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Immobilization of Invertase Through Its Carbohydrate Moiety on *Ocimum basilicum* Seed

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ABSTRACT

Yeast invertase, a glycoprotein, was covalently coupled to *Ocimum basilicum* seeds either through its protein or carbohydrate moiety. Of the various methods investigated, binding of the enzyme through its carbohydrate moiety resulted in the retention of considerably higher amounts of enzyme activity. Immobilized invertase showed a shift in the pH optimum toward the alkaline side without appreciable change in temperature optimum. However, the immobilized preparation was more thermostable than the free enzyme. Invertase bound to the seeds could be used repeatedly for the hydrolysis of sucrose syrups in a batch process without appreciable loss in activity. The seeds could serve as an inexpensive, ready-to-use, natural pellicular polysaccharide support for immobilizing enzymes.

Index Entries: Invertase; immobilization; carbohydrate moiety; epoxy activation; periodate oxidation; *Ocimum basilicum* seeds; pellicular support.

INTRODUCTION

Enzymes and cells have been immobilized on various supports either by adsorption, entrapment, cross-linking, or covalent binding (*1–6*). Among these, covalent binding has been extensively studied for the immobilization of enzymes (*5,6*). Covalent binding of an enzyme to a support

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is normally achieved using some of the nonessential functional groups in the protein. Even though this technique immobilizes the enzyme firmly on the support, it often results in a loss of activity, which has been attributed mainly to either involvement of active sites in bond formation or conformational changes.

Some of these problems can be obviated in the case of glycoprotein enzymes by covalently binding them via carbohydrate moiety (*7,8*). Yeast invertase is a glycoprotein with a mol wt of 250,000; 50% of which is carbohydrate, mainly glucan and mannan (*9*). This paper details studies on covalent binding of invertase through its carbohydrate moiety on *O basilicum* seeds, which can serve as a novel, nontoxic, natural pellicular support.

MATERIALS AND METHODS

Materials

Invertase (250 U/mg), 1,4-butanediol diglycidyl ether (bisoxirane) and glutaraldehyde (25%) were obtained from Sigma Chemical Co. (St. Louis, MO). Ethylenediamine and sodium metaperiodate were procured from SISCO Research Laboratories, India. *Ocimum basilicum* seeds, also known as "Sabja seeds," were purchased from the local market.

Methods

Different methods were investigated for immobilizing invertase on *O basilicum* seeds.

Swelling of Seeds

Ocimum basilicum seeds were soaked in water for 1 h. Under these conditions, 1 g of dry seeds on swelling occupied a vol of 26 mL. In all further studies, the amount of seeds taken is expressed in terms of vol of swollen seeds.

Epoxy Activation of Seeds

Epoxy activation of *O basilicum* seeds was carried out by modification of a method described previously for activation of sepharose (*2,10*). Swollen seeds (50 mL) were mixed with 25 mL each of bisoxirane (70% solution) and 0.6*N* NaOH containing 2 mg/mL of sodium borohydride. The suspension was mixed on a gyrorotary shaker (Lab-Line Instrument Inc., IL) at 120 rpm for 8 h at 25°C. The seeds were then washed with large vol (3 L) of distilled water. The activated seeds were stored in water.

Coupling of Invertase to Epoxy Seeds

Epoxy-activated seeds (50 mL) were suspended in 25 mL of bicarbonate buffer (pH 10) containing 25 mg of invertase. After 8 h of incubation at

room temperature, the enzyme-bound seeds were filtered and washed with water, followed by (0.1*M*) acetate buffer, pH 4.5.

Coupling of Invertase to Epoxy-Activated Seeds Via Ethylenediamine Glutaraldehyde Arm

The epoxy-activated seeds (50 mL) were mixed with 50 mL of 1.0*M* ethylenediamine (pH 11.0 adjusted with 1*N* HCl). After a 24-h incubation at room temperature, the ethylenediamine-bound seeds were separated, washed with water, and then treated with 4 mL of 25% aqueous solution of glutaraldehyde (final concentration of 2%) for 16 h by gently shaking at room temperature. The activated seeds were then washed with excess water and mixed with an aqueous solution containing 25 mg of invertase. The mixture was incubated for 8 h under mild shaking. The seeds were washed with water followed by acetate buffer (pH 4.5) before assaying for activity.

