0273-2289/91/2703—0205$2.40

Entrapment of Proteins by Aggregation within Sephadex Beads

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Received January 12, 1990; Accepted April 9, 1990

ABSTRACT

When a protein is aggregated by chemical crosslinking inside Sephadex beads of appropriate pore size, it gets trapped inside the beads. The above approach was used for immobilization of β-galactosidase, acid phosphatase, trypsin, and concanavalin A. It was found to be a simple, convenient, and fast method for immobilization of proteins.

Index Entries: Entrapment of proteins/enzymes; chemical aggregation of proteins/enzymes; enzyme immobilization; chemical crosslinking.

INTRODUCTION

The use of high concentrations of crosslinking reagents is known to result in formation of insoluble aggregate of enzymes (*1,2*). Chemical aggregation, in fact, has been used by several workers as a simple method of immobilization yielding stable and reusable derivatives (*1–3*). In spite of the simplicity of the aggregation technique, and despite the fact that it is known for a long time, enzyme aggregates have not found much application in enzyme-based industries. One obvious difficulty is that these aggregates are difficult to handle. For example, a column packed with enzyme aggregates would show poor flow rates.

It was felt that if the enzyme aggregation is allowed to take place inside a commercially available polymeric beads, such as Sephadex, the

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aggregates, because of their larger sizes, would not be able to come out of the pores of the beads. Such entrapped aggregates inside Sephadex beads would have excellent flow properties. The experiments described in the following show that this indeed is feasible. This approach may be an elegant and simple alternate way of protein immobilization.

MATERIALS

E. coli β-galactosidase, wheat germ acid phosphatase, trypsin, and *p*-nitrophenyl phosphate (PNP) were purchased from Sigma Chemical Co., USA. Concanavalin A and benzoyl DL-arginine, *p*-nitroanilidine (BAPNA), and *o*-nitrophenyl β-D galactopyranoside (ONGP) were obtained from CSIR center for Biochemicals, India. Glutaraldehyde was bought from Riedel-Dehaenag, Sellza, FRG. Nigrosine was obtained from the BDH Ltd., UK. Sephadex gel filtration media were supplied by Pharmacia Fine Chemicals, Sweden. Coomassie brilliant blue G-250 was a product of E. Merck, FRG. All other reagents were of analytical grade.

METHODS

β-Galactosidase Aggregation within Sephadex G-200

Enzyme solution was prepared by dissolving 200 μg β-galactosidase and 100 mg BSA (inert proteic feeder) (*1*) in 400 μL of sodium phosphate buffer (0.06*M*; pH 7.0, containing 0.003*M* Mg^{2+}—Buffer-A). A 200 μL aliquot of this stock solution was withdrawn and mixed with 5 mg lactose (3). The rest of the enzyme solution was used as control. The enzyme solution containing lactose was used for enzyme immobilization, and was incubated with 1 mL of Sephadex G-200 (equilibrated with buffer-A). After one hour of incubation at 4°C, the supernatant was removed by centrifugation (10,000 rpm for 10 min) and 80 μL of glutaraldehyde (25%) was added into the pellet, and the mixture was kept at 4°C for 1 h. The crosslinking was terminated by adding 4 mL of 0.1*M* lysine solution (in buffer-A). The reaction mixture was kept overnight at 4°C, after which extensive washing was done by the phosphate buffer until the supernatant was free of lactose, protein, and β-galactosidase activity. However, it was observed during the washing that some insoluble β-galactosidase aggregates were also formed outside the Sephadex beads. Thus, after the preparation, the whole mixture was stirred up. The Sephadex beads settled down faster, and some of the insoluble aggregates remained suspended up to 5–7 min. These could be removed by a Pasteur pipet. Repetition of the process separated most of the visually detectable aggregates from the

beads. Now, these beads were suspended in equal vol of buffer and used for further study. This preparation could be used until 3 wk inasmuch as there was no loss in enzyme activity when stored at 4°C, and also there was no leaching observed.

