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Immobilization of Rennet from *Mucor miehei* via Its Sugar Chain. Its Use in Milk Coagulation

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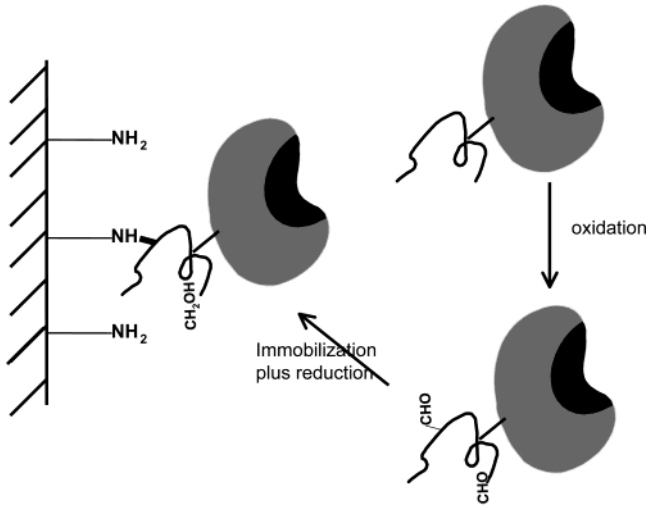
A successful strategy for the immobilization of rennet from *Mucor miehei* has been developed. The strategy is based on the immobilization of the enzyme, via their sugar chains at high ionic strength on aminated supports having primary amino groups with a very low p*K* value. The rennet was covalently immobilized via sugar chains (previously oxidized with periodate), which act as natural spacer arms and allow a very high percentage of rennet activity to be kept against small (H-Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe.TFA (98%)) and macromolecular substrates (k-casein) (78%). The use of tailor-made aminated support was critical to obtain good stability values, because using fully aminated supports achieved much lower thermostability values than using 50% aminated supports. The optimized derivative was utilized to hydrolyze casein in milk. To prevent the coagulation of the milk in the presence of the derivative, the reaction was performed at 4 °C (where hydrolyzed casein did not precipitate). Then the hydrolyzed milk was filtered and latter on heated to 30 °C, achieving a similar aggregate to the one achieved with soluble rennet.

Introduction

The interaction between macromolecular compounds (proteins, nucleic acids, starch, lignin, etc.) and immobilized biomacromolecules is a quite complex process, with interest in biocatalysis and protein purification.[1−6] Calf rennet has been extensively used for cheesemaking all over the world; however, in recent decades due to a shortage of calf rennet on world markets, alternative milk-clotting enzymes of different origins have been investigated.[7−9] Proteolytic enzymes of fungal origin have received considerable attention, specially extracellular enzymes of *Endothia parasitica*, *Mucor miehei*, and *Mucor pusillus*, have been received wide acceptability on the industrial scale due to high milk-clotting and low proteolytic activities.[10−13] Veal rennet or pepsin is very sensitive to milk pH changes, and the high proteolytic activity of trypsin and papain also prevents their use in cheesemaking.[11] Among fungi, an enzyme from *Mucor miehei* is preferred by the industry to produce milk-coagulation because of its R factor (milk clotting activity/proteolytic activity), specificity for certain peptide bonds in casein, good cheese quality and yield.[7,12,14−16]

Immobilization of these enzymes is a very complex task, because it is necessary to achieve a proper orientation of the active center in order to keep the accessibility of the active center to macromolecular substrates. In fact, immobilization on conventional supports drove to dramatic decrease in the expressed activities against casein (about 80%).[17−23] The use of supports activated with aldehyde-dextran has been reported to be a useful tool to solve this problem.[23] However, bearing in mind the glycosylated nature of the rennet from *Mucor miehei*, one feasible option is to oxidize sugar chains from the enzyme and use then as a natural spacer arm, long and flexible, that can allow the functionability of the rennet to be kept against macromolecular substrates[24] (Scheme 1).

Scheme 1. Immobilization of Rennet via Its Sugar Chains on Aminated Supports



Materials and Methods

Materials. Rennet from *Mucor miehei* was kindly donated by Proquiga, S. A. (A Coruña-Spain), Synthetic substrate

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H-Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe.TFA (MW 835.87 g/mol) for hydrolytic activity was purchased from BACHEM Feinchemikalien AG (Switzerland). k-Casein from bovine milk for coagulation activity; sodium acetate, chloride, and bicarbonate buffers; and sodium borohydride were from Sigma Chemical Company (St. Louis, MO). 4% and 6% agarose cross linked beads were a gift from Hispanagar S.A. (Burgos, Spain). Sodium periodate was purchased from Merck (Darmstadt, Germany). All chemicals employed were reagent grade. Pasteurized milk was commercial samples from Pascual S.A. (Aranda de Duero-Burgos, Spain).

