

Research Article



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Covalent immobilization of an alkaline protease from *Bacillus licheniformis*

Bacillus licheniformis'ten bir alkalın proteazın kovalent immobilizasyonu

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Keywords: Cow's milk; Eupergit CM; Immobilization; Protease; Protein; Protein allergy.

Abstract

Objective: Since the soluble enzymes can not be used in repeated reactions and are not stable in operational conditions and not suitable for continuous processes, this study aimed the covalent immobilization of *Bacillus licheniformis* protease (BLP) onto Eupergit CM.

Methods: Optimum conditions for immobilization were determined by changing the conditions individually. The proteins and L-tyrosine were determined by UV/VIS spectrophotometer.

Results: The immobilization resulted in 100% immobilization and 107.7% activity yields. The optimum pH (7–8) and the optimum temperature (70°C) have not changed after immobilization. The K_m values for free and immobilized enzyme were 26.53 and 37.59 g/L, while the V_{max} values were 2.84 and 3.31 g L-Tyrosine/L · min, respectively. The immobilized enzyme has not lost its initial activity during the repeated 20 uses and 20 days of storage. The milk proteins were hydrolyzed in 2 h by using immobilized enzyme. The pH of the milk dropped from 6.89 to 6.53, the color was clearer but there was no change in the smell or the taste.

Conclusion: Consequently, it can be said that the immobilized BLP obtained can be used for industrial purposes.

Özet

Amaç: İmmobilize enzimler, tekrarlanan reaksiyonlarda kullanılmadığı ve kullanım şartlarında kararlı olmadığı ve endüstriyel sürekli süreçler için uygun olmadığından, bu çalışma, BLP'nin Eupergit CM'ye kovalent immobilizasyonunu amaçlamıştır.

Yöntemler: İmmobilizasyon için optimum koşullar, şartlar ayrı ayrı değiştirilerek belirlendi. Proteinler ve L-tirozin, UV/VIS spektrofotometre ile tayin edildi.

Bulgular: İmmobilizasyon % 100 immobilizasyon verimi ve % 107.7 aktivite verimi ile sonuçlandı. Optimum pH (7–8) ve optimum sıcaklık (70°C) immobilizasyon sonrasında değişmemiştir. Serbest ve immobilize enzim için K_m değerleri sırasıyla 26.53 ve 37.59 g/L iken, V_{max} değerleri sırasıyla 2.84 ve 3.31 g L-Tirozin/L · dk idi. İmmobilize enzim, tekrarlanan yirmi kullanım sırasında ve yirmi gün saklama süresinde başlangıç aktivitesini kaybetmedi. İmmobilize enzim kullanılarak süt proteinleri iki saat içinde tamamen hidrolize edildi. Sütün pH'sı 6.89'dan 6.53'e düşerken, rengine biraz açılma oldu fakat koku-sunda ve tadında herhangi bir değişiklik olmadı.

Sonuç: Sonuç olarak, elde edilen immobilize BLP'nin endüstriyel amaçlar için kullanılabileceği söylenebilir.

Anahtar Kelimeler: Eupergit CM; Immobilizasyon; Inek sütü; Proteaz; Protein; Protein alerjisi.

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Introduction

Cow's milk protein (CMP) allergy is the immunological reactions that occur in the body against cow's milk proteins [1]. CMP allergy is one of the most common allergies among infants and seen in 2–5% of all babies [2].

CMP in infant milk formula is used to ensure the protein content. However, non-breastfed babies are commonly fed with cow's milk, especially in rural areas. Considering that more than 138 million babies came into the world each year [3], the CMP allergy which is seen as a world widespread disease can be greatly reduced by consumption of protein-free cow's milk. Proteases are enzymes that convert the proteins to peptides and amino acids by hydrolysis. Therefore, they can be used in the production of cow's milk that does not cause to protein allergy. There are a few studies in the literature related to the hydrolysis of whey proteins using soluble proteases [4–12]. Yet, there is no study in the literature related to the hydrolysis of proteins in the milk using immobilized proteases.

Bacillus licheniformis protease (BLP) (E.C.3.4.21.14) is a serine peptidase which forms peptides by hydrolyzing the proteins. It is used widely in a variety of applications including dietary supplements, food and beverage processes, ingredient development, and protein processing.

BLP has been immobilized by using different supports and methods in a few studies [13–18]. Nonetheless, there has been no study related to the immobilization of BLP on Eupergit CM.

Four different methods of immobilization have been developed according to the nature of the matrix used and the ones intended to be used: cross-linking, adsorption, entrapment, and covalent binding [19]. Hundreds of natural and synthetic supports or matrices have been used for immobilization of industrial enzymes [20]. Choosing the enzyme immobilization matrix and method is related to the nature of the matrix, the simplicity of the method, and the aimed use of the enzyme [21]. The covalent attachment is a more suitable method for all enzymes and for all applications since immobilized enzyme does not easily lose its activity [22].