Coupling of Invertase to Periodate-Oxidized Seeds

Swollen seeds (50 mL) were treated with 50 mL of 0.1*M* $NaIO_4$ for 6 h in the dark on a rotary shaker under mild shaking conditions. The seeds were then washed with distilled water and suspended in an aqueous solution of invertase (25 mg). The suspension was mixed on a shaker water bath for a period of 8 h at 25°C. The seeds were washed as stated above.

Coupling of Invertase Through Its Carbohydrate Moiety

The carbohydrate moiety of invertase was oxidized by mixing 25 mg of invertase dissolved in 25 mL of 50m*M* acetate buffer (pH 5.0) with 3 mL of 50m*M* $NaIO_4$. The suspension was mixed gently (6 h at 4°C) on a Test Tube Rocker (Thermolyne, IA) in the dark. The unreacted $NaIO_4$ was removed by adding 0.1 mL ethylene glycol and mixing for a further period of 30 min. The suspension was then dialyzed against 50m*M* acetate buffer.

Simultaneously, seeds (50 mL) were activated using bisoxirane, and ethylenediamine was bound as described above. The oxidized enzyme was then added to the ethylenediamine-bound seeds, and the suspension was incubated for 8 h with gentle shaking. The supernatant was decanted, and the seeds were washed with water and acetate buffer before assaying for enzyme activity.

Determination of Oxirane Groups

The amount of oxirane groups present on the matrix was determined according to the procedure of Axen (*10*). The epoxy-activated seeds were suspended in 1.3*M* $Na_2S_2O_3$, and the pH was kept constant at 7.0 by addition of 0.1*N* HCl. The amount of oxirane groups present was calculated from the amount of HCl required to maintain neutrality.

Invertase Assay

Invertase was assayed according to the method described earlier (*11*). In the case of immobilized preparation, seeds containing the bound enzyme

Table 1
Techniques for Immobilizing Invertase

Method of Immobilization	(U/g)[a] dry wt of matrix
Control seeds	Nil
Epoxy activated seeds + enzyme	354
Epoxy activated seeds + ethylenediemine glutaraldehyde arm + enzyme	275
Periodate oxidized seeds + enzyme	345
Epoxy activated seeds + ethylenediamine + periodate oxidized enzyme	1000

[a] One U is defined as 1 μmol of sucrose hydrolysed per min.

were stirred (250 rpm) for 30 min at 25°C in sucrose solution (10% in 0.04*M* acetate buffer, pH 4.5). Reducing sugars formed were estimated using dinitro salicylic acid reagent (*12*). Unit of activity has been defined as μmol of sucrose hydrolyzed in 1 min.

RESULTS

Activity retention of invertase immobilized on *O basilicum* seeds using different techniques is shown in Table 1.

Epoxy Activation and Coupling of Ligand

Bisoxirane activates the polysaccharide polymer matrix by imparting active oxirane groups (*10*). In the system used in this study, oxirane activation attained was about 195 μmol of oxirane groups per g dry wt of seeds. Binding of the enzyme directly to the epoxy-activated matrix, however, did not yield high retention in enzyme activity (Table 1).

Immobilization of Invertase Via an Ethylenediamine Glutaraldehyde Spacer Arm

The *O basilicum* seeds could be imparted a free amino group by coupling excess of ethylenediamine to the free oxirane groups present on the epoxy-activated seeds. Treating the seeds with 1.0*M* ethylenediamine was found to be optimal. The seeds could be further activated to contain a free aldehyde group, using a bifunctional reagent such as glutaraldehyde. The enzyme bound to this matrix also showed low retention in activity (Table 1).

