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Calcium Alginate: A Support Material for Immobilization of Proteases from Newly Isolated Strain of *Bacillus subtilis* KIBGE-HAS

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Abstract: Partially purified neutral protease was entrapped in calcium alginate beads and characterized using casein as a substrate. Temperature and pH maxima of the enzyme showed no changes before and after immobilization and remained stable at 50°C and 7.5 respectively. However substrate concentration for maximum enzyme activity of immobilized enzyme was shifted from 0.45% to 0.8%. Reaction time for immobilized enzyme assay was also increased by 5 minutes with reference to soluble enzyme i.e., from 15 to 20 minutes. Enzyme activity was decreased when the concentration of alginate was increased above 2%. Immobilized enzyme retained its activity for longer time and can be reused upto three times. The storage stability of entrapped protease at 4°C was found upto 10 days, while at 30°C the enzyme lost its activity within three days.

Key words: Alginate beads · *Bacillus subtilis* · Immobilization · Protease · GenBank: EU819145

INTRODUCTION

Proteases, due to their pivotal proteolytic activity, have enormous field of research and usage in food, pharmaceutical and detergent industries [1, 2]. This continuous requirement of proteases has made them important industrial enzyme which is about the 60% of total commercial enzymes involve in the industries [3]. Thus different measures have been taken to reduce the cost and increase the utilization of proteases, one of which is enzyme immobilization. Immobilized enzymes are widely used in different industries especially in food and pharmaceutical and offer several advantages over bulk or free enzymes. Advantages include high productivity, automation, continuous processing, precise control of the extent of reaction, easy product recovery and the enzyme does not contaminate the final product [4]. Entrapment of enzyme in calcium alginate is one of the important methods of immobilization. Alginates are commercially available as water-soluble sodium alginates and they have been used for more than 65 years in the food and pharmaceutical industries as thickening, emulsifying and film forming agent. Entrapment within insoluble calcium alginate gel is recognized as a rapid, nontoxic, inexpensive and versatile method for immobilization of enzymes as well as cells [5].

The present research was based on the entrapment of neutral protease obtained from a newly isolated strain of *Bacillus subtilis* KIBGE-HAS in calcium alginate beads and also the characteristic of immobilized enzyme. Repeated use of the immobilized proteases was also studied with storage stability.

MATERIAL AND METHODS

Protease Production: The *Bacillus subtilis* KIBGE HAS was grown in a medium containing 0.1% (w/v) glucose, 2.5% (w/v) peptone, 0.02% (w/v) yeast extract, 0.05% (w/v) K_2HPO_4, 0.01% (w/v) $CaCl_2$ and 0.01% (w/v) $MgSO_4.7H_2O$ for 24 hours at 40°C.

After 24 hours incubation cells were harvested by centrifugation at 10,000 rpm for 10 minutes at 4°C and supernatant (crude enzyme) was used for further studies.

Protease assay: Activity for neutral protease was determined by Anson method [6] with some modifications. Enzyme solution (0.5 ml) was mixed with 5.0 ml substrate (0.45% casein in 50 mM Tris-HCl buffer) at 50°C for 10 minutes and after incubation TCA (110 mM) was added to attenuate the reaction. This mixture was centrifuged at 10000 rpm for 5 minutes and the released

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amino acids were measured as tyrosine by the method of Folin and Ciocalteu [7].

"One unit of protease hydrolyzed casein to produce color equivalent to 1.0 µmol (181 µg) of tyrosine per minute at pH 7.5 at 50°C". The enzyme activity was expressed as U/ml/min."

Partial Purification of Protease by Ammonium Sulphate: The crude enzyme was precipitated by 40% ammonium sulphate saturation at 4°C. The obtained precipitates (partial purified enzyme) were dissolved in Tris-HCl buffer (50 mM, pH 7.5) and used for entrapment.

Immobilization of Protease: The partially purified enzyme solution was mixed with sodium alginate solution (2%) in 1:1 ratio. The protease-alginate mixture was added dropwise into calcium chloride (0.2 M) solution with continuous shaking at 4°C. As soon as the drop of protease-alginate solution mixed with $CaCl_2$ solution, Na^+ ions of Na-alginate were replaced by the Ca^{+2} ions of $CaCl_2$ solution, which finally formed Ca-alginate beads. The beads thus formed were washed 3-4 times with deionized water and finally with 50 m Tris-HCl buffer of pH 7.5. These beads were dried and weighed for further studies.