Eupergit CM, which contains epoxy groups on its surface and consists of porous acrylic microbeads, is a commercial matrix developed for covalent immobilization of enzymes. Eupergit C is an excellent matrix for immobilization of enzymes. It is very stable in wide ranges of pH and at high temperatures, as well as suitable for all kinds of reactors. Epoxy groups on the matrix react with amino, sulfhydryl, and hydroxyl groups of biomolecules depending on pH of the buffer used [23]. Immobilization procedure is quite simple and involves the reaction of Eupergit C beads with aqueous enzyme solution at room temperature or 4°C for 24–20 h. Immobilization of enzymes onto epoxy activated matrix is affected by the amount of Eupergit C, pH and concentration of buffer used, and the duration of immobilization

[24, 25]. These conditions must be optimized for using the immobilized enzymes in industrial applications. Since Eupergit CM has a similar chemical structure to that of Eupergit C, the same procedure is applied for immobilization. Many of the enzymes have been immobilized by using Eupergit C with up to 80% activity yields. Furthermore, in some studies immobilization have been resulted in an increased activity than soluble enzymes [26–31].

In this study, BLP will be tried to be immobilized by using the covalent binding method on Eupergit CM with the highest possible immobilization and activity yields.

Materials and methods

Materials

BLP (Alkaline protease L) which had 392.6 IU/mL protease activity, is a commercial enzyme preparation, was provided as a gift by Bio-Cat (Troy, MI, USA). Eupergit CM was a gift by Röhm and Haas (Darmstadt, Germany). UV-VIS Spectrometer (UV-6300PC) was purchased from VWR (Radnor, PA, USA). pH meter (Hanna HI 2020 edge), was purchased from Hanna Instruments Ltd. (Bedfordshire, UK). Magnetic stirrer (Heidolph MR Hei-Standard) was purchased from Heidolph UK-Radleys (Shire Hill, UK). Pure water appliance (Mini Pure 1, MDM-0170) was purchased from MDM Co. Ltd. (Suwon-si, South Korea). Sensitive scale (Shimadzu-ATX224) was purchased from Shimadzu Corporation (Kyoto, Japan). Orbital shaking heated incubator (Mipro-MCI) was purchased from Protek Lab Group professional laboratory solutions company (Ankara, Turkey). Vacuum pump (Biobase, GM-0.50A) was purchased from Biobase Biodustry Co., Ltd. (Shandong, China). Electrophoresis system (Mini-Protean Tetra Cell) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Bovine serum albumin, sodium hydroxide, sodium dihydrogen phosphate, hydrochloric acid, L-tyrosine and Folin & Ciocalteu's Reagent were purchased from Sigma-Aldrich (Taufkirchen, Germany). Sodium azide was purchased from Merck Millipore (Darmstadt, Germany). Semi-skimmed UHT cow milk was purchased from a local market.

Methods

All experiments were performed in triplicate and the mean values are calculated by using Windows Excell.

Immobilization procedure

Immobilization was performed by reacting 100 mg Eupergit CM with 200 μL free BLP having 78.51 IU activity in 5 mL of sodium phosphate buffer (0.5 M, pH 7.5) at 25°C for 24 h in an incubator with gentle shaking at 0.25155 g. After the immobilization, the beads were filtered and washed with 15 mL of sodium phosphate buffer (0.1 M, pH 7.5) and 15 mL distilled water on a sintered glass filter by suction under vacuum. Then, immobilized enzymes have been stored in 5 mL of sodium phosphate buffer (0.1 M, pH 7.5) in a refrigerator at +4°C until use.

Optimization of immobilization procedure

Optimum conditions for immobilization were determined by changing individually the conditions, (amount of Eupergit CM from 100 to 600 mg, pH from 5 to 10, buffer

BLP having 78.51 IU activity or 0.286 g Eupergit CM carrying 84.06 IU BLP activity at 70°C for 60 min in an incubator with gently shaking. Then, 400 μL of aliquots from the reaction mixture was added to 3600 μL of distilled water and boiled for 10 min to inactivate the enzyme. The amount of formed L-tyrosine was determined by measuring its absorbance using a UV spectrophotometer at 274 nm, according to the method of Esfandiary et al. [34]. One IU is defined as the amount of enzyme forming 1 μmol L-tyrosine from casein per minute, under optimum activity assay conditions.

Calculation of immobilization and activity yields

The immobilization and activity yields were calculated by using following equations.

