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Immobilization of *Bacillus subtilis* α-amylase and characterization of its enzymatic properties

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Abstract

Bacillus subtilis 1 α-amylase was immobilized on different carriers by different methods of immobilization. The immobilized enzymes were prepared by physical adsorption on aminoalkylsilane-alumina (AS-alumina), ionic binding onto DEAE-cellulose, covalent binding on chitin, and entrapment in polyacrylamide had the highest activities. The specific activity of the immobilized enzymes, calculated on bound protein basis, were 13.5–50% of the original specific activity exhibited by the free enzyme. The optimal pH of the immobilized enzymes was shifted to lower values than for the free enzyme. The optimum reaction temperature was determined to be 45 °C for the free amylase, whereas that for the immobilized enzymes was shifted to 60–65 °C. In all cases, Km values for the hydrolysis of starch of the immobilized enzymes were higher than that of the native enzyme. Compared to the free form, the immobilized enzymes exhibited improved thermal stability and higher values of activation energy of denaturation.

Key words: *Bacillus subtilis* – amylase – immobilization – properties

Introduction

Starch is the second-abundant renewable biopolymer present on the earth with more than 109 tons produced annually (Galliard, 1985). Traditionally, starch was, and still is, hydrolyzed to low molecular weight dextrins and glucose using acid, but enzymatic hydrolysis has several advantages. First, the specificity of enzymes allows the production of suga syrups with well-defined physical and chemical properties. Second, the milder enzymatic hydrolysis results in few side reactions and less "browning". Indeed, for the production of glucose syrups from starch, enzymatic hydrolysis is essential.

The hydrolysis of starch to glucose involves many enzymes, the most important of these are α-amylase (α-1,4-glucan-4-glucohydrolase, EC 3.2.1.1), β-amylase (β-1,4-D-glucan maltohydrolyase, EC 3.2.1.2), and glucoamylase (EC 3.2.1.3) and the debranching enzymes (Nigam and Singh, 1995).

Immobilization of enzymes onto water-insoluble supports has become a subject of interest for many industries. The advantages of this technique include the possibility of enzyme re-utilization, enhanced stability, easy separation of catalyst from the reaction mixture and ready application to automated continuous processes (Monsan and Combes, 1988). Since amylases have many applications in the food and fermentation industries, it is not surprising that a number of methods for immobilizing the enzymes have been reported (Fogarty, 1983).

The present paper describes the immobilization of *B. subtilis* 1 α-amylase onto various carriers using physical adsorption, ionic binding, covalent binding and entrapment methods. The properties of the immobilized enzymes were compared with those of the free enzyme to identify the most suitable immobilization method.

Materials and methods

Microorganisms. Bacillus subtilis 1 was isolated from soil plated on agar complex medium containing g/l: soluble starch, 2.0; $(NH_4)_2HPO_4$, 2.0; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5 and agar, 30. Identification of amylolytic isolate was done according to Bergey's Manual of Determination Bacteriology (Buchanan and Gibbons, 1984).

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Culture medium. The medium used for enzyme production was comprised of (g l^{-1}): starch, 30; lactose, 20; peptone, 5; yeast extract, 1; beef extract, 3.0; K_2HPO_4, 1.5; $(NH_4)_2HPO_4$, 5.0; $CaCl_2$, 0.5; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; Na citrate $\cdot 2H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.1; $MnSO_4 \cdot 2H_2O$, 0.001 and 2 ml of Tween-80. The pH was adjusted to 5.0. Cultivation was in 250 ml Erlenmeyer flasks, each containing 50 ml of sterile medium. The inoculum 3%, v/v, (10^6–10^7 CFU/ml) was transferred to the culture medium and the flasks were incubated at 45 °C for 40 h on a rotary shaker. The culture medium was centrifuged (5000 rpm) for 15 min in a refrigerated centrifuge. The clear supernatant was concentrated at room temperature (20 °C) under reduced pressure. The concentrated culture filtrate was fractionated by acetone. The fraction precipitated at 90% acetone showed the highest amylase activity (22.5 unit mg protein^{-1}) was used for the enzyme immobilization.