Calculation:

Initial activity of the free enzyme = 113 U/ml/min
Volume of enzyme solution = 5 ml
Weight of beads formed after immobilization of enzyme solution = 4.7 g
Enzyme solution entrapped in 0.5 g beads = 0.53 ml
Therefore enzyme entrapped = 60 U/0.5g beads (on the basis of free enzyme)
Activity of immobilized enzyme obtained in 0.5 g beads = 27 U/min
Therefore Total Enzyme activity after entrapment = 45%

Statistics: Bonferroni test was applied to locate the source of significant difference. The level of significance was set at $P< 0.05$. Differences between mean values were evaluated by a oneway analysis of variance (ANOVA) (SPSS version 10.0).

RESULTS AND DISCUSSION

Effect of Sodium Alginate Concentration: Various concentrations of sodium alginate (1%-4%) were used to acquire beads with greater stability. The percent entrapped activity was found maximal 45%±2 at 2% (w/v) sodium alginate concentration. Maximum leakage of enzyme from beads occurred at 1% (w/v) sodium alginate concentration owing to the larger pore size of the less tightly crossed linked fragile Ca-alginate beads while at 3% and 4% (w/v) sodium alginate concentration the entrapped activity of the enzyme was found comparatively very low which might be due to the high viscosity of enzyme entrapped beads, which decreased the pores size and thus hindered the penetration of substrate in to the beads. Different researchers also reported that sodium alginate concentration range from 2-3% was suitable for the immobilization of keratinase, lipase and proteases [8-10].

Effect of Calcium Chloride Concentration on Immobilization: Concentration of calcium chloride (0.05-0.3 M) was also varied in order to acquire stable beads capable to secure maximum enzyme and it was found that $CaCl_2$ (0.2 M) retained highest activity of entrapped enzyme and as calcium chloride concentration increased beyond 0.2 M the activity decreased. Roig *et al.* also reported a decrease in the relative enzyme activity of alkaline protease when they increased the concentration of $CaCl_2$ used to form capsule [11]. They observed that the pH of the $CaCl_2$ solution changes with its concentration which might be a factor to affects the activity of entrapped enzyme.

Effect of Temperature and Ph on Activity of Immobilized Protease: Alginate entrapped enzyme was assayed at different temperature and pH, ranging from 20°C to 70°C and pH 6.5 to 8.5 respectively and it was observed that optimum temperature of entrapped enzyme was 50°C while optimum pH was 7.5. The result showed no change in temperature and pH of protease before and after entrapment.

It was reported that the surface of the beads in which the enzyme is localized has a cationic or anionic nature. This charged surface of beads and entrapped enzyme produces a charged microenvironment, which ultimately affects the nature of the active enzyme protein and alters the pH of the entrapped enzyme [12]. But in the present case the surface of beads at pH 7.5 might be neutral and had produced no effect on the active protease, thus the optimum pH of free and immobilized enzyme remained the same. But, when the pH of substrate-immobilized enzyme was increased or decreased from 7.5, a sharp decline in activity was seen, probably due to the charge acquired by the support. Moreover it was observed that when the temperature was raised above its optimum value (50°C) to 70°C, the immobilized enzyme retained 53% of its activity while the free enzyme showed only 40% activity which clearly indicated that the support retained the tertiary structure of enzyme at high temperature. Consistently, Arya and Srivastava (2006) also reported that no change

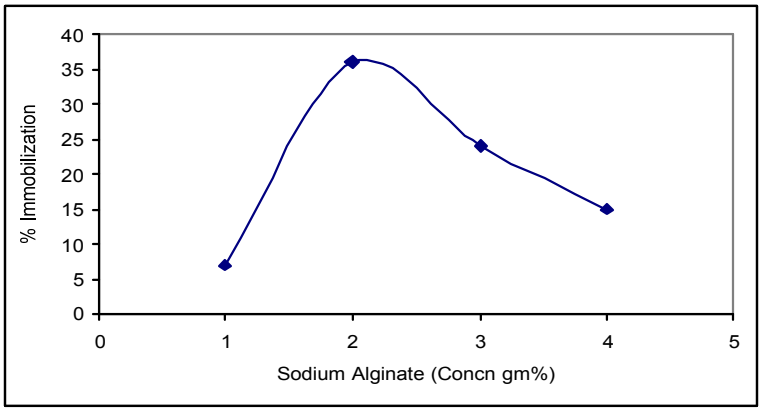


Fig. 1: Percent immobilization of Protease at different concentration of sodium alginate ranging from 1.0% to 4.0%.

