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Immobilization of Thermolysin and Application of the Immobilized Thermolysin to Cheese-making

Se-Eok Yun

Department of Food Science and Technology, Chonbuk National University, Chonju

Thermolysin의 固定化와 固定化 Thermolysin의 Cheese 製造에의 利用

尹 世 億

전북대학교 식품가공학과

Abstract

Thermolysin was immobilized on Dowex MWA-1 with 10 % glutaraldehyde and incorporated into a fluidized-bed continuous coagulation scheme to make Cheddar type cheese. The activity yield of thermolysin was 25%. The immobillized thermolysin was stable at 60℃ in the presence of 1/200 M calcium ions and the half-life value is 16 days at the temperature. Raw milk alkalified to pH 7.0 was passed through a column of thermolysin beads at 55℃, cultivated with *Streptococcus cremoris* and allowed to coagulate. A typical milk curd was formed to make Cheddar type cheese, avoiding troublesome microbial contamination successfully during continuous hydrolysis process. During ripening of this cheese for 6 months at 10℃, its ripening ratio and taste were similar to those of cheese prepared by the traditional method.

Key words: immobilization, immobilized thermolysin, cheese-making

Introduction

Previous reports[1,2] have described that the desirable milk curdling can be possible even if the proteases other than the traditional milk-clotting enzyme, rennet, are employed. This could be achieved by regulating the extents of casein hydrolysis with immobilized proteases. Applications of immobilized proteases in cheese-making have been reported. Immobilized chymotrypsin[3,4], rennet[4,5], pepsin[6,7], and Alkaline-protease[8] have been used in a continuous milk clotting system.

However, the application of these immobilized proteases to cheese-making has been limited with no commercial use. Preparation and utilization of immobilized chymotrypsin and rennet[4]in continuous systems for milk treatment were frustrated by rapid decrease in their milk clotting activity during operation. The possible use of pepsin covalently bound to porous glass or inorganic supports for milk clotting was tried extentively[6,9,10]. The immobilized pepsin column was operated by passing skim milk acidified with phosphoric acid to pH 5.6-5.9 at low temperature (15℃)to avoid coagulation of milk in the column.

However, it may be desirable to treat milk at higher temperatures because milk should be protected from microbial contamination during operation. In a report[8], feasibility of using immobilized Alkaline-protease for cheese-making was examined by operating at a high temperature(50℃) which minimize troublesome microbial growth. By reversing the order of the lactic acid fermentation process and the hydrolysis process of the traditional cheese-making method and by rasing pH of milk to 7.0 during hydrolysis, coagulation of milk in the column could be avoided. In the case, there occurred a problem to be overcome that half-life of the enzyme activity was short at the high operation tempera-

ture(50℃).

In the present work, thermolysin which has the extra thermal stability[11] was immobilized on an anion exchange resin and was incorporated into continuous-flow cheese-making system to improve operational stability.

Materials and Methods

Materials

Crystalline thermolysin from *Bacillus thermoproteolyticus* Rokko and Alkaline-protease from *Bacillus subtilis* were donated by Daiwa Casei Co. (Japan) and Amano Pharmaceutical Co. (Japan), respectively. Rennet was the product of Hansens Laboratorium (Denmark). *Streptococcus cremoris* H-61 was given by the National Institute of Animal Industry (Japan). Dowex MWA -1(20-50 mesh), an anion exchange resin of Dow Chemical Co., was used as a carrier. Skim milk powder and raw milk were the products of Seoul Dairy Co..

Immobilization procedure

Proteases, thermolysin, Alkaline-protease and rennet, were immobilized according to the method proposed by Ohmiya et al[12]. The reaction was allowed to proceed by adding 1m*l* of glutaraldehyde (10%, v/v) to 0.05M buffer solution containing 1g of resin and 1m*l* of enzyme solution(4mg/m*l*),and by stirring the mixture at 10℃ for 1hr. Thermolysin was immobilized in Tris-HCl buffer (pH 7.0) containing 1/200M $CaCl_2$ and Alkaline-protease and rennet were done in $CaCl_2$-free Tris-HCl buffer (pH 7.0). The enzyme-bound particles were well washed, lyophilized, and then stored at below 10℃ until use.