Immobilization Yield (%)

$$= \frac{\text{Enzyme amounts used for immobilization} - \text{enzyme amounts in immobilization filtrate}}{\text{Enzyme amounts used for immobilization}} \times 100 \quad (1)$$

concentration from 0.5 to 2.0 M, and duration of immobilization from 12 to 120 h).

Activity Yield (%)

$$= \frac{\text{Activity of immobilized enzyme}}{\text{Activity of soluble enzyme}} \times 100 \quad (2)$$

Protein assay

The amounts of proteins present in the immobilization buffer before and after the immobilization as well as amounts of proteins present in the semi-skimmed UHT cow's milk before and after the hydrolysis of proteins were determined by using Bradford Protein Assay Method [32]. Accordingly, the 0.1 mL samples were added to 3000 mL Bradford reagents in 10 mL vials and incubated 45 min at room temperature for the completing the formations of color and after then the absorbances were measured at 595 nm by using UV spectrophotometer. The amount of immobilized enzyme protein was assayed from the difference between the amount of protein used for immobilization minus that recovered into the supernatant plus washings [33].

Determination of BLP activity

Casein solutions (1% w/v) prepared 5 mL 25 mM sodium phosphate buffer (pH 7.5) was reacted with 200 μL free

Characterization of free and immobilized enzyme

Effect of pH on enzyme activity

The effect of pH on enzyme activity was investigated by performing the activity assay for the soluble and immobilized enzymes with 1% (w/v) casein solutions at 70°C and different pHs.

Effect of temperature on enzyme activity

The effect of temperature on enzyme activity was found by conducting the activity assay for the soluble and immobilized enzymes with 1% (w/v) casein solutions (pH 7.5) at different temperatures.

pH stability

Two hundred micro liter free BLP having 78.51 IU activity or 0.286 g Eupergit CM carrying 84.06 IU BLP activity

were incubated in sodium phosphate buffer solutions at various pH ranges (5.0–12.0) at room temperature for 1 h, then the remaining activity was determined under standard assay conditions.

Thermal stability

Two hundred micro liter free BLP having 78.51 IU activity or 0.286 g Eupergit CM carrying 84.06 IU BLP activity were incubated in sodium phosphate buffer (25 mM, pH 7.5) at temperatures from 20 to 90°C for 1 h, then the remaining activity was determined using the standart assay method.

Kinetic constants

Initial velocities for kinetic parameters were determined by performing the reactions at different casein concentrations (5 to 25 g/L) for 15 min. K_m and V_m were calculated from Lineweaver–Burk plots by non-linear curve fitting.

Operational and storage stabilities of the immobilized BLP

Operational and storage stabilities of the immobilized enzyme were determined by using the standard activity assay method in repeated batch experiments every 2 days, respectively. The immobilized enzymes used for the determination of storage stability were stored in 0.1 M sodium phosphate buffers (pH 7.5) containing 0.02% sodium azide, in a refrigerator at +4°C until next use. Before each use, the immobilized enzymes were filtered and washed with 15 mL of sodium phosphate buffer (0.1 M, pH 7.5) and 15 mL distilled water on a sintered glass filter by suction under vacuum.

Hydrolysis of milk proteins by using immobilized BLP

0.286 g Eupergit CM carrying 84.06 IU BLP activity was reacted with 5 mL of semi-skimmed UHT cow's milk at 70°C for 2 h and protein content was determined with 30 min intervals by using UV spectrophotometer.

Results

Protein assay

The enzyme concentration in the liquid BLP preparation was calculated as 27.04 mg/mL by using BSA standard

curve equation. In addition, the amount of protein in the semi-skimmed UHT cow was calculated as 3.1%.

Determination of protease activity

Free BLP activity was calculated as 392.6 IU/mL by using Equation 3. Since 27.04 mg of the free protease was present in 1 mL of liquid BLP preparation, the specific activity of free BLP was also calculated to be 14.52 IU/mg. Lastly, the amount of free BLP having 1 IU activity was calculated as 0.069 mg.

$$\text{IU/mL enzyme} = \frac{\text{Released L-Tyrosine } (\mu\text{mol})}{\text{Enzyme used (mL)} \times \text{Duration of Reaction (min)}} \quad (3)$$

Optimization of immobilization procedure

Effect of Eupergit CM amount on immobilization efficiency

Different amounts of support (100–600 mg) for BLP (200 μL) were tested. Table 1 shows that the lowest immobilization yield (71.0%) and highest activity yield (98.2%) were obtained for 100 mg Eupergit CM for 16 h immobilization.