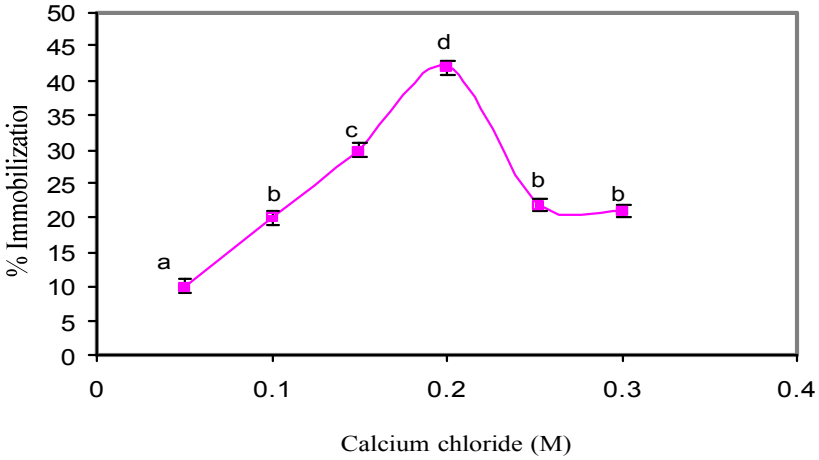


Fig. 2: Effect of calcium chloride concentration on percent immobilization of protease from Bacillus subtilis KIBGE-HAS. Symbols (means± S.E., n = 6) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$)

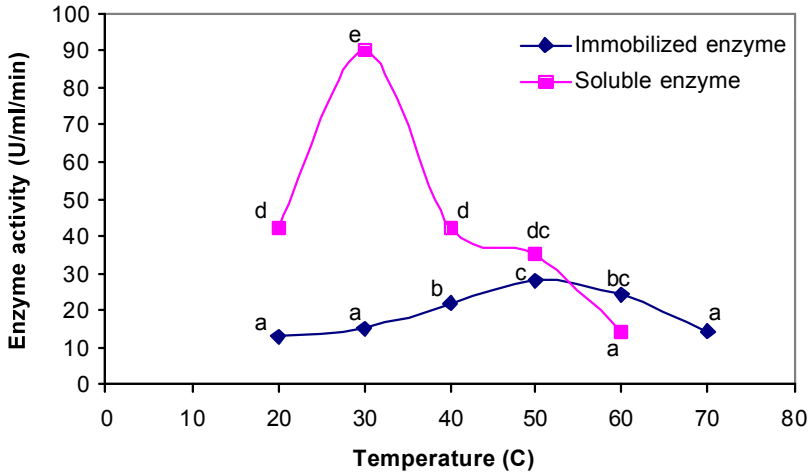


Fig. 3: Effect of different temperature on immobilized and soluble protease activity from Bacillus subtilis KIBGE-HAS. Symbols (means± S.E., n = 6) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$)

