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Adsorptive Immobilization of Intestinal Brush Border Membrane on Triton X-100–Substituted Sepharose 4B

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Abstract

Triton X-100–substituted Sepharose 4B (Sepharose-TX) was used for adsorptive immobilization of intestinal brush border membrane using lactose-phlorizin hydrolase as a representative membrane enzyme. Limited heating of membrane preparations was found to enhance binding. This enhancement is concluded to be owing to a greater availability of the hydrophobic sites, as also confirmed by the 1-anilino-8-naphthalene sulfonate fluorescence studies, for interaction with Triton X-100 moieties on the support. The immobilized preparations obtained by this procedure were found useful in hydrolysis of lactose, involving lactose-phlorizin hydrolase, in continuous operations. It is suggested that the approach may be of general utility for immobilization of biologic membranes by interaction of their extramembrane structures using supports with appropriate hydrophobic groups.

Index Entries: Adsorption; continuous operation; ectoprotein; intestinal brush border membrane; lactase-phlorizin hydrolase; molten globule.

Introduction

Biologic membranes provide selective permeability barriers and the environments for a multitude of functional processes. The latter includes electron transport, transduction, recognition, reception, transportation, and catalysis. Since proximity with phospholipids is normally essential for biologic activity *(1,2)* and stability *(3,4)* of membrane proteins, *in situ* exploitation of their functional properties would provide a desirable possibility. Furthermore, the complicated and tedious task related to their

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isolation and purification would be avoided, and the membrane proteins would function under conditions relatively closer to their natural microenvironments.

Progress in the area of stabilization of biologic activities through immobilization of pure proteins, mainly enzymes, has been extended to whole cells and organelles. A number of such studies (e.g., *[5–7]*) have demonstrated enhanced stability of these structures toward environmental conditions by immobilization. In line with these endeavors, we have recently demonstrated the usefulness of adsorption as a simple and effective method for membrane immobilization *(8–10)*. The procedures followed consisted of participation of general and biospecific interactions and provided stabilized membrane preparations useful for continuous catalytic operations.

In the present investigation, we describe a different approach involving limited denaturation of extramembranous protein structures of intestinal brush border membrane (IBBM) to facilitate their interaction with a hydrophobic adsorbent. The hydrolytic enzymes of this membrane are essential for degradation of nutrients to absorbable units. One of these, lactase-phlorizin hydrolase (LPH) (EC 3.2.1.23/45/46/62), considered the most important glycohydrolase of the mammalian intestine, was chosen to follow immobilization in this study. As a member of a distinct class of membrane proteins termed ectoenzymes, this enzyme is an integral protein that projects its catalytic sites on the outside of the cell. The protein is a single polypeptide chain with an apparent size of approx 135 kDa *(11)*, which can be solubilized from brush border membrane by harsh methods involving either proteolytic *(12)* or detergent *(13)* treatment. The membrane has three other glycohydrolytic activities—sucrase-isomaltase, trehalase, and maltase-glucoamylase—and their catalytic potentials could probably be utilized in immobilized forms.

Triton X-100–substituted Sepharose 4B *(14)* and the process of limited denaturation *(15,16)* have already been found useful for immobilization of soluble proteins with retention of their catalytic potentials.

Materials and Methods

Materials

All biochemicals were purchased from Sigma (St. Louis, MO), and all chemicals were of analytical grade.

Preparation of IBBM

Rabbit (*Oryctolagus cumiculus*) IBBM was prepared from freshly removed small intestine of suckling rabbits. The modified method of Hauser et al. *(17)* was utilized except for the initial step, which involved sonication instead of vibromixing. Sonication was performed on ice three times, for 30 s each with intervals, using an MSE sonicator. The isolation medium consisted of 300 m*M* D-mannitol, 5 m*M* EGTA, and 12 m*M* Tris-

HCl (pH 7.1). $MgCl_2$ at a concentration of 10 m*M* was employed in the precipitation step, which has been reported to provide vesicles, predominantly right side out *(18)*. The IBBM vesicles, which were 0.1–0.3 µm in diameter, looked similar to presentations reported in the literature, when examined under an electron microscope. Specific activity of LPH in the preparations was about 60 µmol of glucose liberated/(min·mg protein), following the assay procedure employed. Fifty-microliter aliquots of such preparations at concentrations of 4–8 mg/mL of protein were then frozen in liquid air until use. Protein determination was made using the Markwell *(19)* and tannic acid *(20)* methods.