Carriers for the enzyme immobilization. Chitin, chitosan, Amberlite-IR 120, Dowex 50W and Dowex 1×4 Cl^- were from Sigma Chemical Co. USA. Acrylamide, bisacrylamide, and Na-alginate were from BDH Chemical Ltd., Poole, England. Sepharose-CL 2B, 4B, 6B and DEAE-Sephadex A-25 were from Pharmacia Fine Chemicals Inc. Uppsala Sweden. Diethylaminoethyl cellulose (DEAE-cellulose DE-52) and Ecteola-cellulose were from Whatman Ltd., Springfield, Mill, Maidstone, Kent, England. All other chemicals were of analytical grade.

Assay for α-amylase activity. This was done according to Bergmann *et al.* (1998) by estimating the released reducing sugars from 1% saline starch in 0.05 M citrate-phosphate buffer (pH 6.0) at 45 °C. Reducing sugars were determined by the method described by Somogyi (1952). One unit of enzyme activity (U) was defined as the amount of the enzyme liberating one μmole of reducing sugars as glucose/min.

Immobilization methods. Physical adsorption. Aminoalkylsilane-alumina (AS-alumina) was prepared by the method of Weetall (1970). Tannin-Sepharose and Tannin-chitosan were prepared by the method reported by Sakai *et al.* (1991). One gram of each carrier (AS-alumina, chitin, chitosan, hydroxyapatite, tannin-chitosan and tannin-Sepharose) was incubated with the enzyme solution (1177.5 U of *B. subtilis* 1 α-amylase in two ml of 0.05 M citrate-phosphate, pH 6.0) at 4 °C for 12 h. The unbound enzyme was removed from the carriers by washing with 0.05 M citrate-phosphate buffer (pH 6.0) until no activity or soluble protein was detected.

Ionic binding. One gram of cation exchanger (equilibrated with 0.05 M citrate-phosphate buffer, pH 6.0) or anion exchanger (equilibrated with 0.05 M phosphate buffer, pH 7.5) was incubated with one ml of the enzyme solution (692.5 U of α-amylase) in the same buffer at 4 °C for 12 h. The unbound enzyme was removed by washing with the same buffer until no activity or protein was detected.

Covalent binding. One gram chitosan was shaken in 2.5 ml of 0.1 M HCl containing 2.5%, (v/v), glutaraldehyde (GA) for 2 h at 30 °C. The solubilized chitosan was precipitated by the addition of one ml 0.1 M NaOH. The precipitates were collected by filtration and washed with distilled water to remove the excess GA. The wet chitosan was mixed with 2.5 ml of enzyme solution containing 1000 U of *B. subtilis* 1 α-amylase. After being shaken for one h at 4 °C, the unbound enzyme was removed by washing with distilled water.

In separate experiments, one gram of chitin, hydroxyapatite, and AS-alumina was treated with 5 ml of 2.5%, (v/v) GA for two h at 30 °C. The carriers were collected by filtration and washed with distilled water to remove the excess GA. Each of the wet carrier was shaken with 2.5 ml of the enzyme solution (1000 U α-amylase) for 2 h at 30 °C and the unbound enzyme was removed as described above.

Cyanogen bromide-activated Sepharose (CNBr-Sepharose)-CL 2B, CL 4B and CL 6B were prepared by the method of March *et al.* (1974) and treated with GA by the method of Gauthier *et al.* (1991). Each carrier (CNBr-Sepharose-CL2B, CL4B, CL6B) was incubated with 10 ml of the enzyme solution (750 U α-amylase) for 2 h at 4 °C. The unbound enzyme was removed as described above.