Table 1: Effect of Eupergit CM amount on immobilization efficiency.^a

Eupergit CM (mg)	Immobilization yield ^b (%)	Activity yield ^c (%)
100	71.00 ± 0.03	98.20 ± 0.04
200	78.40 ± 0.04	96.90 ± 0.03
300	84.60 ± 0.02	96.10 ± 0.04
400	95.10 ± 0.05	95.60 ± 0.02

^aEach value represents the mean for three independent experiments conducted in triplicates. Data was analyzed by using Microsoft Windows Excell.

^bTwo hundred micro liter free BLP having 78.51 IU activity was reacted with different amounts of support in 5 mL of sodium phosphate buffer (0.5 M, pH 7.5) and room temperature with shaking at 0.25155 g in an incubator for 16 h.

^cTwo hundred micro liter free BLP having 78.51 IU activity or 0.286 g Eupergit CM carrying 84.06 IU BLP activity were reacted with 5 mL of 1% (w/v) casein solutions (pH 7.5) at 0.25155 g and at 70°C in an incubator for 60 min.

Effect of immobilization buffer pH on immobilization efficiency

Table 2 shows the influence of pH on immobilization. The highest immobilization efficiency was obtained at optimum pH (7.5) for both soluble and immobilized enzymes.

Effect of immobilization buffer molarity on immobilization efficiency

Table 3 shows the maximum immobilization efficiency obtained with phosphate buffers of 0.5 M.

Effect of immobilization time on immobilization efficiency

The duration of immobilization is of importance: maximum activity yield (107.7%) were achieved for 24 h (Table 4).

Characterization of immobilized enzyme

Optimum pH

According to Figure 1, the optimum pH range of *BLP* (7.0–8.0) was not affected by immobilization. On the other

Table 2: Effect of immobilization buffer pH on immobilization efficiency.^a

Immobilization buffer pH	Immobilization yield ^b (%)	Activity yield ^c (%)
5.0	68.60 ± 0.02	95.30 ± 0.03
6.0	69.40 ± 0.04	96.60 ± 0.05
7.0	70.60 ± 0.02	97.30 ± 0.04
7.5	71.00 ± 0.05	98.20 ± 0.02
8.0	72.50 ± 0.04	97.10 ± 0.03
9.0	73.90 ± 0.03	94.50 ± 0.02
10.0	76.90 ± 0.02	91.80 ± 0.04

^aEach value represents the mean for three independent experiments conducted in triplicates. Data was analyzed by using Microsoft Windows Excell.

^bTwo hundred micro liter free *BLP* having 78.51 IU activity was reacted with 100 mg of supports in 5 mL of sodium phosphate buffer (0.5 M) at different pHs and room temperature with shaking at 0.25155 g in an incubator for 16 h.

^cTwo hundred micro liter free *BLP* having 78.51 IU activity or 0.286 g Eupergit CM carrying 84.06 IU *BLP* activity were reacted with 5 mL of 1% (w/v) casein solutions (pH 7.5) at 0.25155 g and at 70°C in an incubator for 60 min.

Table 3: Effect of immobilization buffer molarity on immobilization efficiency.^a

Buffer molarity (M)	Immobilization yield ^b (%)	Activity yield ^c (%)
0.5	71.00 ± 0.02	98.20 ± 0.03
1.0	68.50 ± 0.04	96.00 ± 0.05
1.5	66.60 ± 0.03	95.40 ± 0.04
2.0	64.40 ± 0.05	94.60 ± 0.02

^aEach value represents the mean for three independent experiments conducted in triplicates. Data was analyzed by using Microsoft Windows Excell.

^bTwo hundred micro liter free *BLP* having 78.51 IU activity was reacted with 100 mg of supports in 5 mL of different concentrated sodium phosphate buffers (pH 7.5) at room temperature by shaking in an incubator at 0.25155 g for 16 h.

^cTwo hundred micro liter free *BLP* having 78.51 IU activity or 0.286 g Eupergit CM carrying 84.06 IU *BLP* activity were reacted with 5 mL of 1% (w/v) casein solutions (pH 7.5) at 0.25155 g and at 70°C in an incubator for 60 min.

Table 4: Effect of immobilization duration on immobilization efficiency.^a

Immobilization duration (h)	Immobilization yield ^b (%)	Activity yield ^c (%)
0	00.00 ± 0.02	00.00 ± 0.04
16	71.00 ± 0.04	98.20 ± 0.03
24	100.00 ± 0.03	107.70 ± 0.05
48	100.00 ± 0.05	102.10 ± 0.02
72	100.00 ± 0.03	97.30 ± 0.04
96	100.00 ± 0.04	94.40 ± 0.03
120	100.00 ± 0.02	92.70 ± 0.05

^aEach value represents the mean for three independent experiments conducted in triplicates. Data was analyzed by using Microsoft Windows Excell.