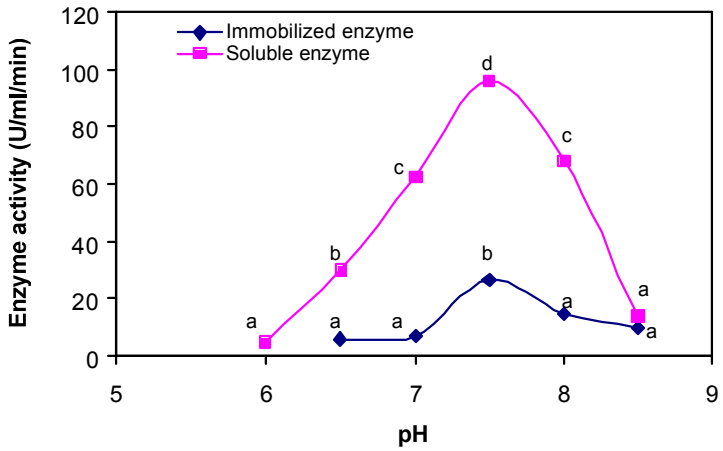
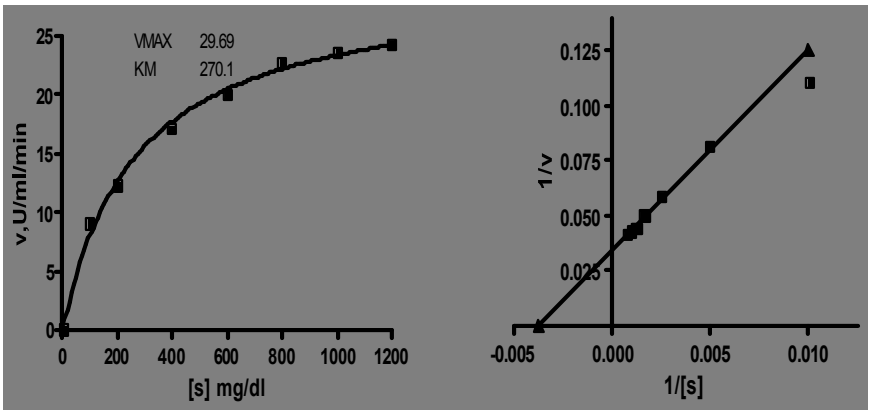


Fig. 4: Effect of pH on immobilized and soluble protease activity from Bacillus subtilis KIBGE- HAS. Symbols (means± S.E., n = 6) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).



(a) (b)

Fig. 5: (a) Substrate Saturation Kinetics of Immobilized proteases (b) Lineweaver Burk plot of Extracellular Immobilized proteases

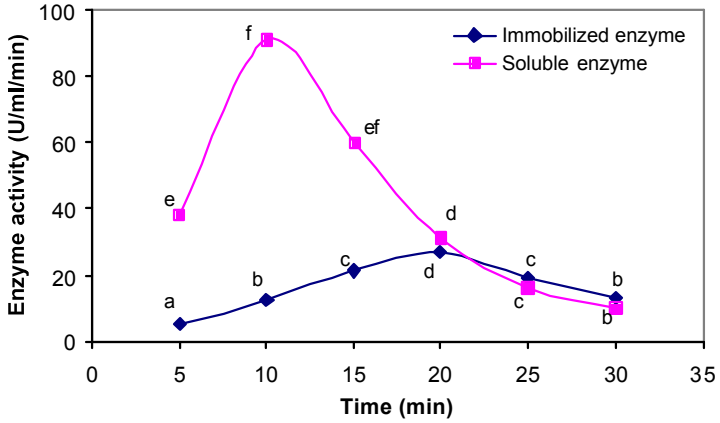


Fig. 6: Effect of reaction time of immobilized and soluble enzyme with substrate on maximum enzyme activity. Symbols (means± S.E., n = 6) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$)

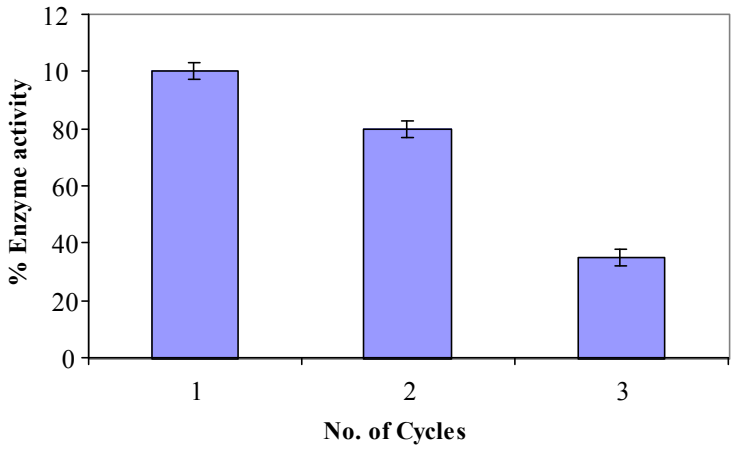


Fig. 7: Repeated use of immobilized protease showing number of times the immobilized enzyme can be used (means±S.E., n = 6).

occurred in the optimum temperature and pH of CGTase before and after entrapment in calcium alginate beads [13]. Similarly, the optimum conditions of α-galactosidase was also not affected by its immobilization in gel fibers of gelatin-alginate and the optimum pH and temperature for free and immobilized enzyme remained same [14].