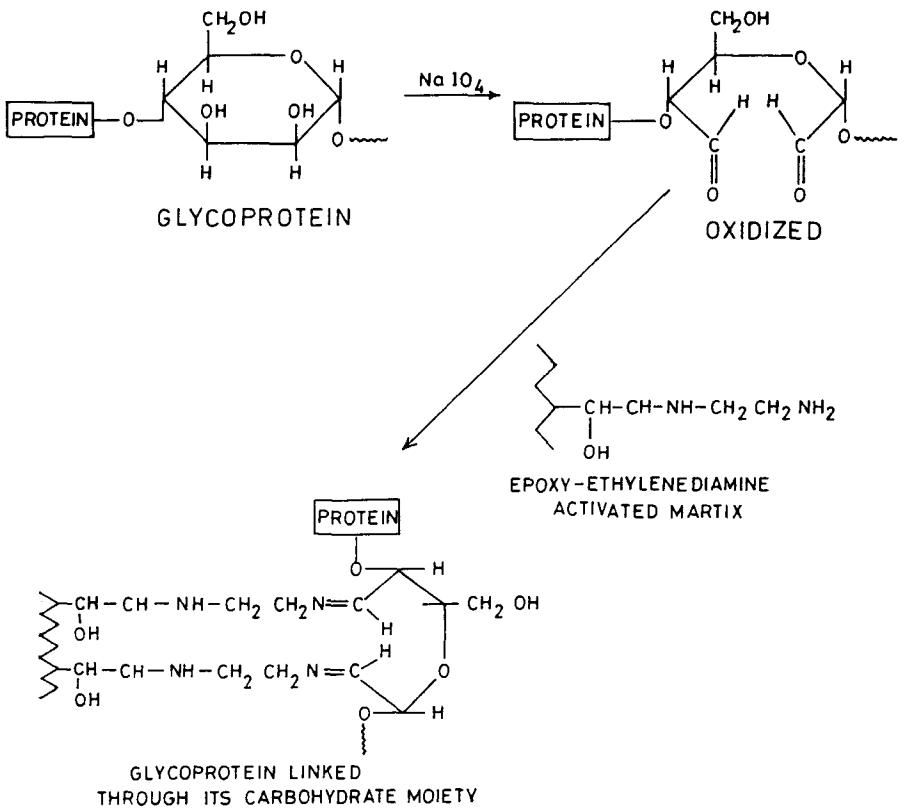

Fig. 1. Coupling of glycoprotein through its carbohydrate moiety.

Periodate Oxidation of Seeds

Treating the swollen seeds with 0.1*M* $NaIO_4$ resulted in partial disruption of the outer pectinous matrix. After oxidation, the vol of seeds decreased from 50 to 12 mL. Immobilization of enzyme through formation of Schiff's base, with the aldehyde groups now present on the matrix, resulted in low yield in enzyme activity (Table 1).

Periodate Oxidation of Enzyme

Immobilization of invertase via its carbohydrate moiety was performed after periodate oxidation of vicinal diol groups of its carbohydrate component capable of forming reactive aldehyde groups (Fig. 1). Under the optimal conditions chosen for oxidizing the carbohydrate moiety of the enzyme, invertase retained about 95% of its original activity. The aldehyde groups on the oxidized enzyme formed stable bonds with the amino groups of the epoxy-ethylenediamine-activated matrix. Thus, washing