Coupling of Triton Glycidyl Ether to Sepharose 4B

Triton glycidyl ether was prepared according to the procedure of Ulbrich *(21)*. The coupling of the product to Sepharose 4B was carried out following the procedure reported previously *(14)* using the same amounts of Sepharose 4B and related reagents.

LPH Activity

Lactose hydrolysis was measured using the coupled enzymatic assay of Rivera-Sagaredo et al. *(22)*. The membrane suspension was diluted with 100 m*M* sodium phosphate, pH 5.5, when required. Normally, 20 µL of membrane suspension was used with an equal volume of lactose solution (200 m*M*) in the same buffer. The mixture was incubated at 37°C for 30 min, and the reaction was terminated by leaving in a boiling water bath for 2 min. Finally, hydrolysis of lactose was determined by the addition of 1 mL of glucose oxidase–peroxidase reagent following a standard procedure *(23)*. In studies related to the stability of LPH activity at different temperatures and pH values, catalytic activities were determined following this procedure, after pretreatment of the immobilized membrane in the various conditions employed.

Determination of Optical Density

Optical density (OD) was determined using 40 µg (protein) of membrane suspension in 0.1 *M* phosphate buffer, pH 7.4, in a final volume of 350 µL. The suspension was continuously heated at a rate of 1°C/min, using a 2400-S Gilford spectrophotometer adjusted at 280 nm, in a total time of 60 min.

Proteinase K Treatment

IBBM (625 µg [protein]) was suspended in 10 m*M* Tris, pH 7.5, and treated with 1.8 U of proteinase K in a final volume of 260 µL at 4°C, normally for 40 min *(24)*. Proteolysis was terminated by the addition of 10 µL of phenylmethylsulfonyl fluoride (2.6 mg/mL in isopropanol). On centrifugation (48,000*g* for 15 min) at 4°C, the corresponding pellets were resuspended in 0.1 *M* sodium phosphate adjusted to pH 5.5 using NaOH.

Gradient Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Kessler et al. *(25)*, using an 8.4–15% gradient.

Fluorescence Measurements

Emission fluorescence measurements were made in a Hitachi (MPF-4) spectrofluorimeter, at room temperature. Slit widths with a nominal band pass of 10 nm were used for both excitation and emission. Excitation wavelength was fixed at 350 nm, and the 1-anilino-8-naphthalene sulfonate (ANS) emission spectra were recorded from 400 to 600 nm.

Immobilization of IBBM

One hundred–microliter volumes of IBBM suspension at known concentrations were added to 100 µL of packed Sepharose-TX, and the mixture was incubated at the desired temperatures for 30–45 min while stirring gently. The mixture was subsequently cooled at 4°C for another 30 min, followed by low-speed centrifugation. The pellets were washed twice (with 100 m*M* phosphate buffer, pH 7.4) to remove unbound IBBM. Protein concentration and enzyme activity were then measured.

Effect of pH on LPH Activity

A buffer mixture containing 33 m*M* each of phosphate, glycine, and citrate was adjusted to the required pH values in the range of 3.0–11.0. Catalytic activities of LPH in free and immobilized IBBM vesicles were then determined.

Continuous Catalytic Operation

Continuous hydrolysis of lactose was achieved using a simple stirred reactor, as depicted in Fig. 1. For each run, 0.5 mL of the immobilized preparation of IBBM was used, and the reaction cocktail was pumped through the reactor at a flow rate fixed at 200 µL/min. Catalytic activity of the collected fractions was subsequently determined in the usual manner.

Results and Discussion

In the course of this investigation, it was found that the extent of adsorption of IBBM vesicles on the hydrophobic matrix could be promoted by incubating the two interacting components at a relatively high and narrow temperature range of 50–55°C (Fig. 2). Later studies showed that this temperature effect was not observed for IBBM vesicles previously treated with proteinase K (Fig. 2), thus suggesting possible involvement of ectoprotein structures in this interaction.