Effect of Substrate Concentration on Activity of Immobilized Proteases: Michaelis-Menten (Km) value of entrapped protease was determined by studying the hydrolysis of different concentrations of casein, ranging from 0.1% to 1.5%. A Lineweaver-Burk plot was drawn between the inverse of different concentrations of casein and reaction velocity to determine the Michaelis-Menten constant (Km) for immobilized protease. The Km and Vmax value for immobilized proteases was found 270 mg/dl and 29.69 U/ml/min respectively while Km and Vmax for free enzyme was 130 and 113 U/ml/min respectively.

This increase in the substrate concentration might be due to the inability of high molecular substrate (casein) to diffuse rapidly into the Ca-alginate matrix [12], which might have caused less access of substrate to the active site of entrapped enzyme and thus decreased the Vmax of immobilized enzyme At the same time, the diffusional resistance encountered by the product molecules might have caused the product to accumulate near the center of the gel to an undesirable high level, leading to product inhibition for some enzymes. An increase in the substrate maxima of immobilized alkaline protease from 3.3 mg/ml to 5 mg/ml of casein was also reported by Sharma *et al.* (2006) when they entrapped an alkaline protease in Ca-alginate beads, isolated from a new strain of *Aspergillus oryzae* [15].

Effect of Incubation Time on Immobilized Enzyme Activity: Effect of incubation time for enzyme assay of entrapped enzyme was noted by varying the time course from 5 to 30 minutes. Maximum activity was found in 20 minutes of incubation, showing an increase by 5 minutes from the free enzyme.

This increase in time is due to the time required by the substrate molecules to penetrate into the beads and reach the active sites of the enzyme. Calcium alginate entrapped dextransucrase took 60 minutes to achieve the maximum enzyme activity, which was 4 time higher than the free enzyme [16].

Reusability of Immobilized Protease: The activity of entrapped enzyme was assayed for four cycles with casein as a substrate, in order to find out the reuse of the entrapped enzyme. The enzyme showed 80% activity during the second reuse and 35% activity on its third use while complete loss in the activity of entrapped enzyme was observed during the forth cycle. This decrease in activity was due to the leakage of enzyme from the beads, occurred due to the washing of beads at the end of each cycle. In another study it was reported that alpha amylase entrapped in Ca-alginate beads was reused for 6 cycles with ~ 30% loss in activity [17].

Storage Stability of Immobilized Enzyme: The immobilized enzyme was stored at two temperatures (4°C and 30°C) and activity was noted for 12 days to determine the storage stability of entrapped enzymes. Beads stored at 4°C showed 35% loss of activity after 2nd days (48 hours) and 89% loss of activity after 10th day (240 hours). Major loss in enzyme activity of immobilized enzyme was observed at 30°C, showing 80% loss in

activity on the 2nd day (24 hours) and no activity was found on the 3rd day. These results suggested that the enzyme is more stable at 4°C as compared with 30°C. Qader *et al.* reported 36% loss in the activity of immobilized dextransucrase at 30°C after 3 hours and 86% loss in activity at 40°C within two hours [16] while Lopez-Munguia *et al.* (1998) reported 96% loss of activity of dextransucrase at 40°C within two hours [18]. Adinarayana *et al.* (2004) reported the stability of alkaline proteases upto nine days after entrapment of cells of *Bacillus subtilis* PE-11 [19].