Entrapment in polyacrylamide. The gel prepared according to the method of Roy *et al.* (1984). The polymerization mixture contained 1.0 ml of the enzyme solution (166.6 U α-amylase), 5.0 ml of 13.6% (w/v) acrylamide, and 4.0 ml of citrate-phosphate buffer (0.05 M, pH 6.0). In separate experiments, the amount of cross-linker monomer (N, N, methylene-bis-acrylamide) added was at the level of 3, 4 and 5% of the total monomer content in separate experiments. The catalyst system consisted of the following: 0.02 ml of N, N, N′, N′, tetramethylethylenediamine (TEMED) and 0.01 g of ammonium persulphate dissolved in 0.2 ml of distilled water, was finally added to the polymerization mixture. The total volume was kept to 10 ml. Finally the mixture was kept in N_2 atmosphere at 20 °C. After polymerization, the gel was washed with citrate-phosphate buffer (0.05 M, pH 6.0), cut into 2×2×2 mm^3 fragments and kept in the same buffer at 4 °C to remove the unbound enzyme.

Entrapment in Ca-alginate. In separate experiments, 10 ml of different concentraiton of sodium alginate solution (3, 4, 5 and 6%, w/v) were mixed with 166 U of α-amylase. The entrapment was carried out by dropping the alginate solutions in 100 mM $CaCl_2$ solution. The

resulting beads (3 mm in diameter) were collected, washed with citrate-phosphate buffer (0.05 M, pH 6.0) and kept in the same buffer at 4 °C for 72 h to remove the unbound enzyme.

Protein estimation. This was done by the method of Lowry *et al.* (1951).

All the results reported here are the mean of at least three separate experiments.

Results and discussion

Immobilization by physical adsorption

Bacillus subtilis 1 α-amylase was immobilized by physical adsorption on AS-alumina, chitin, chitosan, hydroxyapatite, tannin-chitosan and tannin-Sepharose. The immobilized enzyme activities on these carriers were evaluated (Table 1). The enzyme physically adsorbed on AS-alumina showed the highest immobilized activity (139.5 U/g carrier) and the highest immobilization yield (24.6%). In the subsequent experiments, the enzyme immobilized on AS-alumina was selected as an example for immobilization by physical adsorption.

Immobilization by ionic binding

The data of the immobilization of *B. subtilis* 1 α-amylase by ionic binding to various ion exchangers are summarized in Table 2. DEAE-Cellulose showed the highest immobilized activity (170 U/g carrier) and the highest immobilization yield (39.8%), it was therefore seleceted as an example of this group.

Immobilization by covalent binding

The results in Table 3 showed all the carriers used for immobilization by covalent binding through a spacer group (GA) showed a good immobilization yield, especially chitin. This may be attributed to the formation of stable cross-linking between the enzyme and the carrier. In addition, covalent binding through a spacer group increased the local surface are aof the carrier and consequently, reduced the steric hindrance in the immediate vicinity of the enzyme molecule (Kusano *et al.*, 1989, Siso *et al.*, 1990). On the other hand, Shaw *et al.* (1990) reported that the amount of immobilized lipase on different carriers by covalent binding through a spacer group increased by increasing the length of the spacer group. Furthermore, Gauthier *et al.* (1991) reported that increasing the length of the spacer group contributed to the stability of the imobilized protease on agarose by covalent binding. The enzyme covalently bound to chitin showed the highest immobilized activity (335 U/g carrier) and the highest immobilization yield (77.5%) and it was therefore selected for immobilization by covalent binding.

