site (http://davapc1.bioch.dundee.ac.uk/programs/prodrg/).

***β*-Lactoglobulin Succinylation.** Succinylation gradually and specifically blocks the amino groups of β-lg. Succinylated β-lg (β-lg$_{succ}$) was obtained by consecutively adding 5 mg of succinic anhydride per mg of protein (Matheson Coleman and Bell, Ohio, USA) to 100 mL of a 5 mg/mL solution of β-lg (MP Biomedicals, Inc., Ohio, USA) and allowing the mixture to react in constant agitation, maintaining the pH constant at 7.0 with KOH 0.05 N (JT Baker, Xalostoc, Mexico) according to Hollecker and Creighton (*10, 11*). Each time that succinic anhydride was added, a sample was taken in order to test the effect of different degrees of succinylation; each succinylated sample was referred to as a derivate (D); 12 derivates (D1−D12) were obtained, each one with a higher degree of succinylation. The remaining succinic anhydride was eliminated by ultrafiltrating the resulting solution across a 10 kDa cellulose membrane (Pellicon XLPLCGC 10, Millipore, Bedford, Massachusetts, USA). To determine the degree of amino groups blocked in β-lg, succinylation was monitored through Urea−PAGE ($T = 11\%$; $c = 0.4\%$; 8 M urea; pH 4.7) according to Creighton (*12*). Since succinylation changes the net charge of the protein, the amount of amino groups blocked by the reaction was determined from the changes on the R_f of the protein bands.

Enzyme Activity Measurement. A commercial enzyme preparation, Maxilact LX-5000, (Gist Brocades, Delft, The Netherlands) was used as the source of β-galactosidase. All reactions were carried out at 37 °C by dilution 1:400 of Maxilact LX-5000 into 0.05 M phosphate buffer at pH 7.0. A solution of 0.034 M *ortho*-nitro-phenyl-β-D-galactoside (ONPG) (Sigma Chemical Co.St. Louis MO, USA) was used as substrate, and enzyme activity was measured spectrophotometrically at 410 nm on the basis of the release of *ortho*-nitro-phenol (ONP) after mixing 0.2 mL of ONPG solution with 0.1 mL of enzyme solution in 2.7 mL of phosphate buffer. The hydrolysis rate (ν_0) was calculated from the linear portion of data of the ONP production versus time. One enzyme unit (U) was defined as the amount of enzyme that hydrolyses 1 μmol of substrate (ONPG) in 1 min at 37 °C and pH 7.0. Specific activity was calculated dividing by the concentration of protein determined according to Bradford (*13*).

β-Gal activity of Maxilact LX-5000 was measured in the presence of 3 mg/mL of native or succinylated β-lg. Since succinylation yields succinic acid, pH of the succinylated derivates was neutralized with 0.05 M KOH to avoid the effect of acid pH on the activity. A control of β-lg with 0.05 M KOH (β-lg$_{KOH}$) was used to determine the effect of the neutralizer in enzyme activity. All results were compared to a control without protein.

Protein−Enzyme Interaction. This interaction was determined by means of affinity chromatography using an Eupergit (Röhm GmbH & Co., Darmstadt, Germany) support with immobilized β-lg or β-lg$_{succ}$ as ligands. Immobilization was performed following the instructions of the producer. A control without ligand was prepared by blocking the active oxyrane groups of the support with glycine. After immobilization, the amount of immobilized protein was calculated through the difference between the protein in solution before and after interacting with the support. Because of the difference in the available lysines of native and succinylated β-lg, the efficiency of immobilization was different in the cases of both molecules (0.5374 and 0.3157 μmol/g$_{support}$, respectively).

