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Use of Different Adsorbents for Sorption and *Bacillus polymyxa* Protease Immobilization

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Abstract

Proteases constitute one of the most important groups of industrial enzymes, accounting for at least 25% of the total enzyme sales, with two-thirds of the proteases produced commercially being of microbial origin *(1)*. Immobilized enzymes are currently the subject of considerable interest because of their advantages over soluble enzymes or alternative technologies, and the steadily increasing number of applications for immobilized enzymes. The general application of immobilized proteins and enzymes has played a central role in the expansion of biotechnology and synthesis-related industries. Proteases have been immobilized on natural and synthetic supports *(2,3)*.

In the present work, a protease from *Bacillus polymyxa* was partially purified with 80% ammonium sulfate precipitation followed by dialysis and chromatography using a diethylaminoethyl (DEAE)-cellulose ion exchange column. Immobilizaiton was evaluated by using different adsorbents (chitin, chitosan, alginate, synthetic zeolite, and raw zeolite) and the storage stability and recycle of the immobilized protease determined. Immobilization yields were estimated to be 96% and 7.5%, by using alginate and chitosan, respectively, after 24 h. The yield of the immobilization was 17% for alginate at 16 h and the enzyme did not adsorb on the chitin, chitosan, synthetic zeolite, and raw zeolite.

Index Entries: Alginate; *Bacillus polymyxa*; chitin; immobilization; protease.

Introduction

Industrial proteases are used as additives in detergents, and in tanning and food manufacturing (baking, brewing, and dairy industries). The majority of theses enzymes are derived from microbial sources, with a few microbial strains being used for industrial applications. *Bacillus polymyxa* has been shown to produce starch-degrading enzymes that yield maltose in very high yield. In the course of investigations it was reported that this microorganism produced considerable quantities of an extracellular proteases in a basal starch–peptone medium *(4)*.

Immobilization involves the coupling of the enzyme with a selected solid support via covalent and (or) noncovalent interactions. Several

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noncovalent linkages can be specifically favored, including ionic and metal bindings as well as physical adsorption. Immobilization by adsorption results from weak forces between the protein and the solid surface, such as electrostatic and hydrophobic interactions or hydrogen bonds. Adsorption might proceed through conformational rearrangements, depending on the enzyme nature. As adsorptions constitute a mild immobilization process with respect to covalent linkage, it is expected to have less effect on enzymatic kinetic behavior. Despite its simplicity, immobilization by physical adsorption is significantly limited by the tendency of enzyme to desorb from the support *(5–7)*.

The support material can effect the stability of the enzyme and the efficiency of enzyme immobilization, although it is difficult to predict in advance which support will be most suitable for a particular enzyme. The most important requirements for a support material are that it must be insoluble in water, have a high capacity to bind enzyme, be chemically inert and be mechanically stable. Owing to the often conflicting requirements of a good support, various materials have been used. The type of support can however be conveniently classified into one of three categories:

1. Hydrophilic biopolymers based on natural polysaccharides such as agarose, dextran, and cellulose.
2. Lipophilic synthetic organic polymers such as polyacrylamide, polystyrene, and nylon.
3. Inorganic materials such as controlled pore glass and iron oxide.

This work was undertaken to investigate production and immobilization of *B. polymyxa* protease using various carriers using physical adsorption.

Materials and Methods

The compounds obtained from Sigma Chemical Co., UK were bacto tryptone, bacto soytone, bacto agar, peptone, beef extract, ammonium sulfate, manganese chloride, calcium chloride, sodium carbonate, sodium bicarbonate, and diethylaminoethyl (DEAE)-cellulase. Manganese sulfate, acetic acid, tricholoro acetic acid, sodium acetate, boric acid, sodium hydroxide, casein, ammonium sulfate, hydrochloric acid, sodium chloride, ethyl alcohol, ethylenediamine-tetracetic (EDTA), tris, sodium dodecylsulfate, ammonium persulfate, glycerol, and tin sulfate were purchased from Merck Chemical Co., Germany. Acrylamide, *bis*-acrylamide, and glycine were obtained from BioRad Chemical Co., France. Chitin and chitosan were obtained from Fluka, BioChemica, Switzerland. Alginate was purchased from Riedel-de Haen, Germany. Synthetic zeolite and raw zeolite were synthesized.