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REFERENCES

1. Ward, O.P., 1983. Proteinases. In Microbia1 Enzymes and Biotechnology, Applied Science Publishers, London, pp: 251-317.
2. Kalisz, H.M., 1988. Microbial proteases. In Advances in Biochemical Engineering/ Biotechnology, Springer-Verlag, Berlin Heidelberg, pp: 1-65.
3. Rao, M.B., A.M. Tanksale, M.S. Ghatge and V.V. Deshpande, 1998. Molecular and Biotechnological Aspects of Microbial Proteases. Microbiol. Mol. Biol. Rev., 62: 597-635.
4. Swaisgood, H.E. and H.R. Horton, 1989. Immobilized enzymes as processing aids or analytical tools. In *Biocatalyst in Agricultural Biotechnology*, (J.R. Whitker and P.E. Sonnet, eds.) ACS Symposium Series 389, American Chemical Society, Washington, DC, pp: 242-261.
5. Fraser, J.E. and G.F. Bickerstaff, 1997. Entrapment of enzymes and cells in calcium alginate. In Immobilisation of Enzymes and Cells, Humana Press, pp: 61-66.
6. Anson, M.L., 1938. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J. Gen. Physiol., 22: 79-89.
7. Folin, O. and V.J. Ciocalteu, 1929. On tyrosine and tryptophan determinations in proteins. J. Biol. Chem., 73: 627-650.
8. Farag, A.M. and M.A. Hassan, 2004. Purification, characterization and immobilization of a keratinase from *Aspergillus oryzae*. Enzyme Microbial. Technol., 34: 85-93.
9. Doumèche, B., M. Heinemann, J. Büchs, W. Hartmeier and M.B. Ansorge-Schumacher, 2002. Enzymatic catalysis of gel-stabilized two-phase systems: improvement of the solvent phase. J. Mol. Catalysis B. Enz., 18: 19-27.
10. Elibol, M. and A.R. Moreira, 2003. Production of extracellular alkaline protease by immobilization of the marine bacterium *Teredinobacter turnirae*. Process Biochem., 38: 1445-1450.
11. Roig, M.G., D.H. Rashid and J.F. Kenndy, 1995. High-alkaline protease from *Bacillus* PB92 entrapped in calcium alginate gel: Physicochemical and microscopic studies. Appl. Biochem. Biotechnol., 55: 95-121.
12. Norouzian, D., 2003. Review, Enzyme immobilization, the state of art in Biotechnology, Iranian J. Biotechnol., 1: 197-206.
13. Arya, S.K. and S.K. Srivastava, 2006. Kinetics of immobilized cyclodextrin gluconotransferase produced by *Bacillus macerans* ATCC 8244 Enzyme Microbial. Technol., 39: 507-510.
14. Naganagouda, K. and V.H. Mulimani, 2006. Gelatin blends with alginate: Gel fibers for α-galactosidase immobilization and its application in reduction of non-digestible oligosaccharides in soymilk. Process Biochem., 41: 1903-1907.
15. Sharma, J., A. Singh, R. Kumar and A. Mittal, 2006. Partial purification of an alkaline protease from a new strain of *Aspergillus Oryzae* AWT 20 and its enhanced stabilization in entrapped Ca-alginate beads. The Internet J. Microbiol., 2(2).
16. Qader, S.A.U.l., A. Aman, N. Syed, S. Bano and A. Azhar, 2007. Characterization of dextransucrase immobilized on calcium alginate beads from *Leuconostoc mesenteroides* PCSIR-4. Ita. J. Biochem., 56: 158-162.
17. Kumarr, S.S., R.K.S. Vishwanath, S.A. Singh and A.G. Appu Rao, 2006. Entrapment of α-amylase in alginate beads: Single step protocol for purification and thermal stabilization. Process Biochem., 41: 2282-2288.
18. Chellapandian, M., C. Larios, M. Sanchez-Gonzalez and A. Lopez-Munguia, 1998. Production and properties of a dextransucrase from Leuconostoc mesenteroides IBT-PQ isolated from' pulque', a traditional Aztec alcoholic beverage. J. Ind. Microbiol. Biotechnol., 21: 51-56.
19. Adinarayana, K., K.V.V.S.N. Bapi Raju and P. Ellaiah, 2004. Investigations on alkaline protease production with B. subtilis PE-11 immobilized in calcium alginate gel beads. Process Biochem., 39: 1331-1339.