Maxilact LX-5000, determined by electrophoresis to contain a mixture of eight proteins, was used as the β-gal sample. Because of the differences in the amount of protein immobilized, β-gal samples were prepared to have a concentration according to a molar ratio of 0.05 mol β-gal/mol immobilized β-lg or β-lg$_{succ}$. Immobilized ligands and the control were allowed to interact with β-gal by directly adding 0.1 g of support with immobilized ligands to 3 mL of β-gal sample and shaking for 1 h, then the supernatant was separated from the support by centrifugation (3220*g*, 15 min) with a Beckman J2-MI centrifuge (Beckman Instruments, Palo Alto CA, USA), and its specific activity was measured using ONPG as a substrate.

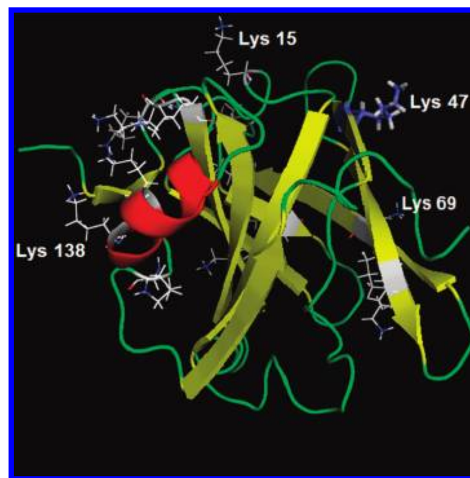

Figure 1. Three-dimensional model of β-lactoglobulin constructed with PyMOL software. Most available lysine residues are shown.

Statistical Analyses. . Each experiment was performed three times. Data were analyzed by means of a variance test (ANOVA); in some cases, a Tukey test was performed. All statistical analyses were carried out using the statistical analysis software Statistica 5.0 (Stat Soft, Tulsa OK, USA), with $p < 0.05$ used as a threshold of statistical significance.

RESULTS AND DISCUSSION

Reactivity of *β*-Lactoglobulin's Lys *ε*-Amino Groups. Previous reports have established that the lactosylation of β-lg diminishes the protein's ability to activate β-gal, probably because of the loss of its capability to bind to the enzyme (*3*); hence, it has been suggested that the same region involved in the binding of lactose could be the one involved in the activation of the enzyme. It has also been reported that β-lg can react with lactose through the first stages of the Maillard reaction (lactosylation) (*4*). In order to evaluate the role of the amino groups of β-lg in the activation of the enzyme, molecular modeling and molecular docking were used to study the interactions between β-lg and lactose.

A 3D model of β-lg was built on the basis of the NMR coordinates of the protein in solution (*9*) in order to study the exposition and possible steric hindrance that the different lysines in the molecule would show when reacting with lactose and/or another protein. Taking into account that pH could affect the exposition of the different amino acids, the model was constructed at pH 7.1, which is the optimum pH of *K. lactis* β-gal and the pH at which all other experiments were performed.

β-Lg has one free amino group and other 15 lysine residues in its molecule, each one with different reactivity. Upon analyzing our 3D model of β-lg (**Figure 1**), it was observed that Lys^{47} and Lys^{138} are very exposed in the molecule, and lysine residues 15 and 69 are also exposed at this pH, suggesting that any of these amino acids could be very reactive.

There are some controversies about which lysines are the most reactive lysines in β-lg. Several authors have reported that Lys^{47} is one of the most exposed and reactive groups in the molecule (*4, 7, 8*) and that this is in fact the first amino acid participating in the lactosylation reaction, while others also

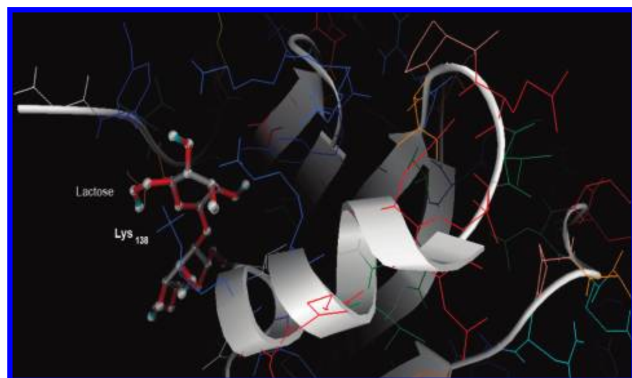

Figure 2. Molecular docking of lactose (gray and red) and β-lactoglobulin showing lactose bound to Lys[138].