Isolation, Screening, and Identification of Protease Producing Strains

Bacterial strains used in the study were isolated from industrial waste discharge samples using enrichment techniques, and screened for their

ability to produce protease. Samples that were collected from different factories in Turkey were screened for microorganisms, especially for the genus *Bacillus*. A 200-µL aliquot of each sample was spread onto different nutrient agar plates containing: 5 g/L peptone; 3 g/L meat extract; 15 g/L agar (pH adjusted to 7.0) and cultured at 37°C for 24 h. After 24 h of incubation isolated colonies were transferred to new agar plates and were grown under same conditions *(8–10)*.

The Gram-stain was used to evaluate the purity and morphology of the isolates. Only Gram-positive spore forming rods were further evaluated. Specific identification of bacterial strains was done using API 50 CH test kits (Biomérieux, France). The API 50 CH test strips were inoculated and incubated according to the manufacturer's instructions. Using this method only one bacterial strain was identified as *B. polymyxa*.

Production of Enzyme

The production of enzyme was carried out by growth in 1-L Erlenmeyer flasks containing 200 mL nutrient broth (5 g peptone, 3 g meat extract, 10 mg $MnSO_4 \cdot H_2O$ for 1 L media) as liquid media. The isolates were cultivated for 20–22 h at 37°C with agitation of 200–250 rpm.

Partial Purification of Protease

After incubation, the culture broth was centrifuged at 4°C and 20,000*g* for 20 min. Ammonium sulfate was added slowly to the solution up to 80% saturation with gentle stirring and left for 30 min at 4°C *(11,12)*. The precipitate formed was collected by centrifugation at 25,000*g* for 30 min, dissolved in a minimum amount of 50 m*M* Tris-HCl buffer, pH 7.5, containing 5 m*M* $CaCl_2$, dialyzed against the same buffer overnight and then lyophilized. The precipitate that was obtained after ammonium sulfate saturation, dialysis, and centrifugation was loaded to a DEAE–cellulose ion-exchange chromatography column (2×60 cm^2) that had been pre-equilibrated with borax-NaOH buffer (0.01 mol/L and pH 9.3). The protein was eluted with the same buffer using a fraction size 10 mL at the rate of 1 mL/min. Fractions containing the majority of the protease activity were pooled for activity assay. The activity of protease enzyme at the end of each step was measured by spectrophotometric method *(13)*.

Protease Assay

Two methods were used to determine protease activity. With the first method activity was determined at 30°C using tubes containing 2.5-mL milk casein in water bath for 5 min. The reaction was carried out by adding 0.5 mL enzyme solution for 10 min. After 10 min, the reaction was stopped by adding 2.5 mL TCA solution, the solution was storage in water bath for 20 min sample *(14)*. With the second method activity was determined at

35°C using tubes containing 3 mL substrate solution incubated in a water bath at 35°C for 5 min. The reaction was carried out by adding 0.5 mL enzyme solution for and incubation 10 min. The reaction was stopped by the addition of 3.2 mL TCA solution, the solution was stored in water bath for 10 min. The rate of casein hydrolysis was determined using absorbance values 275 nm *(12)*. One unit of activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine in 1 min at 30°C (1 U/mL·1 μg tyrosine/min·mL). A tyrosine standard was prepared by dissolving different amounts of tyrosine in TCA solution.

Immobilization Method

B. polymyxa protease was immobilized by adsorption on alginate, chitin, chitosan, raw zeolite, and synthetic zeolite. For the immobilization, 100 mg of the carrier was incubated with 100 U *B. polymyxa* protease dissolved in 1 mL of 0.5 *M* Tris-HCI buffer (pH 7.2) at 4°C overnight under static conditions (in the case of chitosan 200 U of *B. polymyxa* protease was used). The unbound enzymes were removed by washing with Tris-HCl buffer (0.5 *M*, pH 7.2).

Results and Discussion

The specific activity and purification degrees of the partially purified protease are shown in Table 1. The column purification resulted in a 10-fold increase in specific activity compared with the culture supernatant, and a twofold increase compared with ammonium sulfate precipitation. Increases of the partial purified protease activity using ammonium precipitation (twofold increase) are consistent with published literature, which shows a purification (fold) range from 1.9 to 96 *(15,16)*. A 10-fold increase using DEAE-chromatography column is also consistent with reported purification ranges from 7.8 to 555 *(17,18)*.