^bTwo hundred micro liter free *BLP* having 78.51 IU activity was reacted with 100 mg of supports in 5 mL of sodium phosphate buffers (0.5 M, pH 7.5) at room temperature with shaking in an incubator at 0.25155 g for different durations.

^cTwo hundred micro liter free *BLP* having 78.51 IU activity or 0.286 g Eupergit CM carrying 84.06 IU *BLP* activity were reacted with 5 mL of 1% (w/v) casein solutions (pH 7.5) at 0.25155 g and at 70°C in an incubator for 60 min.

hand, the immobilized enzyme is more active than the free enzyme in the pH ranges that is tested.

Optimum temperature

Figure 2 shows that the optimum temperature (70°C) was not affected by the immobilization. It is also clear that the immobilized enzyme exhibits higher activity than the free enzyme at the entire tested temperature range.

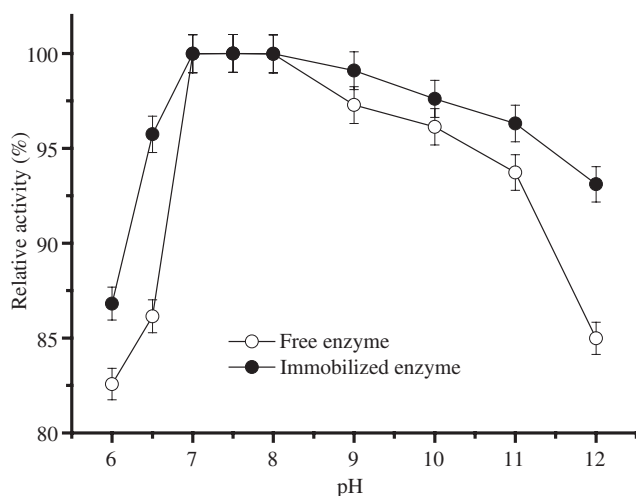


Figure 1: Optimum pH of free and immobilized BLP.

The effect of pH on enzyme activity was investigated by performing the activity assay for the soluble and immobilized enzymes with 1% (w/v) casein solutions, at different pHs, at 70°C. Hundred percent relative activities represent 78.51 IU and 84.06 IU BLP activities for free and immobilized enzymes, respectively.

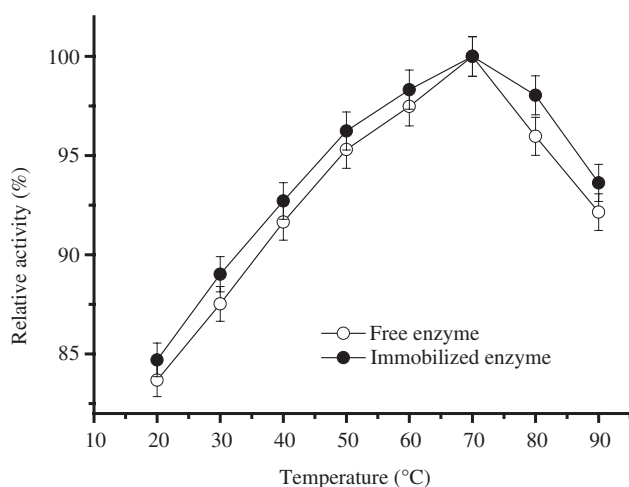


Figure 2: Optimum temperature of free and immobilized BLP.

The effect of temperature on enzyme activity was found by conducting the activity assay for the soluble and immobilized enzymes with 1% (w/v) casein solutions (pH 7.5) at different temperatures. 100% relative activities represent 78.51 IU and 84.06 IU BLP activities for free and immobilized enzymes, respectively.

pH stability

In Figure 3, the immobilized enzyme appears to be more stable than the free enzyme at entire tested pH range.

Thermal stability

Figure 4 shows the effect of temperature on the stability of the free and immobilized enzymes. It is evident that at

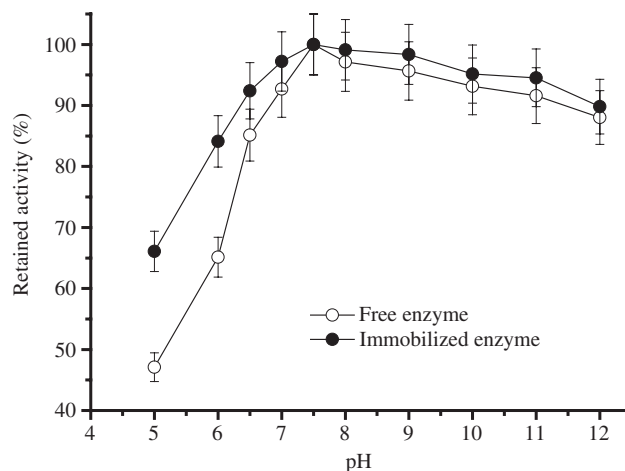


Figure 3: pH stability of free and immobilized BLP.