A number of soluble proteins have been shown to take up intermediate forms under mild denaturing conditions, commonly referred to as molten globule structures *(26,27)*, with provision of hydrophobic micro-

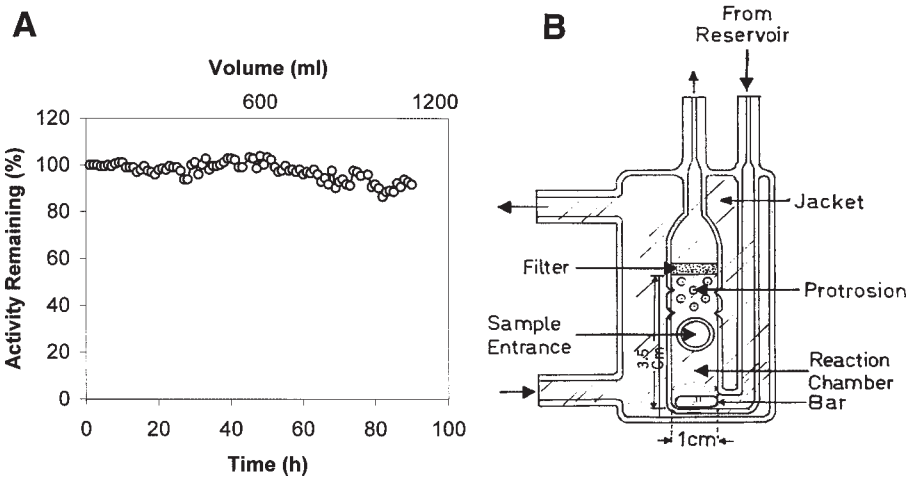

Fig. 1. **(A)** Continuous catalytic operation at 25°C, using immobilized IBBM. Five hundred microliters of packed Sepharose-TX containing immobilized IBBM was used, and LPH activity was determined in the usual manner. **(B)** A homemade vessel was used in this part of the study.

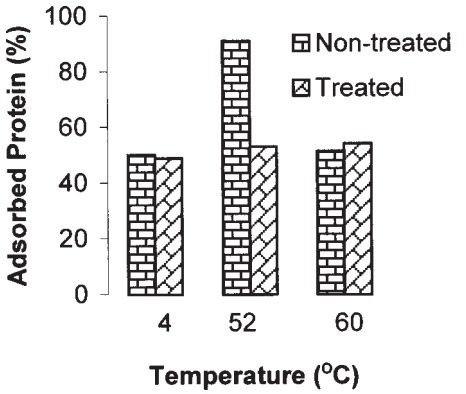

Fig. 2. Adsorptive immobilization of IBBM vesicles on Sepharose-TX using original (nontreated) and proteinase K–treated membranes at three different temperatures. One hundred microliters of an IBBM suspension containing 250 μg of membrane protein (original and proteinase K treated) was used with 100 μL of packed matrix. Percentage protein adsorbed was determined in each case. Further details are described in Materials and Methods.

environments for ligand binding *(28)*. Later reports have indicated that the extramembranous parts of membrane proteins may also take up such intermediate structures *(4)*, shown schematically in Fig. 3. Of these intermediate forms, the molten globule structures reported for acetylcholinesterase *(29,30)*, an externally oriented membrane-bound enzyme, is of relevance to the present investigation. In this connection, it is noteworthy that thermal denaturation has been shown to affect primarily the extramembranous regions of membrane proteins *(4)*.

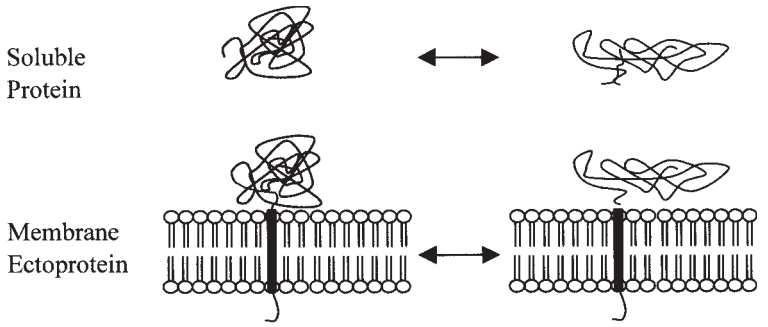

Fig. 3. Schematic representation of limited denaturation of protein structures. For further details see the text.