Determination of enzyme activity

In a routine assay of protease activity, casein (Hammarsten casein, E. Merck, Germany) was used as substrate. The activity was estimated by measuring the amounts of non-protein nitrogenous compounds solbuilized into 5% trichloroacetic acid(TCA): 1m*l* of enzyme solution was added to 5m*l* of casein solution, and after incubation 2m*l* of reaction mixture was combined with an equal volume of 10% TCA solution to remove the unhydrolyzed casein. The amounts of tyrosin liberated from casein in the deproteinized filtrate was determined by optical density measurement at 280 *n*m. For the determination of immobilized protease activity, 5m*l* of casein solution was added to 0.1g of immobilized enzyme beads (wet) and incubated in reciprocal shaker, and then 2m*l* of reaction mixture was treated as described above for the determination of the amounts of tyrosin liberated.

The activity yield was evaluated according to the following equation:

$$\text{activity yield} = \frac{\text{activity in total enzyme beads}}{\text{total activity of free enzyme used for immobilization}} \times 100$$

Preparation of cheese

Normal Cheddar cheese (N-cheese) : To compare the properties of the cheeses prepared by modified method using immobilized thermolysin, normal Cheddar cheese was prepared according to the traditional method[13].

Immobilized rennet cheese (R-cheese) : The pasteurized raw milk(3*l*) containing 0.02% $CaCl_2$ was adjusted pH to 7.0 with NaOH and was supplied at 15℃ at constant rate (0.3 m*l*/sec) upward (fluidized-bed) through the column (1.5× 25cm) in which immobilized rennet beads (20g wet weight) were packed. After appropriate extent of hydrolysis to initiate clotting was attained, the milk effluent from the column was incubated with *Streptococcus cremoris* and was warmed subsequently to 30℃ to form curd.

Checking of the appropriate extent of hydrolysis to initiate clotting was performed by quantifying the nitrogenous compounds solubilized into 5% TCA solution from the effluent by Lowry-Folin method[14].

After about 1 hour incubation, cutting, whey-ing-off, curing and pressing procedures were employed according to the traditional method.

Immobilized thermolysin cheese (TL-cheese) : The pasteurized raw milk(3*l*) containing 0.02% $CaCl_2$ was adjusted pH to 7.0 with NaOH and was pumped peristaltically through the column packed with immobilized thermolysin as described above. The temperature of the column was kept at 55℃ by circulating water through the column jacket from the water bath. The milk effluent from the column was inoculated with *Streptococcus cremoris* after lowering the milk effluent to 30℃ and incubated to form curd. Thereafter, the traditional procedure was followed.

Cheese analysis

Protein, fat and moisture contents in the cheese at the initiation and termination (6-month) of ripening were determined according to standard methods[15]. After 5 month ripening, panal test was performed.

Results

Activity yield

To immobilize protease on 1g of support, 4 mg/m*l* enzyme solution was employed. The activity yields of thermolysin, Alkaline-protease and rennet were 25, 27 and 19%, respectively.

Properties of soluble and immobilized thermolysin

Reaction dependence on pH : The pH profiles for thermolysin in immobilized and free states are shown in Fig. 1. Immobilization shifted the optimal pH from 8.0 to 7.0.

Reaction dependence on temperature : The effects of temperture on the reaction rates of thermolysin in immobilized and free state is shown in Fig. 2. The highest activities of thermolysin in immobilized and free states were 80℃ and 70℃, respectively. The optimum range of temperature was affected by the immobilization treatment.

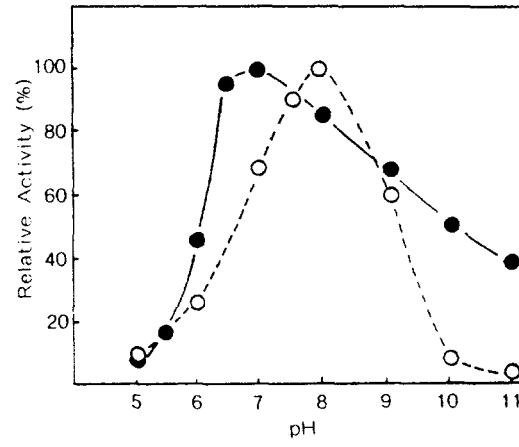

Fig. 1. Optimum pHs of immobilized(●) and soluble(○) thermolysin at 55℃ (substrate: 0.5% casein)

