Biotechnology Letters **23:** 51–54, 2001.

Immobilization of alkaline protease from *Conidiobolus macrosporus* for reuse and improved thermal stability

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Received 27 September 2000; Revisions requested 28 September 2000; Revisions received 24 October 2000; Accepted 24 October 2000

Key words: alkaline protease, detergent-compatibility, polyamide, thermostability

Abstract

Alkaline protease from *Conidiobolus macrosporus* was immobilized on polyamide using glutaraldehyde as a bifunctional agent. The immobilized enzyme was optimally active at a higher temperature of 50 °C than the free enzyme (40 °C) and showed a ten-fold increased thermostability at 60 °C compared to that of the free enzyme. The efficiency of immobilization was 58% under the optimal conditions of pH and temperature. There was a 14-fold decrease in the K_m of immobilized enzyme compared to the free enzyme. The immobilized enzyme was fully active even after twenty-two cycles of repeated use. It retained 80% activity at 50 °C in presence of 8 M urea exhibiting its stability to the denaturant and was compatible with several commercial detergents.

Introduction

Proteases represent one of the three largest groups of industrial enzymes and find extensive applications in food and pharmaceutical industries. Alkaline proteases are important in detergent and leather industries (Rao *et al.* 1998). Microbial proteases are preferred in view of the rapid growth of microbes, limited space required for their cultivation and their ready accessibility to genetic manipulation. Increased thermostability is an important factor for the suitability of an enzyme in industrial applications. The strategies used for improving the thermostability of proteins include use of additives, introduction of disulfide bonds (Perry & Wetzel 1984), site-specific mutagenesis (Imanaka *et al.* 1986) and chemical modification or crosslinking (Braxton & Wells 1992). In all these strategies, however, the recovery yield and reusability of free enzymes as industrial catalysts are quite limited. Therefore, an increased attention has been paid to enzyme immobilization which offers advantages over free enzymes in choice of batch or continuous processes, rapid termination of reactions, controlled product formation, ease of enzyme removal from the reaction mixture and adaptability to various engineering designs (Zaborsky 1973). The fungus *Conidiobolus macrosporus* (NCIM 1298) produces high yields (30 U ml^{-1}) of alkaline protease in short fermentation cycles (48 h) comparable to the bacterial enzyme. Fungal origin of the enzyme offers a distinct advantage for developing ecofriendly technologies in terms of ease of downstream processing as against cost-intensive high-speed centrifugation technologies required for the bacterial enzyme. Recently, as a prelude to its genetic engineering for overproduction, we have shown the presence and proximity of the essential tryptophan of *Conidiobolus* alkaline protease with the active site histidine and cysteine residues (Tanksale *et al.* 2000). The objective of our present work has been to immobilize the enzyme for its repeated reuse and for improving its thermostability with a view of increasing its efficiency for application in detergent industry. The enzyme was immobilized on an inexpensive support, polyamide, using glutaraldehyde as the spacer. The kinetic parameters, reuse potential, and stability of the immobilized enzyme are described.

Materials and methods

Materials

Malt extract, yeast extract, and agar were from Hi Media, India. Peptone, D-glucose, glutaraldehyde, Hammerstein casein were from Difco laboratories, USA; Qualigens ExcelaR, India; Loba chemicals, India and E-Merck, Germany, respectively. All other chemicals used were of analytical grade. Ultrafiltration membranes were purchased from Amicon, USA. Polyamide (100–200 mesh) was a gift from Polymer Division, National Chemical Laboratory, India.

Microorganism and enzyme production

Conidiobolus macrosporus (NCIM 1298) was grown on MGYP medium using soymeal (2% w/v) as an inducer (Tanksale *et al.* 2000). The crude extracellular broth, concentrated by ultrafiltration, was used as the source of the enzyme. The protease activity was determined in 0.1 M Na_2CO_3/$NaHCO_3$ buffer, pH 10, by incubating a suitable aliquot of the enzyme with casein, hemoglobin or ovalbumin (10 mg) in 2 ml at 40 °C for 10 min. One unit of enzyme activity is defined as the amount of enzyme required to cause an increase of one absorbance unit at 280 nm per min at 40 °C (Kunitz 1947). Protein was determined according to the method of Bradford.

Immobilization of the enzyme

All the steps were carried out between 4 to 10 °C. Glutaraldehyde (2% w/v) was added to polyamide (0.5 g) pre-equilibrated with 10 ml of 0.1 M potassium phosphate buffer, pH 7.5 and stirred for 3 h. Excess of glutaraldehyde was removed before the addition of protease. The concentrated enzyme (0.5 ml, 4 mg ml^{-1}) was added drop-wise to the polyamide suspension (12 ml) in 0.1 M phosphate buffer, pH 7.5 under constant stirring. After 6 h, the mixture was centrifuged at 10 000 *g* and the immobilized enzyme was washed twice with the same buffer. The activity of the immobilized enzyme was determined by shaking (200 rpm) in presence of substrate at 40 °C for 20 min.