Trypsin Entrapped Inside Sephadex G-100

Trypsin was similarly entrapped within Sephadex G-100 beads. Here 1 mL Sephadex G-100 beads (equilibrated with sodium acetate buffer, 0.2*M*, pH 5.0, containing 11.5 m*M* Ca^{2+}) were incubated with 200 μL of trypsin solution (containing 50 mg trypsin in above-mentioned sodium acetate buffer), and after one hour of incubation at 25°C, the supernatant was removed by centrifugation (10,000 rpm for 10 min). Glutaraldehyde solution, 120 μL (25%) was added to the pellet. This reaction mixture was again incubated at 25°C for 10 min. Afterward, the reaction was terminated by the addition of 4 mL of lysine (0.1*M* in sodium acetate buffer). This mixture was kept overnight at 25°C and then washed extensively with sodium acetate buffer until the supernatant and washings were free of protein and trypsin activity. Pellet thus obtained was suspended in equal vol of sodium acetate buffer, and was used for further study.

Acid Phosphatase Entrapped in Sephadex G-100

Wheat germ acid phosphatase was also entrapped within Sephadex G-100 beads following exactly similar procedure. Acid phosphatase (50 mg) dissolved in acetate buffer (0.02*M*; pH 5.0) and 1 mL of Sephadex G-100 beads equilibrated in the same buffer was added. Glutaraldehyde solution, 80 μL (25%), was used for aggregation, and the reaction was allowed for 30 min at 25°C. After the washings, pellet was suspended in equal vol of acetate buffer, and was studied further.

Concanavalin A Entrapped in Sephadex G-150 and G-200

Concanavalin A was entrapped in Sephadex G-150 and G-200 in a similar procedure. To 1 mL of Sephadex beads equilibrated in phosphate buffer (0.1*M*; pH 7.0), 1 mL of 6 mg/mL Con A in the same buffer was added. Varying concentrations of glutaraldehyde were added, and the reaction was allowed to occur for 1 h at 4°C. After this reaction was terminated by lysine and the beads were washed with Tris buffer (0.02*M*, pH 7.0 containing 1*M* NaCl).

Known amount of peanut acid phosphatase, a glycoprotein binding to Con A (4), was added to the beads. The unbound enzyme was washed away and, finally, the bound enzyme was recovered with 0.1*M* mannose incorporated in the buffer.

Assays of β-galactosidase, acid phosphatase, and trypsin were carried out as reported by Khare and Gupta (*5*), Kamra and Gupta (*4*), and Rajput and Gupta (*6*), respectively.

Staining of Protein and Microscopic Observation of the Presence of Enzyme Inside the Sephadex Beads

Horigome et al. (*7*) have described a staining procedure by which the protein molecules entrapped within Sephadex beads can be easily visualized.

Sephadex beads containing β-galactosidase activity were incubated overnight with 0.2% aqueous nigrosine solution. Excess dye was removed by washing with distilled water.

Stained beads were spread evenly over a glass slide, and were covered with cover slip carefully. These were observed under a compound light microscope with 100x magnification.

RESULTS AND DISCUSSIONS

We have recently described an aggregate of β-galactosidase that showed adequate activity and enhanced thermal stability as compared to the free enzyme (*3*). This prompted us to think about a method in which the simplicity of aggregation technique could be combined with a practical solution to poor flow rates yielded by enzyme aggregates in general. The present work explores the idea of forming enzyme aggregates within Sephadex beads.

Swollen Sephadex G-200 beads were incubated with β-galactosidase, and chemical aggregation was performed under the conditions optimized for aggregation of soluble β-galactosidase (*3*). Glutaraldehyde was the bifunctional reagent used for extensive crosslinking of the enzyme. After aggregation, these Sephadex beads were repeatedly washed until they were free of the sugar, protein, and β-galactosidase activity. These beads were then assayed for β-galactosidase activity, and it was found that 17% of the total β-galactosidase activity added to the beads right at the beginning was present in the immobilized form.