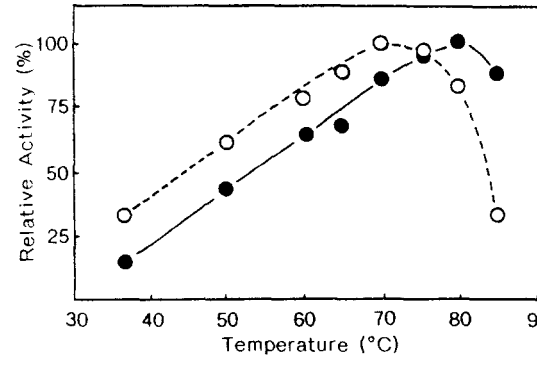

Fig. 2. Temperature optimum for the hydrolysis of casein by thermolysin in immobilized(●) and soluble(○) states at pH 7.0 in 0.05 M Tris-HCl buffer (substrate: 0.5% casein).

Thermostability was characterized by measuring residual activities of enzyme in immobilized and soluble enzymes which were incubated under the various temperatures as shown in Fig. 3. Residual activity was measured at 37℃ after 2hr incubation in 0.05 M Tris-HCl buffer (pH 7.0) containing 1/20 M or 1/200 M calcium ions at the given temperatures. In the presence of 1/20 M calcium ions, immobilized thermolysin retained its initial activity at 75℃, but soluble enzyme lost rapidly its initial activity at higher than 65℃. In the case 1/200 M calcium was added, immobilized thermolysin retained is initial activity at 60℃, while soluble enzyme lost its initial activity at 55℃ and showed sharp decrease in thermostability.

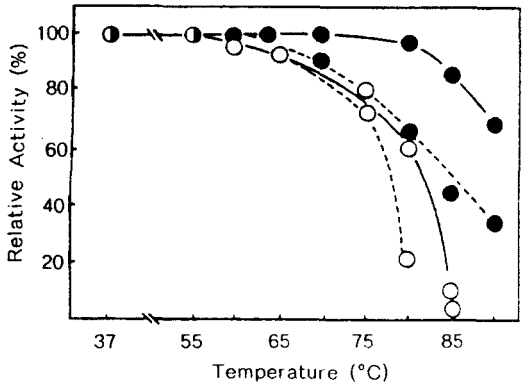

Fig. 3. Thermostability of immobilized(●) and soluble(○) thermolysin at calcium concentration of M/20(——) and M/200(- - - - -) (substrate: 0.5% casein)

Kinetic constants : The Km values for Hammarsten casein were calculated from the Lineweaver-Burk plot as shown in Fig. 4. The Km value of the soluble thermolysin was 0.67, fourtimes larger than that of the immobilized thermolysin.

Operational stability of immobilized thermolysin

Continuous passage of 10% skim milk solution (containing 0.01 M $CaCl_2$) through column of

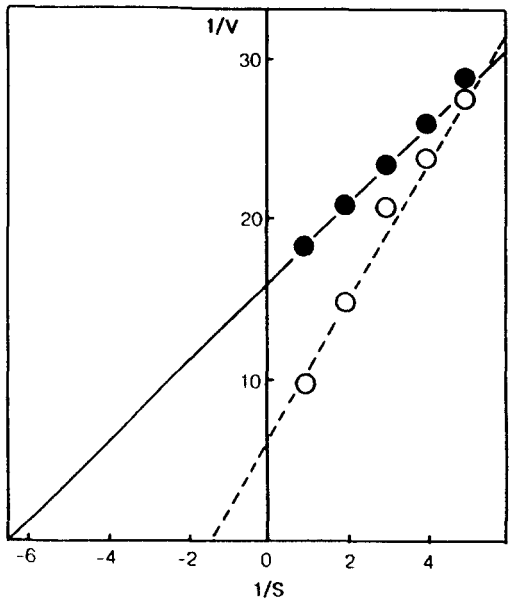

Fig. 4. Effect of substrate concentration on the hydrolysis of casein by thermolysin in immobilized(●) and soluble(○) states. Double reciprocal plots of the velocity vs. the concentrations of casein (S, %) are presented.

immobilized thermolysin resulted in gradual reduction of enzymatic activity. As shown in Fig. 5, the rate of loss of enzymatic activity was depended on the operation temperature. Half-life values of the immobilized thermolysin were 16 days at 60℃ and 1.5 days at 80℃. Whereas those of the immobilized Alkaline-protease were 7.8 days at 45℃ and 1.5 days at 60℃, and those of the immobilized rennet were only 0.13 days at 37℃ and 0.1 days at 45℃.

