

Hydrolysis of Feather Keratin by Immobilized Keratinase

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Keratinase isolated from *Bacillus licheniformis* PWD-1 was immobilized on controlled-pore glass beads. The immobilized keratinase demonstrated proteolytic activities against both insoluble feather keratin and soluble casein. It also displayed a higher level of heat stability and an increased tolerance toward acidic pHs compared with the free keratinase. During a continuous reaction at 50°C, the immobilized keratinase retained 40% of the original enzyme activity after 7 days. The immobilized keratinase exhibits improved stability, thereby increasing its potential for use in numerous applications.

Many techniques for surface derivatization and covalent immobilization of enzymes on different types of porous silica have been developed (2, 6, 11). The immobilization of proteases on solid supports has been widely used in many investigations (1-5). When a protease is immobilized, enzyme autolysis is minimized. Because the proteases exist in the solid phase, the separation of the products, such as amino acids and peptides, is easily accomplished. The immobilized enzyme can be cleaned and reused.

A keratinase from a feather-degrading thermophilic bacterium, *Bacillus licheniformis* PWD-1, was isolated and characterized (8). The purified keratinase, which proved to be a serine protease, demonstrated strong proteolytic activity toward feather keratin and other insoluble and soluble protein substrates. Recently, the keratinase gene was isolated and sequenced (7). In this report, we present the results of immobilization of keratinase on controlled-pore glass beads. Controlled-pore glass beads were chosen as the enzyme support because of their well-defined pore diameter, microbial resistance, excellent mechanical strength, and extraordinary chemical durability.

Keratinase immobilization. Purified keratinase was covalently immobilized on succinamidopropyl-glass by the sequential activation-immobilization procedure developed by Janolino and Swaisgood (6). An excess of purified keratinase (10 mg) was reacted with 4 g of activated glass beads. Approximately 100 µg of purified keratinase was immobilized on each gram of beads. After immobilization, the excess keratinase was collected for reuse. The proteolytic activity of the immobilized keratinase was measured in a 12-ml reaction mixture at 50°C in a rotating 15-ml screw-cap tube (0.2 ml of immobilized keratinase mixed with 1.2 g of feather keratin or casein in 12 ml of 0.05 M potassium phosphate buffer, pH 7.5). Every 30 min, 0.5 ml of the reaction mixture was sampled for free amino group analysis (10). Activity studies indicated that immobilized keratinase hydrolyzes both insoluble feather keratin and soluble casein (Fig. 1).

Heat stability. Casein hydrolysis (9) was used to measure proteolytic activity as described in the following experiments. To compare the heat stabilities of immobilized and free keratinases, a 0.1-ml aliquot of immobilized keratinase (containing 4.5 µg of purified keratinase) and 0.1 ml of free enzyme (containing 2 µg of purified keratinase) were pretreated at 80 or

90°C for different lengths of time and were then cooled to 50°C. A prewarmed (50°C) 1.9-ml casein solution (1.0% in 0.05 M potassium phosphate buffer, pH 7.5) was added to each pretreated enzyme mixture and allowed to react at 50°C for 30 min with mixing. At different time intervals, a 0.5-ml reaction mixture aliquot was taken and added to 1.0 ml of stop solution (0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid). After standing on ice for 1 h, the mixture was centrifuged for 5 min to remove undigested protein. The A_{280} of the supernatant was read against a control prepared with 0.5 ml of a sample taken immediately after the addition of the enzymes. The activities of untreated immobilized and free keratinase were measured in the same manner. The relative activities showed that immobilized keratinase has a higher level of heat stability than does the free enzyme (Table 1).

Durability test. Casein hydrolysis was also used to measure keratinase activities over a 7-day period. Free keratinase (10 µg) and immobilized keratinase (0.3 ml) were added separately into two 50-ml tubes, each containing 0.5 g of casein in 50 ml of phosphate buffer (pH 7.5). Sodium azide (NaN_3) was added in the reaction mixture to a final concentration of 0.02% to prevent bacterial growth. The tubes were incubated at 50°C with rotation. A 0.5-ml aliquot from each reaction mixture was

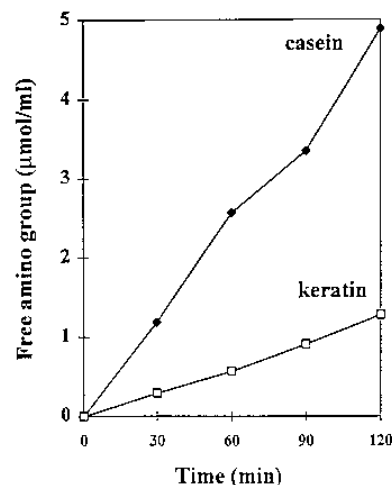


FIG. 1. Hydrolysis of keratin and casein by immobilized keratinase. The amount of free amino acid was measured by the ninhydrin method; each micromole of free amino group represents one micromole leucine equivalent.

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TABLE 1. Remaining relative keratinase activities^a after heat treatment

Treatment	Time (min)	Immobilized keratinase (%)	Free keratinase (%)
Untreated	0	100	100
	80°C		
	1	48	11
	5	26	1
90°C	10	18	0
	1	22	1

^a Enzyme activity was measured by casein hydrolysis. The untreated enzyme activities were changes in A_{280} of 0.41 units per 0.1 ml and 1.03 units per μg for the immobilized and free keratinase, respectively.

taken every 24 h, and casein hydrolysis was measured as previously described. During a 7-day reaction, immobilized keratinase hydrolyzed casein continuously, and it retained 40% of its activity at day 7. However, free keratinase lost its activity in 2 days, probably because of autolysis (8) and denaturation. It is concluded that immobilized keratinase has a higher durability than does free enzyme, allowing an extended reaction period (Fig. 2).