Table 1. Immobilization of *B. subtilis* 1 α-amylase by physical adsoption

Carrier	Enzyme added (U/g carrier) A	Unbound enzyme (U/g carrier) B	Immobilized enzyme (U/g carrier) I	Immobilization yield (%) $= \frac{I}{A-B} \times 100$
AS-alumina	1177.5	610.42	139.5	24.6
Chitin	1177.5	645.0	82.5	15.4
Chitosan	1177.5	615.2	57.5	10.2
Hydroxyapatite	1177.5	1015.0	36.0	22.1
Tannin-chitosan	1177.5	995.0	36.5	20.0
Tannin-Sepharose	1177.5	432.0	118.0	15.8

Table 2. Immobilization of *B. subtilis* 1 α-amylase by ionic binding

Carrier	Added enzyme (U/g carrier) A	Unbound enzyme (U/g carrier) B	Immobilized enzyme (U/g carrier) I	Immobilization yield (%) $= \frac{I}{A-B} \times 100$
Amberlite IR-120	692.5	362.5	30.5	12.0
Dowex 50W	692.5	504.0	21.0	13.7
DEAE-Cellulose DE-52	692.5	265.0	170.0	39.8
Dowex 1 × 4 Cl^-	692.5	309.0	20.5	5.3
Ectula-Cellulose	692.5	100.5	77.5	12.9
DEAE-Sephadex A-25	692.5	200.0	40.15	8.12

Immobilization by entrapment

Immobilization of B. subtilis 1 α-amylase by entrapment was achieved in polyacrylamide (7%; w/v). The amount of cross-linker monomer (N, N, methylene-bis-acrylamide) was added at the level of 3,4 and 5% of the total monomer content (Table 4). The results indicated that a cross-linking concentration of 3% was sufficient for reaching the maximal entrapping activity (105 U/10 ml gel) and the highest immobilization yield (71.04%). Increasing the cross-linker concentration however, resulted in a decrease of the immobilization yield. This may be partially due to the decrease of the porosity of the gel matrix, and hence a diffusion limitation of the substrate and product flux. Similar results were reported by Abdel-Naby (1993) for the immobilization of *Aspergillus niger* xylanase. On the other hand, *B. subtilis* 1 α-amylase entrapped in Ca-alginate showed a gradual decrease in the immobilization yield with the increase of alginate concentration (Table 4). However, it was observed that Ca-alginate was inapplicable to the enzyme immobilization because of the leak out of the enzyme from the gel at the assay temperature (45 °C). In the following experiments α-amylase entrapped in polyacrylamide (3% cross-linking) was used.

Properties of the immobilized enzymes

The immobilized enzymes retained 13.5–50% of the specific α-amylase activity exhibited by the free enzyme (Table 5). This drop in the specific activity could be attributed to the diffusional limitation (i.e., resistance of the substrate to diffuse into the matrix and resistance of products to diffuse out). This diffusional limitation becomes more pronounced with macro-molecular substrates like starch. In addition, multiple fixation of the enzyme to the matrix would also lead to a decrease in the catalytic activity owing to the decrease in the flexibility of the enzyme molecule, consequently, the proper conformational changes required for catalysis was impaired

Table 3. Immobilization of *B. subtilis* 1 α-amylase by covalent binding

Carrier	Added enzyme (U/g carrier) A	Unbound enzyme (U/g carrier) B	Immobilized enzyme (U/g carrier) I	Immobilization yield (%) $= \frac{I}{A-B} \times 100$
Chitosan	1000	507	184	37.32
Chitin	1000	567.7	335	77.5
Hydroxyapatite	1000	812.5	68	36.5
AS-alumina	1000	665.0	172	51.2
CNBr-Sepharose – CL2 B	750	371	169.5	44.72
CNBr-Sepharose – CL4 B	750	421	119.0	36.17
CNBr-Sepharose – CL6 B	750	430.2	115.0	35.93

Table 4. Immobilization of *B. subtilis* 1 α-amylase by entrapment

Carrier	Added enzyme (U/10 ml gel) A	Unbound enzyme (U/10 ml gel) B	Immobilized enzyme (U/10 ml gel) I	Immobilization yield (%) $= \frac{I}{A-B} \times 100$
Chross-linking of* polyacrylamide (%)				
3				
4	166.6	20.22	104.0	71.04
5	166.6	17.0	94.0	62.80
	166.6	14.0	88.1	57.77
Ca-alginate concentration (%)				
3				
4	633.2	584.6	27.6	56.7
5	633.2	570.0	30.2	47.78
6	633.2	560.0	26.2	35.7
	633.2	536.0	21.0	21.6

* Total concentration of acrylamide = 7%.