Methods. *Preparation of MANAE-Agarose Supports.* The protocol was similar to the previously described[25] but using glyoxyl agarose[26] with different activation degrees. 10 mL of agarose (4 BCL) containing the desired amount of glyoxyl groups (20 and 40 μmols) was suspended in 90 mL of 1 M ethylenediamine pH 10.05, and it was gently stirred for 2 h. Then, 1 g of solid $NaBH_4$ was added, and the suspension was reduced for 2 h. The reduced gels, monoaminoethyl-*N*-aminoethyl agarose (MANAE), were filtered and sequentially washed with 100 mL of 0.1 M acetate/1 M NaCl at pH 5.0, with 100 mL of 0.1 M sodium bicarbonate buffer/1 M NaCl at pH 10.0 and finally with 500 mL of deionized water. The full conversion of glyoxyl to MANAE groups permitted that the concentration of amino groups corresponded to the concentration of glyoxyl groups.[25]

Determination of Enzyme Activities. *Hydrolytic Activity with Small Substrates.* The hydrolytic activity was carried out using a synthetic substrate H-Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe.TFA, following the method proposed by Raymond.[27] 20 or 50 μL of enzyme solution or derivative suspension was added to 2 mL of 0.16 mM synthetic substrate in 50 mM sodium citrate pH 4.7 incubated at 25 °C. The amount of *p*-nitro-Phe-Nle released after enzymatic cleavage was estimated by the increment in absorbance at 310 nm ($\xi = 1000\ M^{-1}\ cm^{-1}$.[28] The activity is given in μmol of substrate hydrolyzed per min and per mg of protein under the described conditions. Experiments were carried out at least in triplicate and experimental error was never over ±5%.

Coagulant Activity. 1 mg/mL of k-casein from bovine milk was dissolved in 50 mM sodium citrate buffer and 75 mM sodium chloride at pH 5.3. To start the reaction, 20 or 50 μL of enzyme solution or derivative was added to 2 mL of the substrate solution at 25 °C and the reaction was followed spectrophotometrically by the increment of absorbance at 550 nm promoted by the protein precipitation. Activity was proportional to the inflection point of this curve.[20,23]

Semipurification of the Rennet. The main objective was to eliminate the sugars added to this preparation as stabilizers. A solution of enzymatic extract (8 mg/mL) in 50 mM sodium phosphate buffer at pH 7.0 was mixed with aminated agarose 6 BCL activated with 105 MANAE μmol/g of support, (1:10 (v:v)). The mixture was stirred at 25 °C, and occasionally, samples of supernatant were withdrawn and the hydrolytic activity was assayed spetrophotometrically as described above. When 100% of the enzyme was adsorbed onto the aminated support, this was filtered and washed with distilled water. Finally, the support was re-suspended in 50 mM of sodium phosphate/sodium chloride 0.75 M pH 7.0 (1:10) (v:v) to release the enzyme. Periodically samples of supernatant were withdrawn, and the hydrolytic activity was assayed. The yield was near to 100%.

Oxidation of the Sugar Chain from Mucor miehei Rennet. A semipurified enzymatic solution (8 mg/ml) of *Mucor miehei* was oxidized with an excess of $NaIO_4$ (5 mM) in sodium phosphate buffer 50 mM pH 7.0 for 1 h (sodium periodate remained unconsumed after the reaction). This way, all likely oxidations points in the sugar chain were transformed in aldehydes. Then, the oxidized enzyme was dialyzed against 200 mM sodium phosphate buffer pH 7.0.

Immobilization of Mucor miehei Rennet onto Agarose Amino Support. 10 mL of aminated agarose support 4% beads (4BCL) (20 and 40 amino groups/g of support) were suspended in 100 mL of protein solutions (the enzyme was dissolved in 200 mM sodium phosphate buffer pH 7.0), and the reaction was carried out at 25 °C (relation 1:10 (v/v)).

During immobilizations, samples were withdrawn from the supernatant, and the suspension and enzyme activities were determined following Raymond's method[27] as described above. After 3 h, the derivatives were vacuum-dried and resuspended in 100 mL of 1 mg/mL of sodium borohydride/100 mM sodium bicarbonate pH 10. After 1 h, the derivatives were filtered, washed with an excess of distilled water, and stored at 4 °C.

Protein Determinations. The protein was assayed by absorbance measurements at 280 nm. Total protein concentration was given by the following expression: $C = -0.0008 + 1.9803A_{280}$ where C is the quantities of protein in mg/mL.