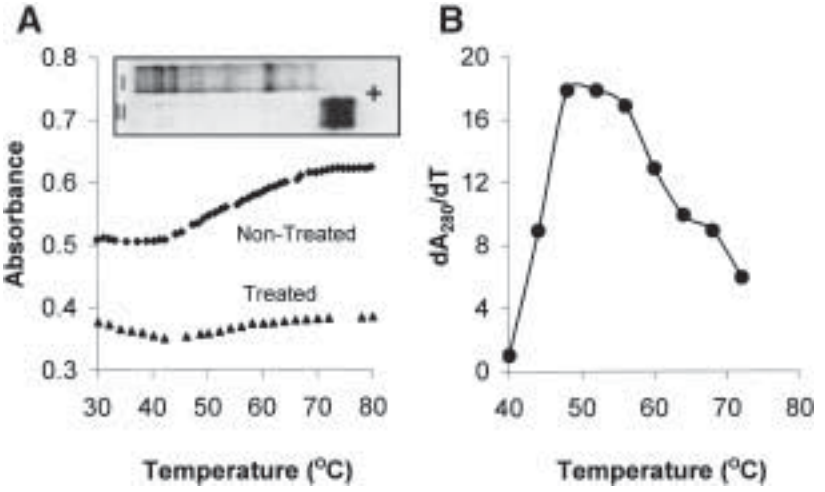

Fig. 4. **(A)** OD measurements of original (nontreated) and proteinase K–treated IBBM membranes, determined at 280 nm. **(Inset)** Coomassie brilliant blue staining of gradient SDS-PAGE of original (I) and protease-treated (II) IBBM proteins. The band observed in II represents proteinase K and the digestion products of higher molecular weight proteins in the membrane. **(B)** First derivative of hyperchromicity with respect to temperature. The heating rate was adjusted at 1°C/min. For further details, see Materials and Methods.

In recent studies involving interaction of soluble proteins with hydrophobic matrices, we encountered the very effective utility of reversible heat denaturation for immobilization of carbonic anhydrase *(15)* and acidic denaturation for urease *(16)*, known to take up the intermediate structures already discussed. Influenced by this background, we suspected that improvement in IBBM immobilization at relatively high temperatures was probably owing to a greater availability of the hydrophobic sites of membrane components. Support for this contention was provided by the nature of temperature dependency of near ultraviolet (UV) measurement and extrinsic fluorescence studies (Figs. 4 and 5). Involvement of extra-

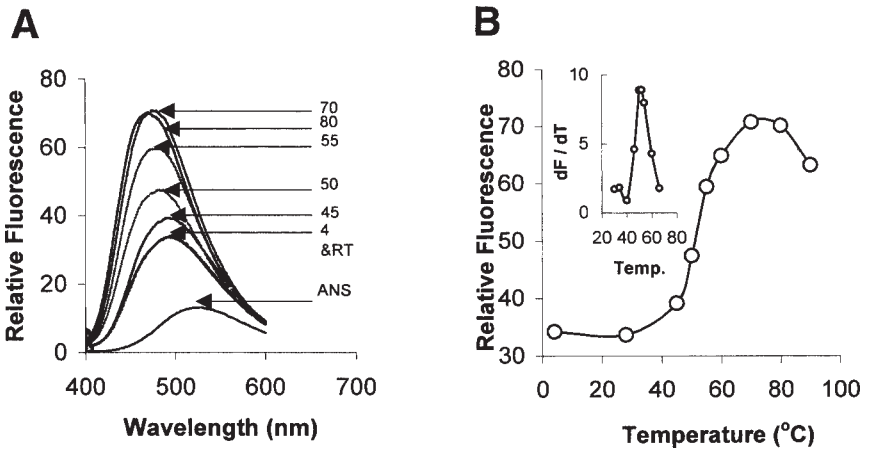

Fig. 5. **(A)** Extrinsic fluorescence intensity determination using ANS as a hydrophobic reporter probe. One hundred forty micrograms (protein) of IBBM suspension was employed in a final volume of 2 mL using an ANS concentration of 50 μM, and the mixture was incubated at the indicated temperatures for 30 min, followed by cooling at 4°C. Excitation was fixed at 350 nm, and emission spectra were obtained as indicated, at 25°C. **(B)** Maximum fluorescence intensities obtained from the data presented in (A) plotted against the temperature of incubation. **(Inset)** First derivative of change in fluorescence intensity with respect to temperature vs temperature. For additional details, see Materials and Methods.