consider Lys[138] as one of the most reactive ones (*6*). Fogliano et al. (*14*), however, reported Lys[100] as the most reactive one; they also suggested that the reactivity of the different Lys ε-amino groups in the protein depends on the conditions of the medium in which it is dissolved. Our results show that even though there are several lysine residues in the outer face of the protein, at pH 7.1 the most exposed ones are Lys[47] and Lys[138], which are the ones more likely to interact with another protein.

Molecular docking of lactose and β-lg (**Figure 2**) showed that the amino acid with most favorable interaction energy at pH 7.1 was Lys[138], which suggests that the most probable site of interaction between the protein and the sugar is this amino acid. The docking program used was blind, which means that it had no previous information about which amino acids are known to react with the sugar, and therefore, it calculates the interaction energies with all the amino acid residues of the protein. Hence, it was remarkable that among all the amino acid residues, lysines were the ones with lowest interaction energies ranging from −8.2 to −4.0 kcalmol^{-1}, and among those, Lys[138] showed the highest probability to be the binding site with a $\Delta G_{\text{binding}}$ of −4.7 kcal mol^{-1}. This indicates that lysine residues easily react with lactose through a nucleophilic attack and that their exposure at different conditions facilitates or inhibits this interaction.

Jiménez-Guzmán et al. (*3*) reported that when β-lg is lactosylated its interaction with β-gal diminishes and so does its activating effect on the enzyme; nevertheless, it is not known whether the interaction is diminished because of the steric hindrance by lactose inhibiting the protein's interaction through different amino acid residues of the same region or if the amino groups of β-lg that are blocked by lactose are in fact important for the protein−enzyme interaction. Because of this, succinic anhydride was chosen as an electrophile to react with β-lg; this is a very small molecule, which as long as the protein is not denatured, would not cause steric hindrance for any other groups to interact with β-gal.

In order to support that succinic anhydride reacts with the same amino acids as lactose, molecular docking of succinic anhydride and β-lg was done (**Figure 3**). Results showed that Lys[138] was the only amino acid with favorable interaction energy at pH 7.0 ($\Delta G_{\text{binding}} = -6.6$ kcal mol^{-1}). This is the same amino acid interacting with lactose, suggesting that succinic anhydride could be a small molecule that simulates the reaction with lactose without causing steric hindrance. Hence, succinic anhydride was used as a model to study the effect of blocking the amino groups of β-lg on β-gal activity.

Effect of Succinylation of Amino Groups of β-Lactoglobulin on β-Galactosidase Activity. Succinylation gradually and specifically blocks the amino groups of β-lg without changing

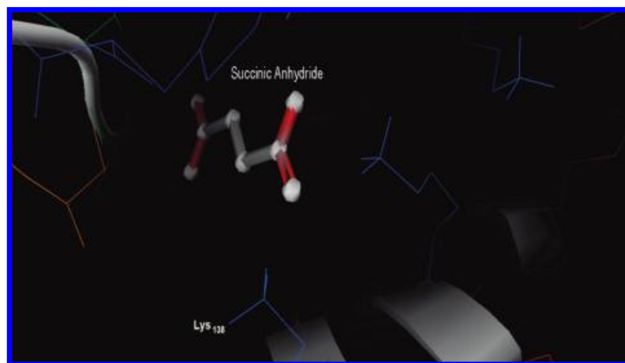

Figure 3. Molecular docking of succinic anhydride (gray and red) and β-lactoglobulin showing succinic anhydride bound to Lys[138].