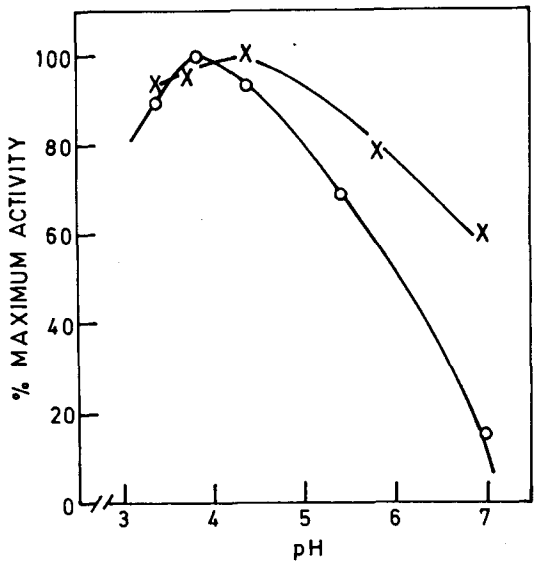

Fig. 2. The pH activity profile of invertase immobilized on *O basilicum* seeds. The buffers used were acetate in the pH 3.4–5.4 range and phosphate in the 5.8–6.9 range. Experimental details are stated in the text. Activity obtained at optimum pH was taken as 100%. O, soluble enzyme; X, immobilized enzyme.

the enzyme bound seeds with buffer containing 0.5*M* NaCl did not elute the enzyme.

Of the various techniques discussed above, invertase immobilized via its carbohydrate moiety was found to retain the maximum activity (Table 1). This preparation was therefore used for further characterization.

KINETIC PROPERTIES OF INVERTASE IMMOBILIZED ON *O BASILICUM* SEEDS

pH Activity Profile

The pH activity profile of invertase in the free and immobilized form has been shown in Fig. 2. The pH optimum of the soluble and immobilized preparation is 3.8 and 4.4, respectively. The decrease in activity in the alkaline range was more rapid for the native invertase as compared to the immobilized preparation.

Temperature Activity Profile

The temperature activity profile of invertase (free and immobilized) was studied by carrying out the enzyme assay at the desired temperature. No appreciable change in the temperature optimum was observed on immobilization (Fig. 3). However, immobilized enzyme was found to retain greater activity at higher temperatures compared with free enzyme.

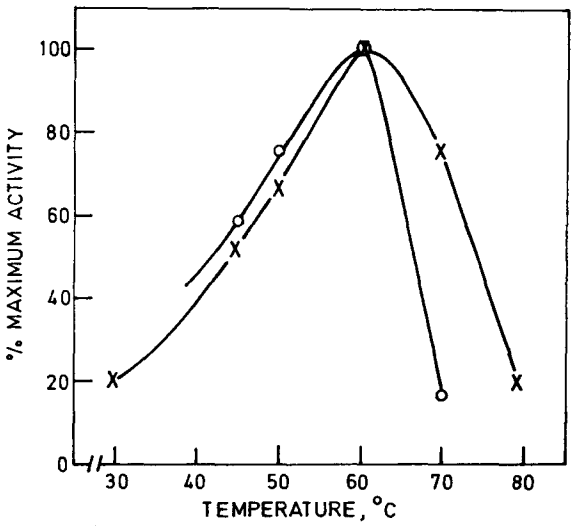

Fig. 3. Effect of temperature on invertase activity. The enzyme assay was carried out as indicated in the text. Activity obtained at optimum temperature was taken as 100%. O, soluble enzyme; X, immobilized enzyme.

Thermostability

Samples of invertase, free or immobilized on *O basilicum* seeds, were suspended in acetate buffer at the desired temperature in a water bath (30 min) and then cooled rapidly by immersion in ice. The residual enzyme activity was then assayed at 25°C. The immobilized preparation was found to be more thermostable when compared to the free form (Fig. 4). Thus, even at a temperature of 70°C, the immobilized enzyme retained 78% of original activity; the free enzyme retained only 20% of the activity.

Reuse

Invertase-bound seeds (15 mL) were suspended in 100 mL of 10% sucrose in 0.04*M* acetate buffer (pH 4.5) for 30 min at 25°C. The enzyme-bound seeds were retrieved by filtration using a sieve, washed extensively with water and buffer, and reused in fresh batches of substrate. Intermittently, when not in use, the seeds were stored in 0.1*M* acetate buffer (pH 4.5) at 0–5°C. The enzyme-bound seeds could be reused for more than 10 batches without appreciable loss in activity (Table 2).