These beads containing entrapped β-galactosidase inside in form of aggregate showed an increase in K_m towards ONGP ($6.32 \times 10^{-4} M$) (Fig. 1) as compared to the native soluble enzyme ($2.64 \times 10^{-4} M$). Such an increase in K_m is generally observed for immobilized systems, and was only to be expected in view of both aggregation and subsequent entrapment. However, it is noteworthy that this K_m value for the entrapped aggregates was quite comparable to the corresponding K_m value ($6.02 \times 10^{-4} M$) in case of simple aggregates reported earlier (*3*).

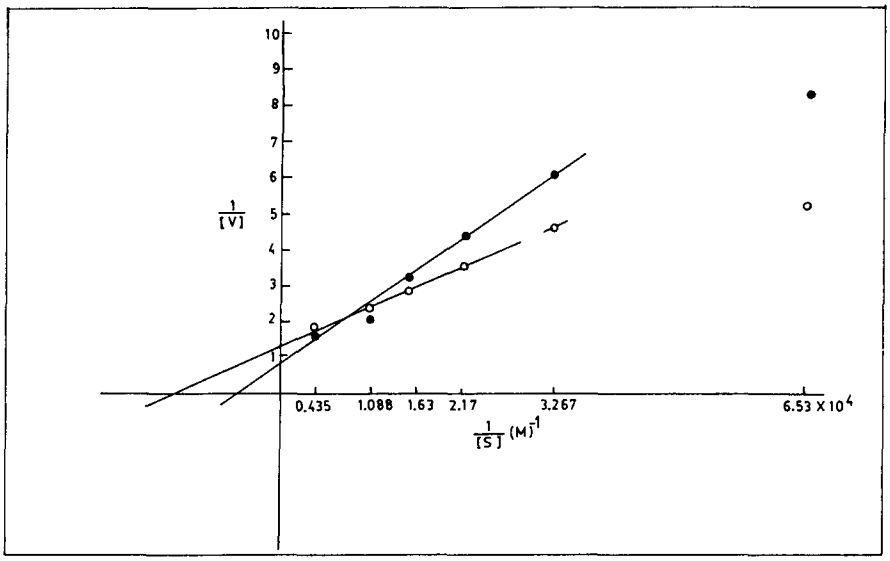

Fig. 1. K_m determination of β-galactosidase. K_m values were determined from the Lineweaver-Burk plot under the following experimental conditions: ONGP as substrate in sodium phosphate buffer (0.3*M*) containing 0.003*M* Mg^{2+}, pH 7.5, 25°C. O--O; Native β-galactosidase. ●--●; β-galactosidase aggregates.

The beads containing the aggregated enzyme were subjected to heat treatment in order to assess the thermal stability of entrapped β-galactosidase. β-Galactosidase within the beads had higher thermal stability than the native enzymes (Figs. 2,3). At 55°C, immobilized enzyme lost no activity for 8 h, whereas soluble enzyme lost 80% activity during this time.

The thermal stability of the entrapped aggregated enzyme was quite similar to the aggregated enzyme, as reported earlier (*3*). Thus, entrapment inside the carbohydrate matrix did not enhance the stability further.

At this stage, it was thought appropriate to test the general validity of this approach by trying it out with other enzymes. Trypsin was aggregated inside Sephadex beads of various pore sizes by using the conditions described by Rajput and Gupta (*2*). It was confirmed that an appropriate pore size range existed for the successful entrapment of the enzyme aggregates inside the beads. Thus, Sephadex G-75 and G-100 (fractionation range 3000–70,000 and 4000–150,000, respectively) gave maximum yield for trypsin (mol wt 24,000) (*8*) (Table 1).

When similar experiments were performed with wheat germ acid phosphatase (mol wt 80,000) (*9*), Sephadex G-100 was found to be most suitable in obtaining a maximum yield of enzyme entrapped inside the beads (Table 2).