Two hundred micro liter free BLP having 78.51 IU activity or 0.286 g Eupergit CM carrying 84.06 IU BLP activity were incubated in buffer solutions at various pHs (5.0–12.0) of at room temperature for 1 h and the remaining activity was determined under standard assay conditions.

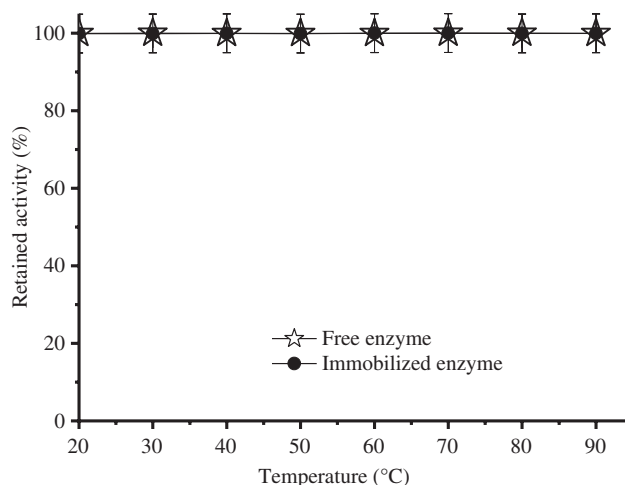


Figure 4: Thermal stability of free and immobilized BLP.

Two hundred micro liter free BLP having 78.51 IU activity or 0.286 g Eupergit CM carrying 84.06 IU BLP activity were incubated in buffer solutions (25 mM, pH 7.5) at temperatures from 20 to 90°C for 1 h and then the remaining activity was determined using the standard assay method.

the entire tested temperatures of free and immobilized enzymes, their stability is greatly preserved.

Kinetic constants

The Michaelis-Menten (K_m) and maximum velocity (V_{max}) constants of the free and immobilized enzymes were calculated from the Lineweaver-Burk Graph by non-linear curve fitting (Figure 5). Immobilization increased the value of the K_m constant from 26.53 g/L to 37.59 g/L while

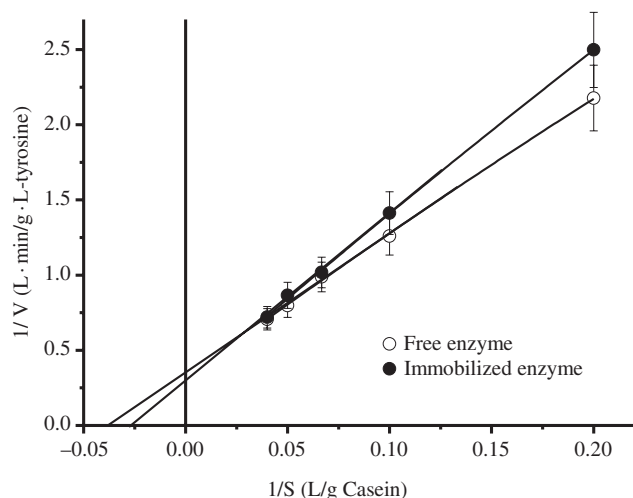


Figure 5: Lineweaver–Burk plots of free and immobilized BLP. Initial velocities for kinetic parameters were determined by performing the reactions at different casein concentrations (5–25 g/L) for 15 min.

increased the V_{\max} from 2.84 g L-Tyrosine/L·min to 3.31 g L-Tyrosine/L·min.

Operational and storage stabilities of the immobilized BLP

As seen in Figure 6, the immobilized enzyme has not lost its activity during the repeated 20 uses under optimum conditions. According to Figure 7, the immobilized enzyme has not lost its activity for 20 days under optimum storage conditions either.

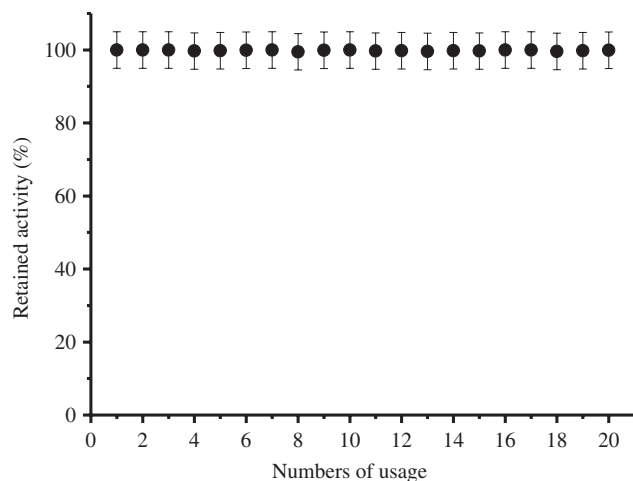


Figure 6: Operational stability of immobilized BLP. Operational stability of the immobilized enzyme was determined by using the standard activity assay method, in repeated batch experiments.