In the present study a partially purified protease from *B. polymyxa* was immobilized on alginate, chitin, chitosan, raw zeolite, and synthetic zeolite by physical adsorption. The carriers were incubated with 100 U and 200 U protease dissolved in Tris-HCl buffer at +4°C for 16 h. The highest bound enzyme and immobilization yield was found with alginate. A low immobilization yield (17%) and bound enzyme (171.38 U/g solid) was detected with alginate. It was determined that protease enzyme was not adsorbed on the chitin, chitosan, synthetic zeolite, and raw zeolite *(19,20)*.

Table 2 shows that an immobilization yield (5.8–96%) and bound enzyme (52.1–950 U/g solid) was achieved using raw zeolite, chitin, chitosan, and alginate. The enzymes physically bound to alginate showed the highest immobilization yield (96%) and the highest activity per gram carrier (950 U/g solid). The activity recovery of the immobilized protease increased with prolonged reaction times with the highest activity recovery obtained the immobilization was allowed to proceed for 24 h. Activity

Table 1
Protein Purification of Partial Purified Protease Enzyme From *B. polymyxa*

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold
Crude homogenate	9797.6	155,467	15.9	1
40% saturation	8812.8	141,073	16	1
80% saturation	210	6369	30.3	2
DEAE-cellulose ion exchange chromatography	2.5	403.5	161.4	10

Table 2
Immobilization of *B. polymyxa* Protease (24 h)

Solid	Protease enzyme (U/g solid)	Bound enzyme activity (U/g solid)	Unbound enzyme activity (U/g solid)	Yield
Alginate	1000	950	10.97	96
Chitin	1000	65.36	74.2	7.5
Chitosan	2000	91.34	131.09	4.9
Raw zeolite	1000	52.1	107.52	5.8
Synthetic zeolite	1000	–	–	–

Table 3
Effect of Time on the Immobilization of *B. polymyxa* Protease

Solid	Protease enzyme (U/g solid)	Activity (U/g solid) 20 d after	Protected activity (%)
Alginate	950	611	64
Raw zeolite	52.1	32.8	62

recovery decreased if the reaction time was longer because of the increasing process deactivation. The immobilization yield was found as 7.5% and 5.8% for chitin and raw zeolite, respectively *(21,22)*.

Immobilization by physical adsorption for 24 h showed considerable bound enzyme activity (good loading efficiency) and immobilization yield. This good loading efficiency for the immobilization might have been owing to the formation of stable cross linking between the carrier and the enzyme through a group (alginate).

Storage stability is a major concern in enzyme preservation. The storage stability of the *B. polymyxa* protease was examined for 20 d at +4°C as shown in Table 3. It was found that protease immobilized on alginate and synthetic zeolite maintained their activities 64 and 62%, respectively. After

storage for 20 d, the immobilized protease still maintained about 64% of its initial activity, whereas the native enzyme only maintained about 40% of the initial activity.

Conclusions

Physical adsorption of enzymes onto solid supports is probably the simplest way of preparing immobilized enzymes. The method relies on nonspecific physical interaction between the enzyme protein and the surface of the matrix, brought about by mixing a concentrated solution of enzyme with the solid. A major advantage of adsorption as a general method of immobilization enzymes is that usually no reagents and only a minimum of activation steps are required. As a result, adsorption is cheap, easily carried out, and tends to be less disruptive to the protein than chemical means of attachment, the binding being mainly by hydrogen bonds, multiple salt linkages, and Van der Waal's forces.

In this study, a protease was partially purified from *B. polymyxa* using 80% $(NH_4)_2SO_4$ precipitation, dialysis and DEAE-ion exchange chromatography. Immobilization was achieved on alginate, chitin, chitosan, raw zeolite, and synthetic zeolite by noncovalent absorption. The maximum yield of absorption was 17% for 16 h and 96% for 24 h onto alginate. It was found that immobilized enzyme was stable to 20 d. Having storage stability the practical applications of enzyme is a potential advantage. The detailed study of immobilization on the same support with the different methods, such as the covalent attachment, and the usage of this immobilized protease, which has high storage stability in the batch, and continuous processes will be further investigated.

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