Table 5. Properties of free and immobilized α-amylase

Property	Free enzyme	Carrier of immobilized enzyme			
		AS-alumina	DEAE-Cellulose	Chitin	Polyacrylamide
Specific activity (U/mg protein)	22.58	3.1	5.1	8.3	11.2
Optimum pH	6.0	5.75	5.75	5.25	5.5
Optimum temperature (°C)	45.0	60	60	65	65
Q 10, 45–55 °C	–	1.35	1.11	1.07	1.11
Activation energy (Kcal/mol)	24.27	26.46	29.65	36.81	38.8
Thermal stability at 60 °C, 60 min (Residual activity, %)	26.93	70.53	76.42	98.0	88.1
Activation energy for denaturation (Kcal/mol)	31.83	33.81	43.78	99.55	89.5
Km (mg/mL)	2.72	4.0	4.76	4.3	5.26
V_{max} (μmol/min)	28.57	4.0	5.26	11.76	15.0

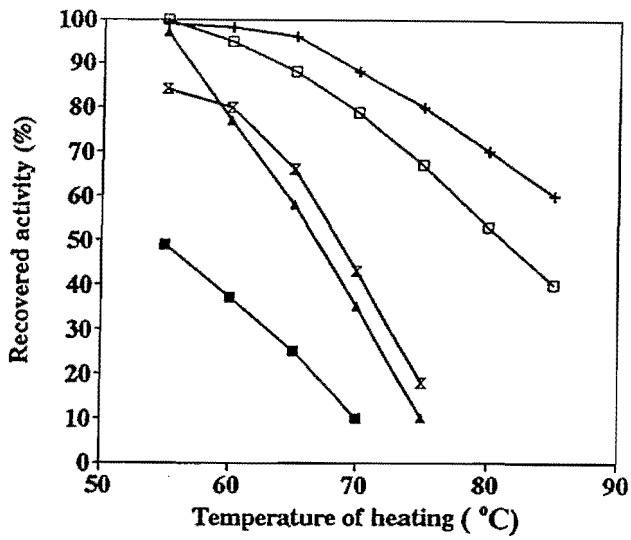

Fig. 1. Thermal stability of free and immobilized enzymes.
Free enzyme (-■-)
Immobilized enzyme on As-alumina (-⧖-)
Immobilized enzyme on DEAE-vcellulose (-▲-)
Immobilized enzyme on Chitin (-⋆-)
Immobilized enzyme on polyacrylamide (-⊟-)

or prevented (Siso *et al.*, 1990). The decrease of the specific activity of amylases after the immobilization was similarly reported (Gottschalk and Jaenicke, 1991, Roy *et al.*, 1995).

The positively charged supports such as used in the present study (AS-alumina, DEAE-Cellulose, chitin, chitosan) could affect the apparent pH profile of the enzyme. The optimum pH of the immobilized enzymes was shifted to acidic range (optimum pH 5.25–5.75) in comparison to the free enzyme (optimum pH 6.0) (Table 5). These results are in agreement with the general observation that the cationic supports shift the pH-activity curves of enzymes attached to them towards low pH values (Goldstein *et al.*, 1964). Similar shifts of the optimal pH were reported for other immobilized glucoamylase (Xiao *et al.*, 1991) and pullulanases (Kusano *et al.*, 1989).