DISCUSSION

Success of any immobilized enzyme process will depend to a great extent on the selection of a proper technique as well as a support. The technique used should be mild enough to retain as much of the activity as possible on immobilization. In addition to various other aspects, the sup-

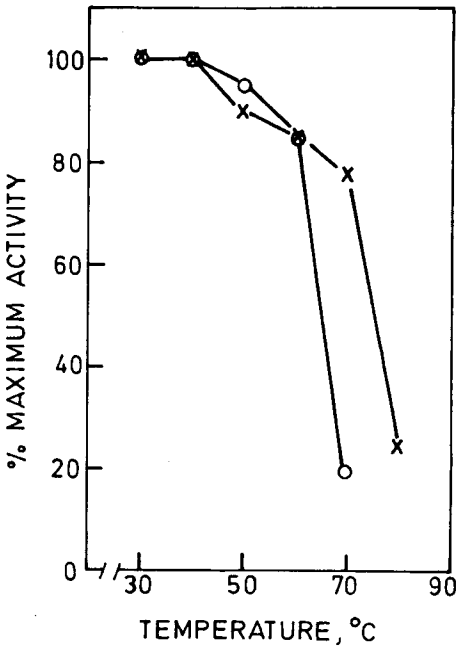

Fig. 4. Thermostability of invertase. Maximum activity was taken as 100. Experimental details as stated in the text. O, soluble enzyme; X, immobilized enzyme.

Table 2
Reuse Stability of Invertase Coupled
Through Its Carbohydrate Moiety to *O basilicum* Seeds

Reuse no.	Total reducing sugar (g)	% Maximum activity
1	6.1	100
2	5.9	96
3	5.7	94
4	5.8	96
5	5.6	91
6	5.8	95
7	6.0	100
8	5.6	91

[a]Total reducing sugars formed at the end of reuse (30 min.) was estimated in each of the fresh batches. Experimental details as mentioned in the text.

port used also should have a large surface area and functional groups for binding enzymes, should exhibit minimal diffusional problems and good flow properties, and should be inexpensive and readily available (*13*). Nontoxicity of the support is of prime importance when using immobilized enzymes for applications in the food industry, as in this study.

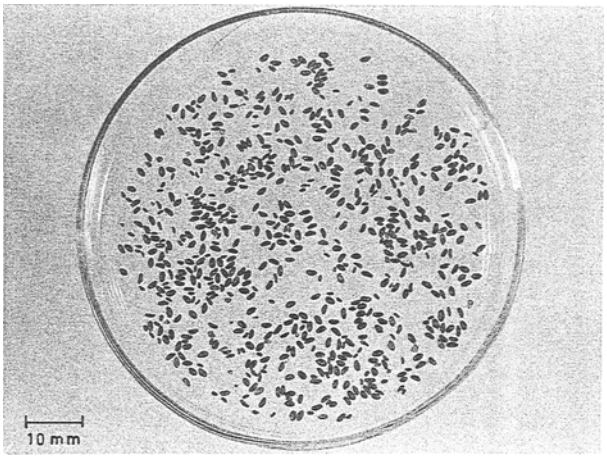

Fig. 5. Dry seeds of *O basilicum*.

Polysaccharide supports, including cellulose, dextran, starch, agar, agarose, alginate, and their derivatives, have been extensively investigated for the immobilization of enzymes (*14*). This is mainly because such supports contain a large number of functional groups that can be activated using different techniques for covalent binding of enzymes and other biomolecules. Another important attribute is their hydrophilicity, an important factor for the preservation of enzyme activity. However, in addition to the very high cost of some, the short comings of currently used polysaccharide supports include low mechanical stability and low temperature stability.