The above data is understandable since if the pore size is too small, the soluble enzyme does not enter the beads and hence, it cannot be trapped

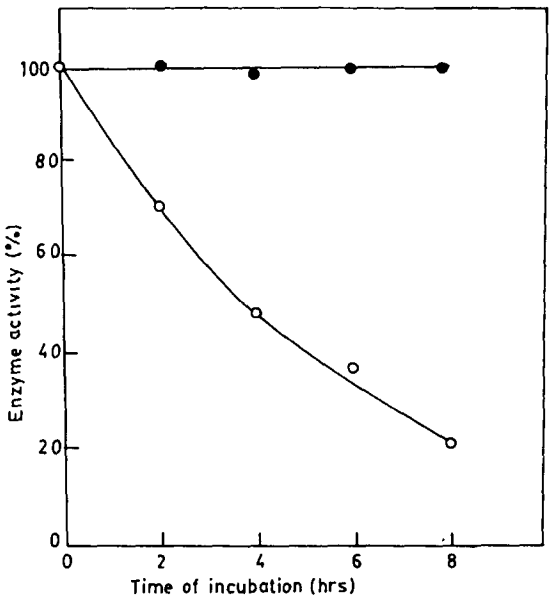

Fig. 2. Thermal stability of β-galactosidase aggregates entrapped within Sephadex G-200 beads at 55°C. Sephadex beads containing entrapped enzyme aggregates were incubated at 55°C. Simultaneously, a native enzyme control having identical activity and 50 mg BSA was also given the same treatment. Enzyme activity toward ONGP was determined in the aliquots (200 μL) and withdrawn at various time intervals. O--O; Free native β-galactosidase. ●--●; β-galactosidase aggregates entrapped within Sephadex beads.

by aggregation. On the other hand, if the pore size is too big, even the aggregated enzyme cannot be retained by the beads. Incidentally, all this data strongly indicates that at least, most of the activity observed in the preparation belonged to the trapped enzyme.

In order to optimize the activity of the immobilized acid phosphatase, varying concentrations of glutaraldehyde were used for aggregation, and 3% concentration was found to be best (Fig. 4). As higher protein concentration is known to increase intermolecular crosslinking (*10*), acid phosphatase concentration was varied and it was found that beyond 50 mg/mL, increasing protein concentration did not yield immobilized preparations with greater enzyme activity (Fig. 5).

There was an enhancement in the thermal stability of both trypsin and acid phosphatase entrapped within the beads. Thus, immobilized trypsin, when incubated at 45°C for 2 h, lost no activity, whereas the soluble enzyme lost 20% of its activity in the same amount of time (Fig. 6). In the case of the acid phosphatase, the immobilized enzyme was stable at 60°C, whereas the soluble enzymes lost 35% activity in 30 min at this temperature (Fig. 7).

In order to further test the generality of the method, we tried entrapping a lectin concanavalin A (Con A) and checked whether this entrapped

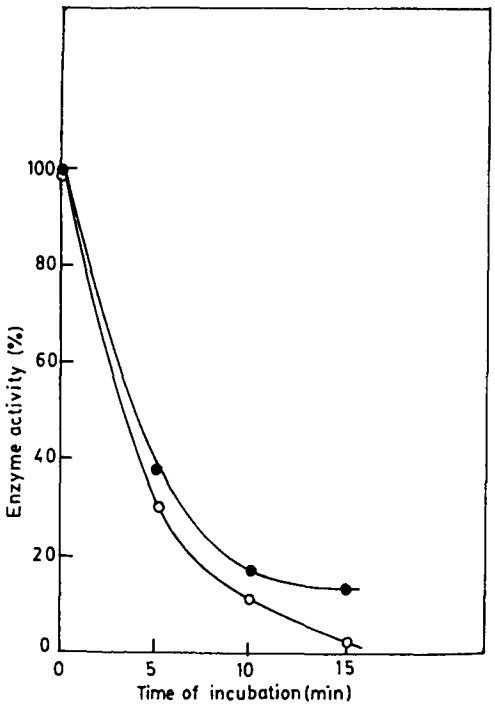

Fig. 3. Thermal stability of β-galactosidase entrapped within Sephadex G-200 beads at 60°C. Thermal stability was also determined at 60°C for various time intervals as described in the legend to Fig. 2. ○--○; Native β-galactosidase. ●--●; β-Galactosidase entrapped within Sephadex beads.