High- and low-pH treatments. Glycine buffer (0.1 M; pH 2, 3, or 4) or 0.05 M phosphate buffer (pH 5.5) was employed for the pretreatment of keratinase. For free keratinase, 2 μg of keratinase in 5 μl of distilled H_2O was mixed with 45 μl of buffer. After 30 min at room temperature, 450 μl of potassium phosphate buffer (0.1 M; pH 7.5) was added to each solution to bring the final pH to 7.5. A 1.5-ml aliquot of casein solution (1% in 0.1 M potassium phosphate buffer) was added to each solution, and this was followed by incubation at 50°C for 30 min. Enzyme without low-pH treatment was used as a control. Immobilized keratinase (0.1 ml) was incubated in 0.5 ml of glycine or phosphate buffers of various pHs for 30 min. Afterwards, the beads were washed with 1 ml of potassium phosphate buffer (pH 7.5) and resuspended in 0.5 ml of potassium phosphate buffer for casein hydrolysis. For pH 12 pretreatment, keratinase was incubated in 0.01 M NaOH (final concentration) for 30 min and then an equivalent concentration of HCl was added to neutralize the solution. Pretreated enzymes were then used for measuring casein hydrolysis. The results (Table 2) indicated that high-pH (pH 12) treatment does not decrease keratinase activity of both forms but acid pH does.

The two forms of keratinase were also tested against feather keratin and casein in phosphate buffers (pHs ranging from 5 to

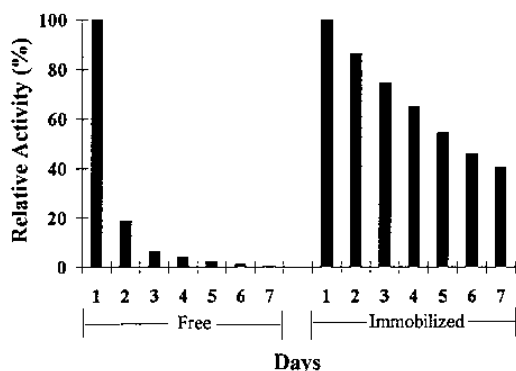


FIG. 2. Long-term hydrolysis of casein by immobilized and free keratinase. Values for casein hydrolysis on day 1 were changes in A_{280} of 1.26 and 6.69 units for immobilized and free keratinase, respectively.

TABLE 2. Comparison of caseinolytic activities after various pH treatments

pH	Immobilized keratinase		Free keratinase	
	ΔA_{280} ^a	Activity (%)	ΔA_{280}	Activity (%)
12	0.43	103	1.10	100
7.5	0.42	100	1.10	100
5.5	0.42	100	1.10	100
4.0	0.40	96	0.92	84
3.0	0.18	42	0.09	8
2.0	0.10	23	0.06	5

^a ΔA_{280} , changes in A_{280} , relative to value before treatment.

9.5). The caseinolytic activities of immobilized and free keratinase were measured by the methods as previously described except various 0.05 M phosphate buffers were used (Fig. 3). Keratinolytic activities were determined by using the ninhydrin method (10) to measure the release of free amino groups (8). An optimum pH of 7.5 was observed for feather hydrolysis with both immobilized and free keratinase. This result agrees with the previously reported optimum pH for azokeratin hydrolysis (8). However, when casein was used as the substrate, both forms of keratinase demonstrated the highest activity at pH 8.0. The shift in pH optimum is most likely due to differences in substrate solubility, steric hindrance, etc. But, immobilization of keratinase does little to change the enzymatic properties.

In conclusion, immobilized keratinase is able to hydrolyze both soluble casein and insoluble feather keratin. With increased heat stability and pH tolerance, immobilized keratinase could be exploited in some industrial applications. For example, a bioreactor with immobilized keratinase can convert ground feathers to peptides and amino acids which can be separated by filtration and ion-exchange chromatography. It is likely that immobilized keratinase can be used as a structural probe in studies of structure-activity relationship and its specificity. The results of the present study indicate that the level of keratinase attachment is low; therefore, further studies on im-

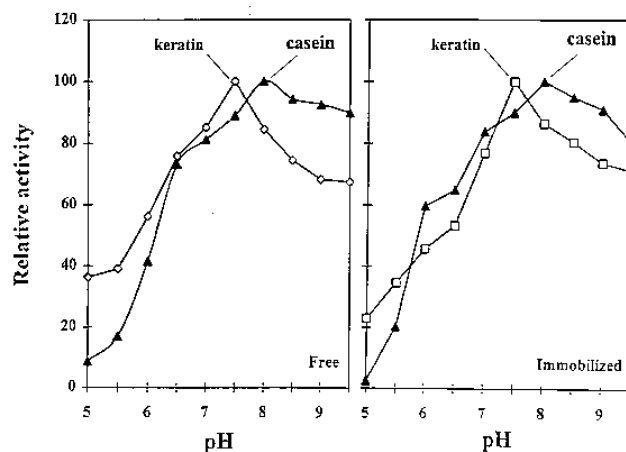


FIG. 3. Alteration of pH optimum for keratin and casein hydrolyses. The highest caseinolytic activities at pH 8 were changes in A_{280} of 0.39 unit per 0.1 ml and 1.23 units per μg for immobilized and free keratinase, respectively. The highest keratinolytic activities at pH 7.5 were 0.27 micromole leucine equivalent per 0.1 ml and 2.89 micromole leucine equivalents per μg for immobilized and free keratinase, respectively.

proving keratinase binding to various carriers are currently in progress.

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