In view of this, the swollen seeds of *O basilicum* could serve as a useful, alternative polysaccharide support for immobilizing enzymes. The mucilaginous layer of the swollen seed is a pectinous matrix with a large capacity for hydration (Figs. 5,6). The mucilage consists of a thread-like microfibrillar structure, with a large surface area as observed under a microscope (Fig. 7). Because of its open structure with an inert hard core, this particular morphology can be useful as a natural pellicular support for immobilizing enzymes. Using synthetic pellicular supports, which consist of enzymes immobilized only in the porous outer shell of the carrier particle, recently has been gaining importance in enzyme immobilization. Such supports, because of their large particle size with lesser diffusional or mass transfer problems, have been recommended for use in packed-bed reactors (*15,16*). To date, most of these synthesised supports are expensive and tedious to prepare.

Invertase could be coupled to the seeds either through its protein or carbohydrate moiety. However, coupling of the enzyme via its protein moiety resulted in a lesser amount of activity retention as compared to binding via carbohydrate moiety. Biomolecules can be covalently linked

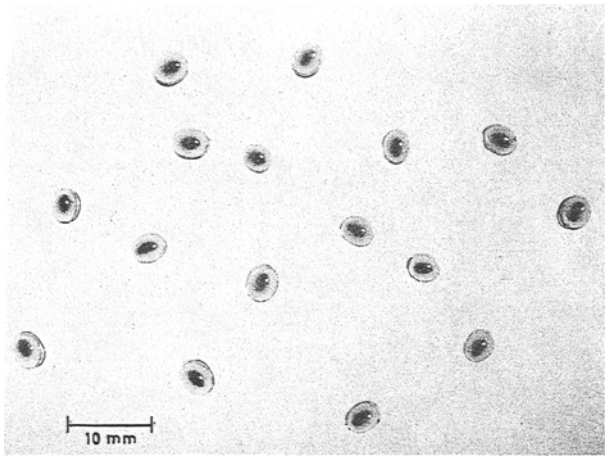

Fig. 6. Swollen seeds of *O basilicum.*

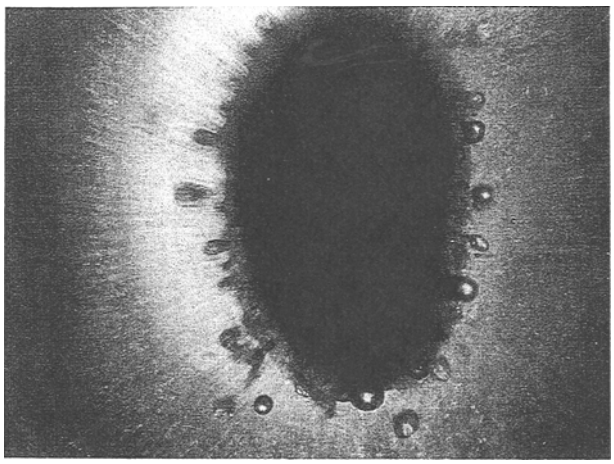

Fig. 7. Microscopic view of the seed.

to epoxy-activated supports through the free NH_2 groups. The efficiency of linkage is pH- and temperature-dependent. Coupling is performed in the pH range of 9–13, but the stability of the ligand and the matrix at the operating pH could be a limiting factor. Immobilizing invertase directly onto the epoxy-activated seeds resulted in low retention in activity, which could be attributed, in addition to other factors, to possible inactivation occuring at an alkaline pH. Deactivation of invertase with time at a higher pH of the medium during immobilization on diazonium salt of 4-amino-benzyl cellulose has been reported by Simionescu et al. (*17*).

The alternate method of binding invertase to the epoxy seeds via an ethylenediamine glutaraldehyde arm did not improve the retention yield of enzyme activity. Glutaraldehyde is known to inactivate enzymes by

binding either to essential -NH_2 or -SH groups. It has been reported that the active form of invertase requires an unprotonated acidic group COO- for substrate binding and a protonated imidazole residue, which is apparently involved in substrate cleavage. It also has been suggested frequently that an -SH group is involved in the catalytic action of invertase (*9*). Woodward and Wiseman (*18*) have also reported on invertase inactivation when crosslinked with glutaraldehyde as a result of the modification of some of the amino groups involved in the maintenance of enzyme in the active state.