Properties of the immobilized enzyme

Optimum pH and temperature of the enzyme were determined by estimating the enzyme activity in the pH range of 7 to 12 and in a temperature range of 40 to 80 °C at pH 10.0. The pH stability was determined by incubating the immobilized enzyme with 0.05 M buffer of desired pH for 1 h at 25 °C. The temperature stability was determined by incubating the enzyme at temperatures ranging from 40 °C to 80 °C at pH 7.5 for 1 h. Residual activity was determined at pH 8.0 at 50 °C and compared with the control sample kept at 4 °C at pH 7.5. The kinetic constant (K_m) of the enzyme was determined from the Lineweaver–Burk plots using casein (0.025–10 mg). The immobilized enzyme was incubated with the substrate at 50 °C for 20 min. It was recovered by centrifugation and washed with buffer for the next reuse. The immobilized protease (1 U each) was incubated at 30 °C in absence and presence of commercial detergents (7 mg ml^{-1}, which is the concentration, required to simulate washing conditions) for zero to 60 min. The effect of denaturants was studied by incubating the free and immobilized enzymes with urea (0–8 M) or SDS (0–0.3%) for 30 min. The residual activity was determined under the optimal conditions of pH and temperature.

Results and discussion

Optimization of the immobilization conditions

Conidiobolus macrosporus produces high yield of protease (30 U ml^{-1}). The crude extracellular broth consists of five different alkaline proteases as visualized by the gel-X-ray film contact print technique (Tanksale *et al.* 2000). To utilize the activity of all these proteases in the commercial application, the crude culture filtrate was used for immobilization on polyamide. Glutaraldehyde acts as a spacer arm for the immobilization of alkaline protease on polyamide. Maximum immobilization was obtained with glutaraldehyde at 1.76% (w/v) and by stirring the enzyme with polyamide for 6 h. The efficiency of immobilization was 58% when the activity was determined under the optimum conditions of pH (8.0) and temperature (50 °C).

Characterization of the immobilized enzyme

The thermal stability of the immobilized enzyme is one of the most important criteria for its application. The native *Conidiobolus* protease showed maximum activity at 40 °C whereas temperature optimum for the immobilized enzyme was 50 °C (Figure 1). The immobilized enzyme showed 10 times higher activity at 60 °C compared to the native enzyme and exhibited higher thermostability (Table 1). Covalently-bound

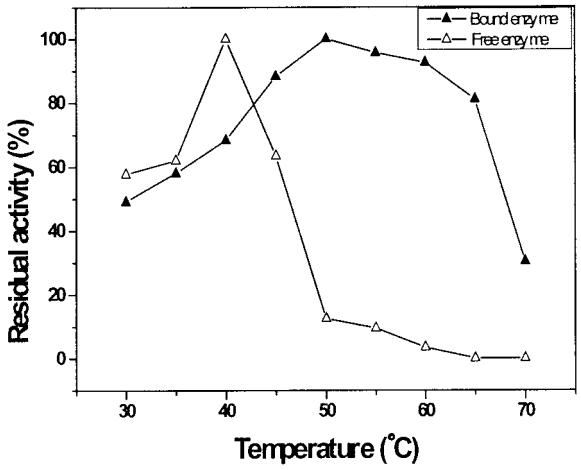

Fig. 1. Optimum temperature of the immobilized alkaline protease. The free (△) and the bound (▲) enzyme (0.3 and 1 U, respectively) were incubated at different temperatures (30–70 °C) at pH 10.0 for 20 min and the residual activity was determined.

Table 1. Comparison of the properties of free and immobilized enzyme.

Enzyme	Free	Immobilized
	Activity (%)	
At 60 °C	5	50
In presence of		
Urea (8 M) at 50 °C	17	80
Ariel	47	60
Rin Shakti	76	84
Surf Excel	80	54
K_m (mg ml^{-1})	102	7

immobilized enzyme system is more resistant to heat and denaturing agents than its soluble form. This confers a good operational stability to the immobilized preparation, i.e., the ability to maintain a constant level of activity over a significant period during actual use, resulting in an easier process control. This higher stability can be attributed to the prevention of autodigestion and/or thermal inactivation because of the fixation of enzyme molecules on the surface of polyamide and can be exploited for its application in detergents.