Thermal Stability of Rennet Preparations. Enzyme derivatives or soluble enzyme were incubated on sodium acetate buffer at pH 5.0 or sodium phosphate buffer pH 7.0, at 50 °C. Periodically, samples of the suspension and soluble enzyme were withdrawn, and their remaining enzyme activities were determined spetrophotometrically at 310 nm as described above.

SDS−PAGE Analysis. Experiments of SDS−PAGE were performed as described by Laemmli:[29] in a SE 250-Mighty small II electrophoretic unit (Hoefer Co.) using gels of 15% polyacrylamide in a separation zone of 9 cm × 6 cm and a concentration zone of 5% polyacrylamide. Gels were stained with Coomassie brilliant blue method. Low molecular weight markers from Pharmacia were used (14 000−94 000 D).

Milk-Clotting Activity. The assay was performed in eppendorf tubes. Commercial samples of milk were used. 50 μL of derivative was added to 1 mL of milk, and the reaction was carried out initially at 4 °C to prevent the coagulation of casein at different periods of time (from 5 to 30 min). The samples were filtered and finally incubated at 30 °C for 30 min. After that, the eppendorf tubes were inverted to allow nonclotted milk to drain. Clotting activity was confirmed by the presence of a white coagulate at the bottom of the well and was recorded taking a photo against a black background with a digital Olympus Camedia C-350 camera. At the end, the coagulated samples of milk were centrifuged. The soluble fraction (supernatant = whey permeate) was analyzed by SDS−PAGE, and the solid fraction was

Table 1. Activity of Different Rennet Preparations Immobilized on Different Aminated Supports with (H-Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe.TFA) and k-Casein[a]

oxidation of the enzyme	support	immobilization yield (%)	expressed activity (%) small substrate	expressed activity (%) k-casein
no	MANAE 40	0.1	Nd	Nd
yes	MANAE 40	100	93	78
no	MANAE 20	0.1	Nd	Nd
yes	MANAE20	100	98	88

[a] The reaction was carried out at pH 7.0, sodium phosphate buffer 200 mM at room temperature. Enzyme loading was 7.8 mg/g of support. Other specifications were described in the Materials and Methods section.

weighted to determine the yield of cheesemaking. Moreover, SDS-PAGE of the samples were realized.

Results

Optimization of the Immobilization Protocol. The oxidation of the sugar chain of rennet had no effect on enzyme activity; the enzyme maintained 100% of the activity after oxidation.

Then, the oxidized enzyme was immobilized on two aminated supports, one fully activated (with 40 μmols of MANAE groups/g of support) and the other activated only 20 μmol of MANAE groups/g of support.

Under the immobilization conditions, the non-oxidized enzyme could not be adsorbed in both supports (Table 1). However, immobilization was quite rapid when using the oxidized enzyme, although somehow slower in the less activated one, but all enzymes were immobilized on the support. After reduction with sodium borohydride, the activity was maintained under similar values, and the desorption of the enzyme was not observed even if the derivative was boiled in the presence of SDS, showing that the enzyme was covalently bond to the support via very stable secondary amino linkages.

Using 8 mg of enzyme per gram of support, the recovered activity was very high when using both supports. This high activity recovery was observed using both the small synthetic substrate and the k-casein (Table 1). Although the activity was slightly higher using the lowly activates support when using k-casein as substrate (88% versus 78%).

Both derivatives kept their activities fully unaltered between 4 and 37 °C for one week (results no shown). However, inactivations at higher temperatures (50 °C) permitted us to observe that the enzyme immobilized on the lowest activated support yielded the highest stability at pH 5 and 7 (Figure 1).

The only difference between both derivatives is the amount of MANAE groups below the enzyme that could promote ionic interactions between the enzyme and the support surface. Apparently, these interactions promote a negative effect on the enzyme stability.

Loading of the Support. Figure 2 shows that it was possible to immobilize up to 11.4 mg of enzyme/g of support. Using higher amounts of enzyme, the immobilization yield decreased, reaching around 11 mg of immobilized enzyme/g of support as the maximum amount of immobilized enzyme. This is the expected behavior when using a pure enzyme, after covering the enzyme surface, because the rest of the enzyme is unable to react with the support.