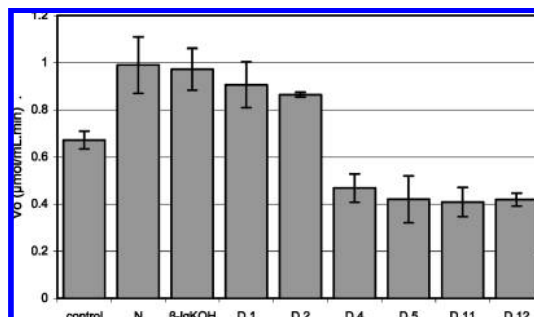

Figure 4. Activity of β-galactosidase in the presence of either β-lg or its different succinylated derivates. Control refers to the activity measured in buffer solution without protein; N refers to native β-lg; β-lg$_{KOH}$ refers to the neutralized native β-lg solution; D1−D12 refer to the different succinylated derivates of β-lg.

its native conformation at least until half the amino groups have been succinylated (*11*). In order to determine if amino groups of lysine actively participate in the activation of the enzyme, β-gal activity was measured in the presence of either native or blocked β-lg and compared with a control without protein; since succinylation yields succinic acid, pH of the succinylated derivates was neutralized using KOH, and a control of β-lg with KOH (β-lg$_{KOH}$) was also used to determine the effect of the neutralizer on the activity.

The presence of native β-lg or β-lg$_{KOH}$ (**Figure 4**) increased the hydrolysis rate with respect to the control in about 147% ($p < 0.001$), confirming the activating effect of β-lg reported by Jiménez-Guzmán et al. (*3*); the activity measured in the presence of β-lg$_{KOH}$ was not significantly different ($p > 0.1$) from that of native β-lg, suggesting that KOH had no effect on enzyme activity. When activity was measured in the presence of the first succinylated derivates, the activating effect was still observed (**Figure 4**, bars D1−D2). Nevertheless, in the presence of the rest of the succinylated derivates, activity decreased reaching the same value of the control without protein, showing no significant difference with it ($p > 0.05$) (**Figure 4**, bars D4−D12).

To determine the degree of amino groups blocked in the different succinylated derivates, succinylation of β-lg was monitored through 8 M Urea−PAGE. According to Hollecker and Creighton (*11*), succinylation of each amino group causes a change in the migration of the protein (R_f) due to the change in the protein's charge. When analyzing the R_f values of the different derivatives (**Figure 5**), it was observed that from D1 to D12, the amount of amino groups blocked varied from one to five. Furthermore, results showed that in the first two succinylated derivates even though a band of succinylated β-lg with one Lys ε-amino group blocked can be observed (**Figure**

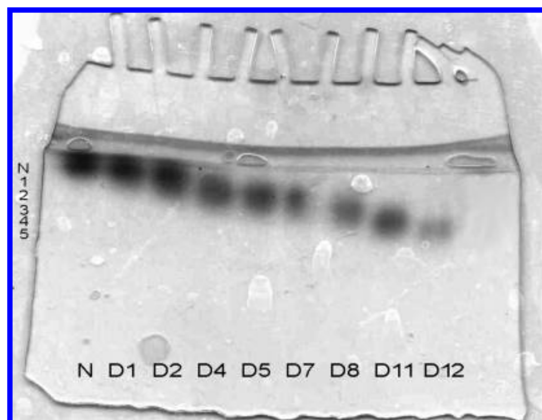

Figure 5. Urea−PAGE electrophoretic pattern of either native β-lg (N) or its different succinylated derivates (D1−D12). The numbers on the left side specify the number of lysine ε-amino groups that are blocked in each derivate.

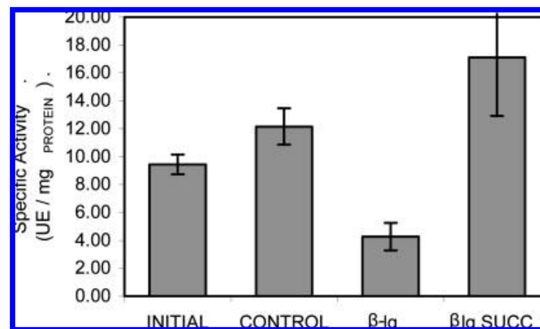

Figure 6. Specific activity of β-galactosidase after affinity chromatography using β-lg or succinylated β-lg as ligands showing the differences in the binding of β-galactosidase by the proteins. Initial refers to the specific activity of the solution before affinity chromatography. Control refers to the specific activity obtained when no ligand was bound to the support.