Immobilization of enzyme on supports in general is known to alter its kinetic and stability characteristics, which have been attributed to various factors. In this study, the pH optimum was shifted to the alkaline side. This could be partly attributed to the presence of free carboxyl groups on the support as the matrix consists of a considerable amount of unesterified galacturonic acid (*19*). Shift in pH optimum to the alkaline side with enzyme bound to polyanionic supports has also been reported (*20*). The higher thermal stability observed on immobilization can be a positive attribute as higher temperatures enhance reaction rates and minimizes microbial contamination.

The enzyme-bound seeds were found to be mechanically very stable. Continuous, vigorous stirring on a magnetic stirrer did not affect mechanical stability of the support. The swollen seeds could also be lyophilized and reswollen. No physiological change, structural alterations, or microbial growth was seen in seeds stored at room temperature for a study period of even 8 mo. The seeds are also very cheap. They are nontoxic, and swollen seeds are used in certain food and pharmaceuticals formulations (*21*). *Ocimum basilicum* seeds could thus serve as a useful natural pellicular support for immobilizing enzymes.

REFERENCES

1. Kamath, N., Melo, J. S., and D'Souza, S. F. (1988), *Appl. Biochem. Biotechnol.* **19,** 251.
2. Melo, J. S., D'Souza, S. F., and Nadkarni, G. B. (1986), *Biotechnol. Lett.* **8,** 885.
3. D'Souza, S. F., Melo, J. S., Deshpande, A., and Nadkarni, G. B. (1986), *Biotechnol. Lett.* **8,** 643.
4. D'Souza, S. F. (1989), *Indian J. Microbiol.* **29,** 83.
5. Hartmeier, W. (1986), *Immobilized Biocatalysts: An Introduction,* Springer-Verlag, Berlin, p. 26.
6. Mosbach, K. (1987), *Methods in Enzymology,* vol. 135, Academic, New York, p. 130.
7. Marek, M., Jary, J., Valentová, O., and Vodrážka, Z. (1983), *Biotechnol. Lett.* **5,** 653.

8. Marek, M., Valentová, O., and Kas, J. (1984), *Biotechnol. Bioeng.* **26,** 1223.
9. Lampen, J. O. (1971), *The Enzyme,* vol. 5, Boyer, P. D., ed., Academic, New York, pp. 291–306.
10. Sundbfrg, L. and Porath, J. (1974), *J. Chromat.* **90,** 87.
11. D'Souza, S. F. and Nadkarni, G. B. (1980), *Enzyme Microb. Technol.* **2,** 217.
12. Miller, G. L. (1959), *Anal. Chem.* **31,** 426.
13. Messing, R. A. (1975), *Immobilized Enzymes for Industrial Reactors,* Messing, R. A., ed., Academic, New York, pp. 63–78.
14. Porath, J. and Axen, R. (1976). *Methods in Enzymology,* vol. 44, Mosbach, K., ed., Academic, New York, pp. 19–45.
15. Powell, L. W. (1984), *Biotechnology and Genetic Engineering Reviews,* vol. 2, Russell, G. E., eds., Intercept, Newcastle upon Tyne, UK, pp. 409–438.
16. Horvath, C. (1974), *Biochim. Biophys. Acta* **358,** 164.
17. Simionescu, C., Popa, M. I., and Dumitriu, S. (1987), *Biotechnol. Bioeng.* **29,** 361.
18. Woodward, J. and Wiseman, A. (1978), *Biochim. Biophys. Acta.* **527,** 8.
19. Pilnik, W. and Voragen, A. G. J. (1970), *The Biochemistry of Fruits and Their Products,* vol. 1, Hulme, A. C., ed., Academic, London, pp. 53–80.
20. Goldman, R., Goldstein, L., and Katchalski, E. (1971), *Biochemical Aspects of Reactions on Solid Supports,* Stark, G. R., ed., Academic, New York, pp. 1–78.
21. (1966), *The Wealth of India-Raw Materials,* vol. 7, Publication and Information Directorate, CSIR, New Delhi, pp. 81–84.