Table 1
Effect of the Sephadex Pore Size
on the Yield of Entrapment of Trypsin Inside the Beads

Trypsin was entrapped inside the Sephadex beads as described in the method section. However, Sephadex beads of different pore sizes were used and the enzyme activity entrapped within the Sephadex beads in each case was determined towards BAPNA as the substrate. The activity values are expressed in terms of percentage activity assuming the total activity added right at the start of immobilization to be 100%.

Type of Sephadex	Enzyme activity immobilized inside the beads (%)
G-25	1.8
G-75	20.3
G-100	25.8
G-200	15.0

Table 2
Effect of the Sephadex Pore Size
on the Yield of Entrapment of Acid Phosphatase Inside the Beads

Acid phosphatase was incubated within Sephadex beads of various pore sizes and the entrapment of the aggregates was attempted as described in the method section. The enzyme activity entrapped inside the beads was determined towards PNP as the substrate. The activity values are expressed in terms of percentage activity assuming the total activity added right at the start of immobilization to be 100%.

Type of Sephadex	Enzyme activity immobilized inside the beads (%)
G-25	4.8
G-100	24.8
G-200	2.8

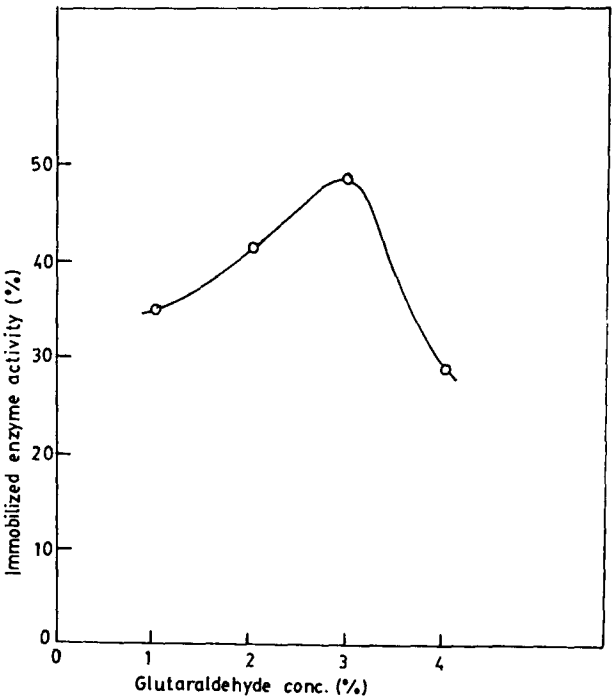

Fig. 4. Effect of glutaraldehyde concentration on the yield of acid phosphatase aggregates entrapped within Sephadex G-100 beads. Acid phosphatase (50 mg in 200 μL sodium acetate buffer, 0.2*M;* pH 5.0) was incubated with 1 mL of Sephadex G-100 beads in various sets. Varying concentration of glutaraldehyde was added to different sets. The rest of the procedure was as described in the method section, and the phosphatase activity in the beads was determined using PNP as the substrate. The percentage immobilized activity is calculated by taking the enzyme activity before the reaction started as 100 (i.e., at 0% glutaraldehyde concentration, the activity was 100%).