5, D1−D2), native β-lg was still present at high concentrations, while in the rest of the succinylated derivates (**Figure 5**, D4−D12), native β-lg was not observed. Moreover, D2 and D4 contain two, and two and three lysine amino groups blocked, respectively, and the main difference found between them was the presence of native β-lg in D2. When comparing with the activation results, it seems clear that while native β-lg was still present in the reaction medium, enzyme activation was observed (D1 and D2), but when all of β-lg is succinylated in at least one amino group (D4−D12), the activating effect was lost. At this point, the most reactive amino groups of β-lg were blocked and stable, suggesting that the loss in activating capacity is due to the blocking of its amino groups.

Even though in the first succinylated derivatives some amino groups are already blocked, the activating effect is still present. This could be due either to the fact that even small amounts of β-lg are able to activate the enzyme or to the fact that more than one Lys ε-amino is involved in the interaction with the enzyme.

Effect of Succinylation on the Interaction of *β*-Lactoglobulin with *β*-Galactosidase. Jiménez-Guzmán et al. (*3*) demonstrated that the activating effect of β-lg could be due to the interaction of β-lg with β-gal. In order to study the effect of Lys ε-amino groups in the interaction of β-lg with β-gal, the capacity of β-lg$_{succ}$ (the equivalent to D12) to bind to β-gal was compared with that of native β-lg through affinity chromatography, immobilizing either β-lg or β-lg$_{succ}$ on Eupergit and allowing the immobilized ligands to interact with a solution of Maxilact LX-5000. A control was used in which no protein was immobilized, but the reactive oxyran groups of the resin were blocked with glycine.

Specific activity relates the activity ratio to the amount of protein present in a given sample and therefore helps to establish the proportion of enzyme among other contaminating proteins. When the interaction with the ligand is unspecific, any of the proteins can be bound to the support, but their proportions in the eluting mixture will not change; however, when there is a specific interaction between one protein (in this case β-gal) and the ligand, its proportion in the eluting mixture will diminish. Results showed that the specific activity of the control was not significantly different from that of the initial β-gal solution ($p > 0.1$) meaning that β-gal was not specifically bound to the support without protein (**Figure 6**). When the same solution of β-gal (Maxilact LX-5000), containing eight proteins visible by electrophoresis, was mixed with the support containing native β-lg as ligand, the specific activity of the supernatant decreased significantly (70% ± 1.3, $p < 0.01$) with respect to the control (support without ligand) and the original solution, showing that β-gal bound specifically to β-lg. When β-lg$_{succ}$ was used as ligand, even though some of the protein in Maxilact LX-5000 was bound to the column, the specific activity of the supernatant was not significantly different from that of the control ($p > 0.1$) (**Figure 6**). This can be explained by the unspecific adsorption of the protein in the resin, but there is no evidence of a specific interaction with β-gal.

The fact that no interaction and no activation are detected when Lys ε-amino groups of β-lg are blocked suggests that these groups could be involved in the protein's ability to bind to the enzyme and hence its capability to activate β-gal. The interaction of the protein with β-gal could induce some structural changes of the enzyme, and according to Tello-Solís et al. (*15*), small conformational changes (in that case induced by pH) can give up important activation of *K. lactis* β-galactosidase.

Consequently, it has been clearly demonstrated that blocking Lys ε-amino groups diminishes the interactions between β-lg and β-gal, thus causing the loss of its activating effect. It is clear that amino groups from lysine are essential for this effect; nevertheless, at this point it is not possible to define whether this effect is due to a single lysine residue such as Lys^{138} or Lys^{47}, or to more than one of them. Further work is being done to clarify this issue.

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Received for review December 28, 2007. Revised manuscript received April 30, 2008. Accepted May 8, 2008.

JF703785P