When the enzyme loading is increased, the activity of the immobilized derivative was increased until it reached a maximum loading capacity of the support (11.4 mg/g). However, to define the optimal derivative, we have also considered the percentage of expressed activity. Using the small substrate, the recovered activity was similar, with just a very slight decrease in the recovered activity at high

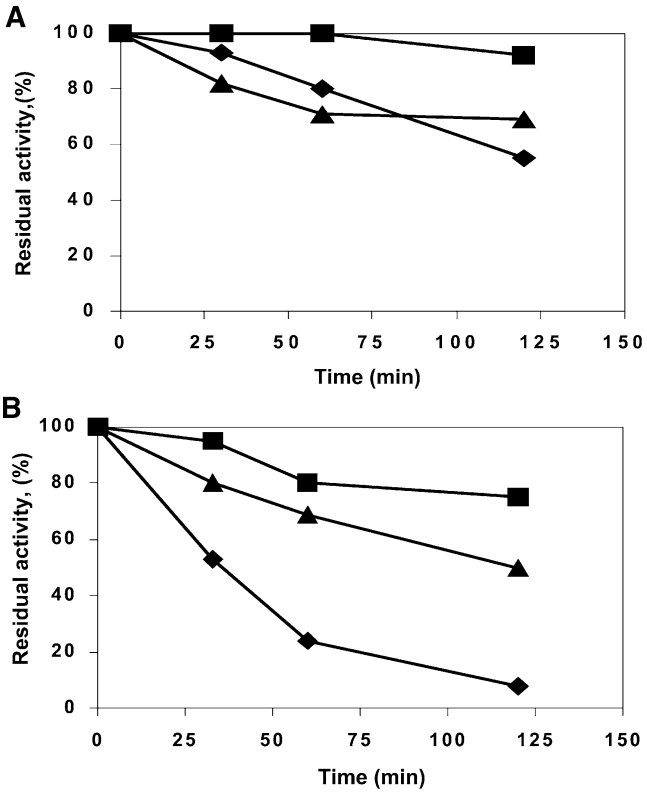

Figure 1. Thermal inactivation of rennet derivatives at pH 5.0 (Figure 1A) and pH 7.0 (Figure 1B). Derivatives were incubating at 50 °C as described in the Methods section; following the activity with H-Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe.TFA. Symbols: (◆) Soluble rennet. (■) Derivative prepared with 20 μmol of MANAE groups per g of support. (▲) Derivative prepared with 40 μmol of MANAE groups per g of support.

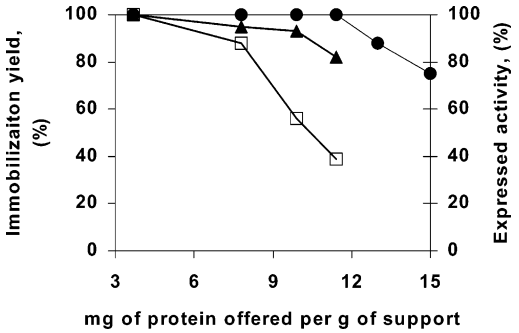

Figure 2. Loading capacity of rennet on MANAE supports. A different amount of proteins was added to test the maximum loading capacity of the support. Experiments were carried out as described in methods. Symbols: (●) Immobilization yield. (▲) Activity expressed with the synthetic substrate (H-Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe.TFA). (□) Activity expressed with k-casein.

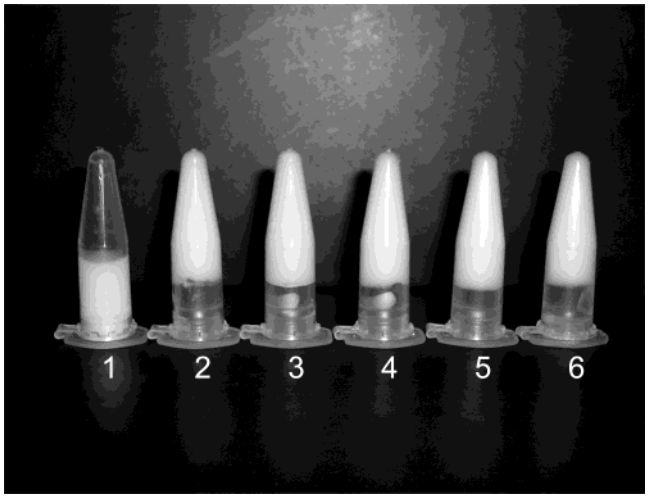

Figure 3. Milk-clotting activity of soluble or immobilzed *Mucor miehei* rennet. The amount of enzyme or derivative used in each eppendorf tube is the same for all of them (50 μL/mL of milk). Other details of the experiments were carried out as described in the Methods section. 1. Untreated milk. 2. Milk incubated with soluble *Mucor miehei* rennet at 30 °C for 5 min. 3. Milk incubated with *Mucor miehei* rennet derivative 4 °C for 5 min, filtered, and heated at 30 °C for 30 min. 4. Milk incubated with *Mucor miehei* rennet derivative 4 °C for 10 min, filtered, and heated at 30 °C for 30 min. 5. Milk incubated with *Mucor miehei* rennet derivative 4 °C for 20 min, filtered, and heated at 30 °C for 30 min. 6. Milk incubated with *Mucor miehei* rennet derivative 4 °C for 20 min, filtered, and heated at 30 °C for 30 min.