Preparation of cheeses and their ripening

When milk clotted as a result of proteolytic action of thermolysin in both soluble and immobilized states, the extent of proteolysis was 2-3% at the initiation of clotting as described in the previous report[1]. Accordingly, the flow rate of milk passed through the immobilized column was controlled so as to attain the same extent of

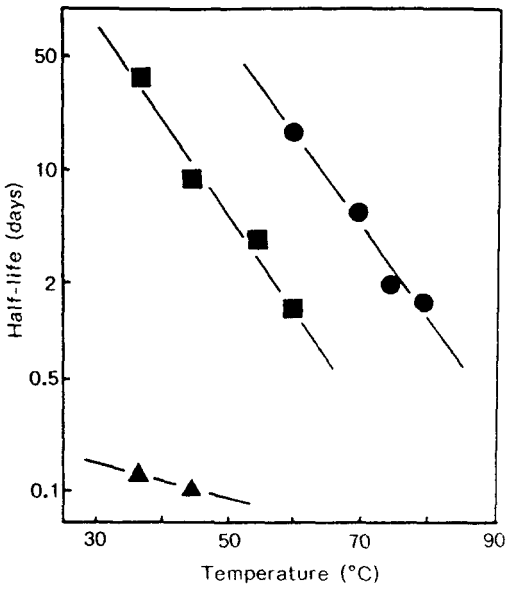

Fig. 5. Deactivation of immobilized enzymes during continuous treatment with skim milk through an immobilized enzyme column at various temperatures.
▲: immobilized rennet, ■: immobilized Alkaline-protease, ●: immobilized thermolysin

Table 1. Composition(%) of cheese prepared using immobilized protease

Component	N-cheese	R-cheese	TL-cheese
Moisture	45	44	46
Protein	22	22	19
Fat	19	18	17

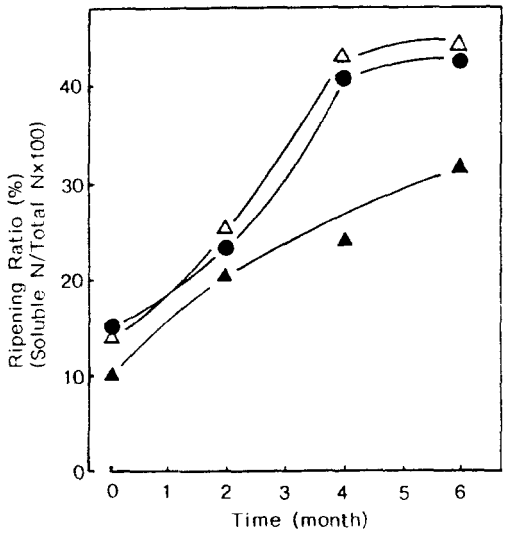

Fig. 6. Changes of ripening ratio in N-cheese(△), TL-cheese(●) and R-cheese(▲) during ripening at 10℃ for a given period.

hydrolysis.

The milk began to coagulate when lactic acid fermentation proceeded enough to lower the pH of the milk to 6.2. It took about 2hrs to form hard curd enough for cutting procedure. The curd made by the immobilized thermolysin was slightly softer than that obtained using soluble or immobilized rennet. The appearance and hardness of TL-cheese were comparable to those of R- and N-cheeses. Protein and fat contents in TL- and R-cheeses were some what lower than those in N-cheese, while moisture content was same in all cheeses (Table 1). The difference of curd preparation method did not significantly affect these compositions.

Ripening ratios (Fig. 6) of TL- and N-cheeses increased to about 40% durng ripening for 6 months and were higher than that of R-cheese. Acidity increase of these cheeses was revealed after their ripening for 3 months but not in the case of R-cheese. The taste of TL-cheese was not inferior to that of R- and N-cheeses. No bitter taste was detected in any of the three kinds of cheeses through the ripening period.

Discussion

Characteristics of immobilized thermolysin

Milk clotting enzymes such as rennet, pepsin, Mucor rennin, trypsin, thermolysin and Alkaline-protease were immobilized on an anion exchange resin, Dowex MWA-1 and incorporated into a fluidized-bed for continuous milk

coagulation process. Operated at 55℃, only thermolysin retained sufficient activity toward skim milk to warrant further studies.