The free and the bound enzyme were stable (>50% activity) over a wide pH range of 5 to 12. The maximum stability of the free enzyme was at pH 7.5 whereas that of the bound enzyme was at pH 8. The free enzyme exhibited maximum activity at pH 10, whereas the bound enzyme exhibited maximum activity between pH 8 to 9, which can be explained on the basis of partitioning of hydrogen ions. Since polyamide is positively charged, it has a tendency to concentrate [OH] ions around it, thus increasing the pH around the enzyme. In order to obtain the pH value for optimum activity in the vicinity of the enzyme, the external bulk phase value of pH must be lower than the enzyme's intrinsic pH optimum. Hence, the pH optimum shifted towards more acidic values of pH and as a result, the optimum pH of the enzyme is lowered by 1–2 units. A shift of 0.5 units in the optimum pH towards alkaline side was observed in case of an alkaline protease immobilized on epichlorohydrin-activated cellulose beads (Chellapandian & Velan 1998).

The kinetic studies of the soluble and the immobilized alkaline protease showed that there was a 14-fold decrease in the apparent K_m of the immobilized enzyme indicating increased affinity for the substrate (casein). The alteration in the kinetic constant can be attributed to the change in the microenvironment around the enzyme. The covalent binding of the enzyme may reduce the diffusion barrier for the substrate and facilitate the access of the active site of the enzyme to the substrate. Substrate specificity determines the suitability of the enzyme preparation for its biotechnological exploitations. Both native and immobilized protease preparations were able to hydrolyze other substrates such as hemoglobin and ovalbumin besides casein, exhibiting broad substrate specificity. The immobilized enzyme exhibited higher efficiency (9.4 U ml^{-1}) of hydrolysis of hemoglobin than casein (5.1 U ml^{-1}) at 50 °C.

Effect of denaturants

There was an increase in the activity of both free and polyamide-bound enzyme with increasing concentration of urea. Maximum 43% increase in the activity was observed at 3 M urea for the free enzyme. The bound enzyme showed 47% increase in the presence of 4 M urea at 28 °C. Increase in temperature to 40 °C led to inactivation of the free enzyme with increase in urea concentration, whereas the immobilized enzyme continued to show increased activity. Further, increase in temperature to 50 °C led to a complete loss of activity of the free enzyme, whereas the immobilized enzyme was able to retain 80% of the activity in presence of 8 M urea. Thus, the immobilized enzyme was stable in the presence of urea at higher temperatures. Both free and polyamide-bound enzymes exhibited a simi-

lar pattern of inactivation due to SDS. They retained about 25% of the original activity in the presence of 0.3% SDS at 28 °C. The loss of activity may be attributed to the binding of negatively charged detergent to the positively charged alkaline protease. The immobilized enzyme retained 25% and 15% of its activity compared to 13% and 0% for the free enzyme in the presence of 0.3% SDS at 40 °C and 50 °C, respectively, indicating higher stability of the bound enzyme compared to the native enzyme to the denaturant at increased temperature.

Compatibility with detergents

Due to their environmentally friendly nature, alkaline proteases have potential application in detergent industry for partial or total replacement of toxic chemicals. An ideal detergent enzyme should be stable and active in the detergent solution and should have adequate temperature stability to be effective in a wide range of washing temperatures. Both the free and bound proteases showed excellent compatibility with commercial detergents available in the Indian market such as Ariel, Rin Shakti, and Surf Excel. The immobilized enzyme retained 84% of its activity after incubation with Rin Shakti at 28 °C for 1 h, whereas the free enzyme retained 76% of its activity under similar conditions. Both the enzymes retained more than 50% of their activity in presence of Ariel and Surf Excel. The enhanced stability for the immobilized enzyme can be attributed to its covalent binding with the polyamide, which offers greater protection against autodigestion and inactivation. The polyamide-bound enzyme is stable over a wide range of pH and exhibits a broad substrate specificity that may be effective in washing of a variety of stains. These properties are also shown by the free enzyme. Earlier, the alkaline protease from *Conidiobolus* sp. (NCL 86.8.20) has been shown to be compatible with the detergents like Nirma, Snow-white, Revel, Wheel etc. (Phadatare *et al.* 1993). However, the free enzyme is stable only upto 40 °C, whereas, as shown in the present work, the immobilized enzyme from *Conidiobolus macrosporus* is stable upto 60 °C, which is an additional advantage for its potential use in washing powders.

Reuse of the immobilized enzyme

The reuse of the immobilized enzyme is very important from the point of view of reducing the cost of the enzyme, which is an important factor while considering its suitability for commercial application. The immobilized alkaline protease retained its activity without any significant loss even after the reaction was repeated twenty-two times indicating excellent performance of the polyamide-bound enzyme.

In conclusion, the alkaline protease covalently immobilized on an inexpensive support, polyamide, exhibits several improved properties such as a higher optimum temperature, increased thermal stability, enhanced affinity to the substrate, resistance to denaturants, excellent compatibility with detergents and retention of activity even after twenty-two reuses.

Acknowledgements

The award of SRF to AMT by CSIR, New Delhi and the financial support by DBT, India is gratefully acknowledged. We thank Prof V. Sitaramam for useful discussions.

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