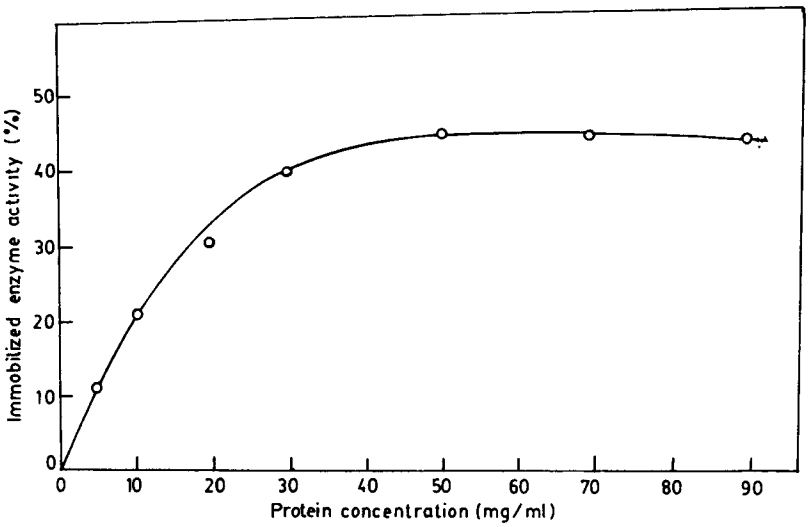

Fig. 5. Effect of protein concentration on the yield of acid phosphatase aggregates entrapped within Sephadex G-100 beads. Varying concentrations (in the range of 0–90 mg/mL) of acid phosphatase (in 200 μL sodium acetate buffer, 0.2*M*, pH 5.0) were incubated with 1 mL of Sephadex G-100 in different sets, and the aggregation within the beads was performed as described in methods. The activity entrapped within these beads was determined using PNP as the substrate. The protein concentrations shown on the *x*-axis refer to the concentrations in 200-μL buffer aliquots that were added to 1 mL Sephadex G-100. The immobilized enzyme activity (%) shown on the ordinate was calculated as follows:

$$\text{Immobilized enzyme activity (\%)} = \frac{\text{activity observed in beads containing entrapped enzyme (p)}}{\text{enzyme activity added to the Sephadex beads in the beginning of that particular experiment (q)}} \times 100$$

q, thus was not a fixed amount but varied from 0 to the activity of 90 mg/mL enzyme solution.

lectin would constitute an affinity medium for Con A-specific glycoproteins. Table 3 shows that with Sephadex beads of different pore size (G-150 and G-200) and varying concentrations of glutaraldehyde, Con A entrapment could be confirmed as judged by binding of a known Con A-interacting glycoprotein (*4*). The control experiment without Con A showed no binding of the glycoprotein, and confirmed that the observed glycoprotein in other cases was not an artifact.

We believe that this approach may have a great potential in developing lectin based affinity media for glycoconjugates, and so on, since it would have the following added advantage. If a low mol wt lectin is entrapped by this method in a suitable gel filtration beads, this affinity medium would be able to discriminate between two glycoproteins, both

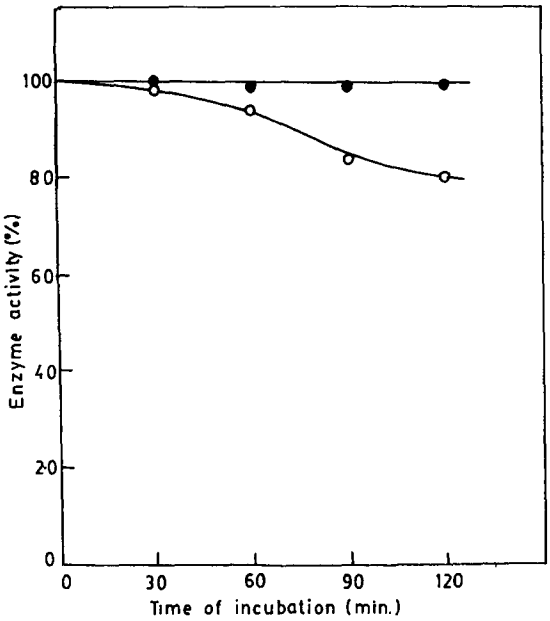

Fig. 6. Thermal stability of trypsin entrapped within Sephadex G-100 beads. Sephadex G-100 beads containing trypsin activity were incubated at 45°C. Aliquots were withidrawn at various time intervals and the activity toward BAPNA was determined in each case. Simultaneously, a native trypsin control having identical activity was subjected to the same treatment for comparison. ○--○; Native trypsin. ●--●; Trypsin aggregates entrapped within Sephadex beads.