enzyme loading (for 11.4 mg/g of enzyme recovery, 80%). However, with k-casein, the specific activity of the immobilized enzyme sharply decreased when the loading increased (Figure 2). Thus, while using 3.7 mg of protein/g of support, around 100% of the immobilized activity was expressed, whereas using 11.4 mg/g, the recovered activity was only 40%. This may be due to some diffusion limitations promoted by the high activity of the derivative that prevent the observation of the whole activity of the immobilized enzyme. In fact, the mechanical breaking of the support permitted us to recover some of the activity.

Thus, as a compromise between volumetric activity and expressed activity, we propose the use of enzyme loadings of 7.8 mg/g of support to have a good expressed activity (around 60%). This derivative expressed a volumetric activity only 5% lower than the derivative with the maximum loading, but expressed around 50% more of the immobilized enzyme activity.

Use of the Immobilized Rennet to Coagulate Milk. The optimal derivative was used to coagulate milk. If the reaction was carried out directly at 30 °C, a rapid coagulation of the milk was observed, similar to that achieved with the soluble enzyme. However, the derivative was trapped by the aggregates and was not possible to separate the biocatalysts from the coagulate. Obviously, this is not a good situation, because it is convenient to prevent the coagulation of the milk in the presence of the enzyme derivative to recover it, prevent the contamination of the coagulate by the biocatalysts, and permit the reuse of the enzyme derivative.

However, it has been reported that the milk coagulation is only produced over 15 °C.[20] Therefore, a new strategy was designed. The derivative was offered to the milk at 4 °C at different times and then filtered at this low temperature, and the hydrolyzed milk was heated at 30 °C. Rapid coagulation of the milk could be observed (see Figure 3 and Table 2), and a coagulate with similar apparent properties to the one obtained by using soluble rennet at 30 °C during all of the process was obtained. Figure 4 shows the decrease of nonproteolyzed proteins after incubation of milk and soluble purified k-casein as substracts with *Mucor miehei* rennet immobilized derivative.

The proposed protocol is as follows: 2.5 g of optimal rennet derivative was added to 50 mL of milk at 4 °C, stirring the system for 50 min. Afterward, the product was filtered to recover the derivative, and the filtered product was heated at 30 °C, until coagulation was produced (coagulation occurs in only a few minutes).

Table 2. Weight of Milk Clotting Obtained after Different Treatments with Soluble and Immobilzied *Mucor miehei* Rennet[a]

treatment	weight of milk clotting (mg)[b]
hydrolysis with soluble rennet 30 °C	162
hydrolysis with immobilized rennet at 4 °C	
5 min	162
10 min	163
20 min	162
30 min	170

[a] The weight of coagulated milk was obtained by centrifugation of the coagulate samples of milk and determined as described in Methods. [b] This value was obtained from 1 mL of milk.

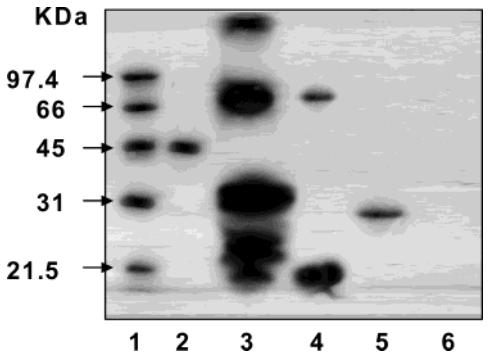

Figure 4. SDS−PAGE analysis of proteolytic activity of commercial milk and isolated milk k-caseins treated with *Mucor miehei* rennet derivatives (15% polyacrylamide gels). Lane 1. Molecular weight markers. Lane 2. Soluble extract of *Mucor miehei* rennet. Lane 3. Milk incubated with *Mucor miehei* rennet immobilized derivative. Lane 4. Milk whey after incubation of commercial milk with *Mucor miehei* rennet immobilized derivative (supernatant). Lane 5. Commercial k-casein before digestion with *Mucor miehei* rennet derivative. Lane 6. Commercial k-casein after incubation with *Mucor miehei* rennet derivative. The gel was stained with Coomassie blue. The positions of molecular mass markers (SDS−PAGE; Bio-Rad) are indicated on the left.

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