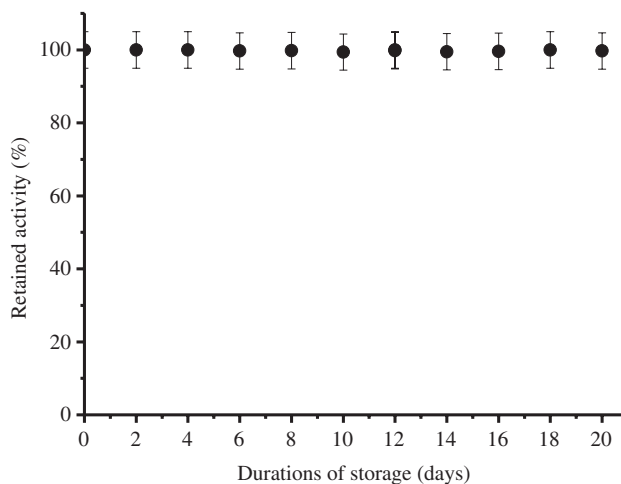


Figure 7: Operational stability of immobilized BLP. Storage stability of the immobilized enzyme was determined by using the standard activity assay method every 2 days.

Hydrolysis of milk proteins by using immobilized BLP

Changes in the protein concentration of the semi-skimmed UHT cow's milk during hydrolysis was evaluated by using a graph showing protein concentration against the time course (Figure 8). As seen in that figure, all proteins available in the semi-skimmed milk have been completely hydrolyzed in 2 h. This result is the highest degree of hydrolysis obtained compared to the previous findings in the relevant literature. Additionally, the pH of milk has decreased from 6.89 to 6.53 after hydrolysis. It

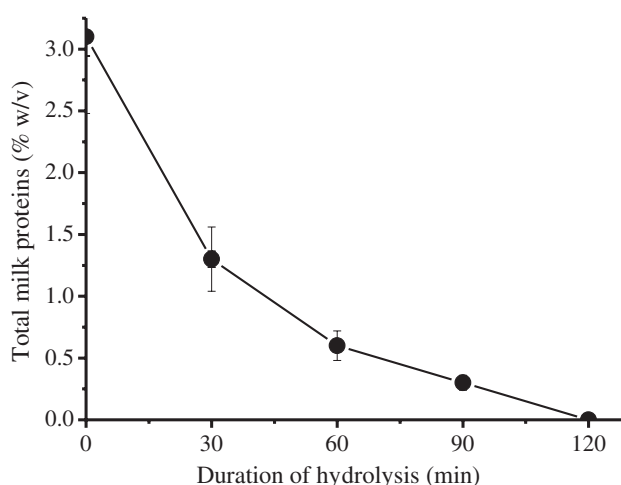


Figure 8: Hydrolysis of semi-skimmed UHT cow's milk proteins by using immobilized BLP. 0.286 g Eupergit CM carrying 83.3 IU BLP activity was reacted with 5 mL of semi-skimmed UHT cow milk at 70°C for 2 h and protein content was determined with 30 min intervals by using UV spectrophotometer.

was also observed that the milk was slightly transparent after hydrolysis. On the other hand, no change in the taste and smell of the milk has been detected at the end of the hydrolysis.

Discussions

The highest immobilization efficiency achieved for 100 mg of Eupergit CM can be seen from Table 1. Usage of higher amounts of Eupergit CM has resulted in the low activity yield. This is possibly due to the result of the deterioration of the three-dimensional structure of enzyme molecules upon multipoint attachment of enzyme molecules to the support. Moreover, the reaction of epoxy groups on support with amino acid residues associated with the active site of enzyme molecules can lead to decrease of the activity. As seen in Table 2, the highest activity yield in the immobilization with Eupergit CM has been obtained at the optimum pH (7.5) of the enzyme. The epoxy groups on Eupergit C can react with various reactive groups of enzymes in a wide pH range (0–12). Nevertheless, the highest activity yield is usually achieved at the optimum pH range in the immobilization of enzymes with Eupergit C [35]. Eupergit CM has a similar chemical structure to that of Eupergit C, but only beads and pore diameters, and the number of epoxy groups they contain differs. According to Table 3, when the molarity of immobilization buffer increased, activity yield has decreased while the immobilization yield has increased. Binding and activity yields in covalent enzyme immobilization on synthetic carriers, such as epoxy carriers, were often affected by the properties of the salts and their concentrations [36]. High salt concentration often increases the efficiency of binding due to the exposure of buried amino acid residues [37]. As it can be seen in Table 4, activity yield has decreased by increasing the duration of immobilization. This probably is due to changing the three dimensional structure of the enzyme, upon multipoint attachment of the enzyme to the support. By optimizing the immobilization conditions, 100% of immobilization yield and 107.7% of activity yield were achieved. Similar results are frequently encountered in the literature as well [26–31].