The stability of immobilized thermolysin was increased by adding large amounts of calcium ions. It is known that calcium ions contribute to the stability of thermolysin[11]. Similarly calcium ions affected noticeably the activity yield of the immobilized thermolysin. Large amounts of calcium (at least 1/200 M) were required to immobilize thermolysin with a high activity and be stable at the concentration of 1/500 M calcium. It was assumed that more calcium was required to protect the enzyme protein from inactivation by glutaraldehyde. Calcium ions affected more noticeably on the thermostability of immobilized thermolysin than soluble one (Fig. 3).

The affinity of the enzyme toward the substrate casein was evaluated as Km values (Fig. 4). Km value was markedly increased as the result of immobilization, which is influenced by the speed of diffusion of the substrate through the carrier. The same result was reported in immobilized Alkaline-protease and rennet[12].

One of the major problems in continuous coagulation of milk has been the rapid loss in activity of immobilized enzyme preparations; a half-life of 4-8hr has been reported employing pig pepsin immobilized by covalent binding to porous glass[6]. In the present paper, immobilized thermolysin showed exceedingly long half-life even at high temperature (Fig. 5). The high thermostability and long half-life of immobilized thermolysin shed some possibility to be used in more efficient continuous coagulation process in high temperature.

Preparation of cheese using immobilized thermolysin

Separation of the hydrolytic and clotting phases at curd formation was successfully accomplished by reversing the order of the lactic acid fermentation process and the protease treatment process of milk, i.e., rennetting. By applying more thermostable immobilized thermolysin, column operation at high temperatures (50 or 55℃) was effective in preventing microbial infection in the enzyme column, maintaining longer retention of operational stability than Alkaline-protease and rennet (Fig. 5).

As the second step, lactic acid fermentation in the milk was initiated by *Streptococcus cremoris*; within 1hr milk began to coagulate. After clotting, it took about 1hr to obtain desirable firm curd to employ cutting procedure. In the traditional procedure using rennet, the similar firm curd can be obtained when the clotted milk is incubated for 30-40min. The delay in curd formation can be resulted from the fact that the pH of milk was raised to 7.0 at the first step and increasing rate of curd tension decreased when other coagulant than rennet is used[1].

By passing the milk through a reactor containing an immobilized protease, protease would not be incorporated into the cheese, enabling control of ripening. The ripening ratio of TL-cheese was higher than that of R-cheese, though both TL- and R-cheeses did not include the clotting enzyme. Whereas the ripening ratio of TL-cheese was almost same to that of N-cheese which contains the rennet. It seems that a slight excess of casein hydrolysis by thermolysin compared with that by rennet makes the casein in TL-cheese be more easily hydrolyzed by the protease of lactic acid bacteria than casein in R-cheese, accelerating the ripening of TL-cheese.

Under the conditions in this study, it may be desirable to treat milk at 50 or 55℃ although it is known that it may not be desirable to treat milk at higher temperature because of effects on whey proteins. Virtually immobilized thermolysin might be used successfully in continuous coagulation system since troublesome microbial growth would be minimized and since no contamination of milk with protease would occur thereby avoiding excessive proteolysis leading to bitterness and defective texture in cheese.

요 약

Thermolysin을 Dowex MWA-1에 10% glutaraldehyde로 고정화하였으며, 이 고정화 thermolysin을 사용하여 連續的凝乳를 行하였다. Thermolysin의 固定化收率은 25%이었다. 고정화 thermolysin은 1/200 M Ca^{++} ion의 존재하에서 60℃의 고온에서도 안정하였으며 이 온도에서의 half-life는 16일이었다. 원료 milk를 pH 7.0이 되도록 조정하여 55℃로 유지된 고정화 thermolysin column을 통과시켜 분해시킨 후, *Streptococcus cremoris*를 접종하여 凝乳시켜 curd를 얻었으며, 이렇게 하므로써 미생물오염이 방지될 수 있었으며 연속적응유를 효과적으로 행할 수 있었다. 고정화 thermolysin을 사용하여 얻은 cheddar type의 cheese는 rennet을 사용한 전통적인 방법으로 만든 cheese와 거의 비슷하였다.

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