Table 3
Binding of Peanut Acid Phosphatase
to Con A Entrapped Within Sephadex Beads

Con A was entrapped within sephadex beads as described in the method section. Peanut acid phosphatase was added (0.1 enzyme units) to 1 ml of Con A-Sephadex beads. The unbound enzyme was washed away and the bound enzyme was eluted with 0.1M mannose. The amount of eluted enzyme is expressed in terms of percentage of the total enzyme added. In the control experiment Con A was eliminated and the procedure was repeated exactly.

Type of Sephadex	Glutaraldehyde	Acid phosphatase activity eluted with mannose	
		from Con A Sephadex	from Sephadex (control)
G-150	0.05%	44%	0
	0.10%	44%	0
	0.50%	40%	0
G-200	0.05%	24%	0
	0.10%	40%	0
	0.5%	14%	0

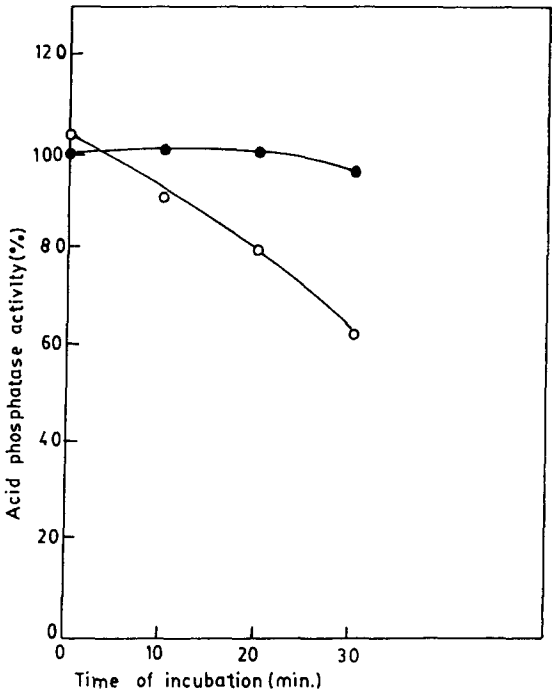

Fig. 7. Thermal stability of acid phosphatase entrapped within Sephadex G-100 beads. Sephadex G-100 beads containing the entrapped aggregates and a native control (having identical enzyme activity dissolved in equal vol of buffer) were incubated at 60°C. The acid phosphatase activity was determined in the aliquots withdrawn at various time intervals. ○--○; Native acid phosphatase. ●--●; Acid phosphastase aggregates entrapped within Sephadex beads.

having affinity for the lectin but having large differences in mol wt, so that the one whose mol wt exceeds the exclusion limit of the gel filtration beads would not bind.

When this work was in progress, we came across the work of Horigome et al. (*7*), who have shown that the enzyme molecules immobilized on the surface of Sephadex beads could be distinguished from those present inside by nigrosine staining. The entrapped aggregates of β-galactosidase inside Sephadex G-200 were stained by this technique and examined under a high resolution light microscope. This confirmed that enzyme aggregates were indeed entrapped inside the beads. At the same time, this also showed that some traces of the enzyme aggregates outside the beads were present in the preparation. All simple and obvious approaches like density gradient centrifugation failed to separate these traces of aggregates from the beads.

However, the overall preparation, when packed inside a column reactor, was found to give excellent flow rates (*11*). Thus, even if a small percentage of enzyme activity (which is difficult to quantify) did originate in the traces of aggregates present outside the beads, the approach out-

lined here does constitute a simple way of obtaining enzyme aggregates with excellent flow properties when packed in a column. Moreover, as pointed out earlier, the very fact that right pore size of beads was found to be critical for obtaining beads with adequate enzyme activity shows that bulk of the enzyme activity does, in fact, belong to the trapped aggregates, and not free aggregates.

The low yield of entrapment of the aggregates also needs to be improved. However, the results described above show that this approach is a viable alternate to existing techniques of enzyme immobilization.

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