Optimum pH range of BLP has not changed after the immobilization (Figure 1). This result partially overlaps with the findings of some studies in the related literature on the immobilization of BLP [13] that reported the optimal pH of the BLP (Subtilisin Carlsberg) immobilized on glutaraldehyde-activated silica was 8.0, which was silanized with 3-aminopropyltriethoxysilane. The optimal pH for the immobilization of *Bacillus licheniformis* CMIT 1.33 protease

using silica gels, ceramic matrix, and entrapment method decreased to 8.0 from 9.0 [16]. In the adsorption of protease from *Bacillus licheniformis* B 40 on granular ceramics, the optimum pH of the enzyme (7.0) did not change [17]. On the other hand, both free and immobilized enzymes are more active at the alkaline pHs than at the acidic pHs. The activity of an enzyme is highly related to the chemical structure of amino acid available in the active site. The ϵ -amino group of histidine and the carboxyl group of aspartic acid present in the active site of the *Bacillus licheniformis* alkaline protease is more protonated in the acidic pH range than in the alkaline pH ranges; therefore activity may be lower at the acidic pHs. We see in Figure 2 that the optimum temperature of BLP also has not changed after the immobilization, and the immobilized enzyme exhibit more activity than the free enzyme at the entire tested temperatures. Generally, at high temperatures, immobilized enzymes exhibit higher activity than the free enzymes thanks to immobilization increasing the thermal stability of the enzyme. According to Figure 3, it is also seen that the immobilized enzyme is more stable than the free enzyme at all tested pH values. Probably this is a result of strengthening the three-dimensional structure of the enzyme, after binding of the enzyme molecules to the matrix via covalent bonds that is the strongest bond. In Figure 4 is seen that both of free and immobilized BLP are stable also at high temperatures. It is generally known that immobilization improves the thermal stability of enzymes. In addition, the thermophilic character of BLP may also have been contributed to this as well.

According to Figure 5, immobilized BLP has a higher K_m (37.59 g/L · min) and a higher V_{max} (3.31 g L-Tyrosine/L · min) values, respectively than the free enzyme. Comparison of kinetic constants for free and immobilized enzymes supplies the information about effectiveness and performance of immobilized enzymes. Similar results are also seen in the literature. For example, the immobilization of alcalase alkaline protease from *Bacillus licheniformis* on the magnetic chitosan nanoparticles resulted in similar pattern of Lineweaver-Burk Plot to our results. K_m values for free and immobilized enzyme were 10.6 and 12.8 mg/mL while V_{max} were 3.86 and 4.02 U/mg, respectively [31]. In another study, the immobilization of alkaline protease Esperase on Eudragit S-100 by covalent binding method increased the K_m value of the enzyme from 4.2 mg/mL to 5.2 mg/mL [14]. In the stabilization of *Bacillus licheniformis* ATCC 21415 alkaline protease by immobilization and modification, the K_m value had increased from 4.8 mg/mL to 5.0 mg/mL and 5.3 mg/mL, respectively, after immobilization and modification [15]. The increased K_m value implies the immobilized enzyme has lower affinity to the substrate than soluble enzyme. This result probably caused from

steric hindrance of the active site by the support, or the loss of enzyme flexibility necessary for substrate binding [14, 15]. On the other hand, the increased V_{\max} value may be due to hydrophobic character of matrix which supplies the optimum microenvironment for active sites of the enzymes [28]. There are also some similar studies that reported the increased V_{\max} values after immobilization in the literature [26–31].

It is seen in Figures 6 and 7 that the immobilized BLP has not lost its activity during 20 repeated usage and 20 days of storage. According to these results, it can be said that the immobilized BLP has a high usage and storage stability.

As seen in Figure 8, immobilized BLP has been successfully used in the hydrolyzing of all proteins available in the semi-skimmed UHT cow's milk without causing any important changes in its properties.

Consequently, it can be said that immobilized BLP obtained in the present study can be used in the industrial production of cow's milk without milk protein allergy causing effects.

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