The apparent maximum temperature of activity was relatively sharp to about 45 °C for the free enzyme. However, the optimal reaction temperature of the enzymes immobilized on AS-alumina (physical adsorption) and DEAE-Cellulose (ionic binding) was shifted to 60 °C. A higher optimal temperature (65 °C) was found with the enzymes covalently bound to chitin or entrapped in polyacrylamide (Table 5). These results indicated that the enzyme becomes more stable when immobilized by covalent binding to chitin or entrapment in polyacrylamide. The Q10 values at 45–55 °C were 1.35, 1.11, 1.07 and 1.11 for the enzymes immobilized on AS-alumina, DEAE-Cellulose, chitin and polyacrylamide, respectively (Table 5). The activation energies of the enzymes immobilized on AS-alumina, DEAE-cellulose, chitin, and polyacrylamide obtained from Arrhenius plots were 26.46, 29.65, 36.81 and 38.8 Kcal $mole^{-1}$, while the activation energy of the free enzyme was lower (24.27 kcal/mole). The higher values of activation energy obtained for the immobilized enzymes indicate that the applied immobilization procedures introduced changes in the structure of the enzyme molecule which impeded the enzyme catalyzed reaction (Krajewska, 1990). Similar results were reported previously (Siso *et al.*, 1990). In contrast, cases where enzyme activation energy decreased after the immobilization have also been reported (Kusano *et al.*, 1989).

The thermal stability of the free and the immobilized enzymes were evaluated (Fig. 1). In general, the immobilized enzymes were more thermostable than the free enzyme. Thus after heat treatment of the enzymes at 60 °C for 60 min, the immobilized enzymes retained 70.5 to 98.0% of the original activity, while the free enzyme retained only 26.9% of the original activity. A greater evaluation for the thermal stability was

found after heating the enzymes at 75 °C for 60 min. The enzymes covalently bound to chitin and entrapped in polyacrylamide retained 86.27 and 53.3% of the original activity whereas the enzymes immobilized on AS-alumina (physical adsorption) and DEAE-cellulose (ionic binding) retained only 18.1 and 10.9% of their original activities. Using Arrhenius equation, the activation energy of thermal denaturation of the enzymes immobilized on AS-alumina, DEAE-cellulose, chitin and polyacrylamide were 33.81, 43.78, 99.55 and 89.5 kcal/mole, respectively (Table 5). These and the above results indicated that *B. subtilis* 1 α-amylase becomes more thermostable when immobilized by covalent binding on chitin or by entrapment in polyacrylamide.

The kinetic constants K_m (Michaelis constant) and the V_{max} (the maximum reaction rate) for the hydrolysis of starch of the immobilized enzymes were determined (Table 5). In general, the K_m values exhibited by the immobilized enzymes (K_m = 4.0–5.26 mg/ml) were higher than that recorded for free enzyme (K_m = 2.7 mg/ml). This increase of the Km values is most likely a consequence of either conformational changes in the enzyme introduced by the immobilization procedure and/or lower accessibility of the substrate to the active sites of the immobilized enzymes. The latter may have resulted either from diffusional resistance of the matrix or steric hindrance in the immediate vicinity of the enzyme molecules. Consequently, the maximum rate of the reaction catalyzed by the immobilized enzyme was lower than for the free enzyme. Increased Km values of amylases after the immobilization process were similarly reported (Siso *et al.*, 1989, Gottschalk and Jaenicke, 1991).

Conclusion

In this study *B. subtilis* 1 α-amylase was immobilized on various carriers by different methods of immobilization including: Physical adsorption on AS-alumina, ionic binding on DEAE-Cellulose, covalent binding on chitin and entrapment in polyacrylamide. A considerably good loading efficiency but low thermal stability was exhibited by the enzymes ionically bound on DEAE-Cellulose. A lower loading efficiency and thermal stability was shown by the enzymes physically adsorbed on AS-alumina. A moderate loading efficiency but good thermal stability was exhibited by the enzymes entrapped in polyacrylamide. On the other hand, the enzymes covalently bound to chitin showed the highest loading efficiency and thermal stability. It is suggested that chitin and polyacrylamide are good candidates for the support of α-amylase immobilization.

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