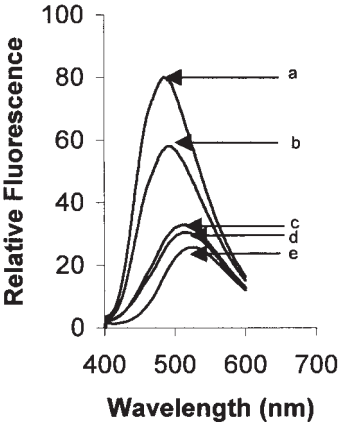

Fig. 6. Effect of proteinase K treatment of IBBM on ANS binding at 52°C and subsequent fluorescence emission spectra at 25°C. Samples were treated with proteinase K for different times: (a) 0 min; (b) 10 min; (c) 20 min; (d) 30 min; and (e) free ANS.

membranous structures in interaction with ANS was further indicated by the dependency of ANS fluorescence on the extent of proteolysis brought about by treatment of IBBM vesicles with proteinase K (Fig. 6).

It is noteworthy that for the temperature range in which immobilization (Fig. 3), UV absorbance (Fig. 4), and fluorescence (Fig. 5) were affected,

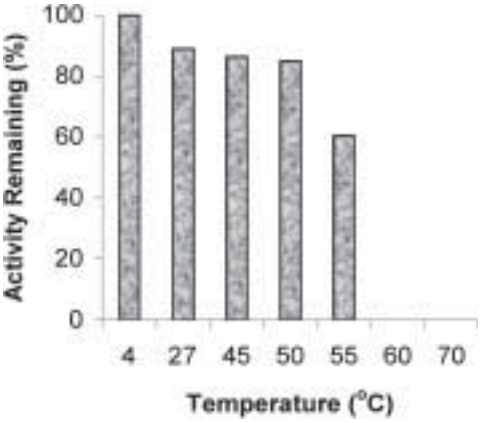

Fig. 7. Catalytic activity of LPH in IBBM vesicles preincubated at different temperatures. IBBM (in 0.1 *M* phosphate, pH 7.4) was incubated at a concentration of 1 mg/mL of protein at the indicated temperatures for 2 h. Each sample was then placed on ice for 30 min followed by activity determination in the usual manner.

irreversible thermoinactivation of LPH was also observed (Fig. 7). The latter clearly suggested the occurrence of some crucial events related to the structural properties of the ectoproteins of IBBM.

The continued increase in ANS fluorescence at temperatures higher than 55°C was unexpected and at first appeared in discord with other experimental findings discussed earlier. However, it is suggested that at temperatures >55°C, a number of events may occur in the membrane, providing new opportunities for ANS binding. It should be pointed out that the probe may interact with both protein and phospholipid components of membrane. Furthermore, for interaction with Triton X-100 the proteins should not only possess hydrophobic patches on their surface but should also form crevices large enough for accommodating the bulky 1,1,3,3-tetramethylbutylphenyl head group of the detergent *(14)*. Accordingly, it is not surprising that certain structural changes occurring in IBBM may be detrimental to interaction of Triton X-100 and not of ANS in the experimental conditions utilized in the present study.

An increase in the availability of hydrophobic sites in protein structures observed at high temperatures (such as those indicated in Fig. 5) is not totally surprising. For example, in a study related to formation of molten globule intermediate structures in equinatoxin II, it was observed that even at 95°C, the protein is not completely denatured and may undergo further conformational transitions at higher temperatures *(28)*. Resistance to irreversible thermoinactivation of thermophilic proteins may also be of relevance to this discussion.

The relationship between the degree of adsorption and total IBBM concentration in the form of suspension was determined (Fig. 8). As shown in Fig. 8, adsorption of increasing quantities of IBBM vesicles to a fixed amount of the matrix is represented by a curve indicating saturation at high

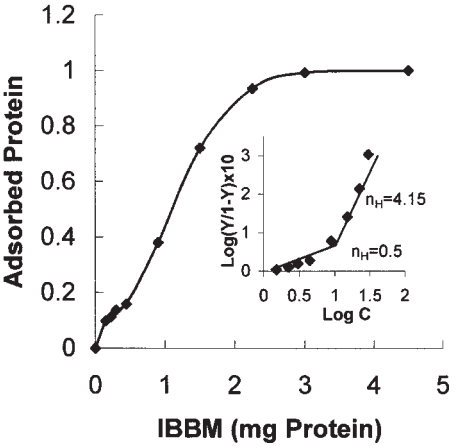

Fig. 8. Adsorption of IBBM vesicles on Sepharose-TX as a function of IBBM concentration. One hundred microliters of the packed matrix was incubated with different concentrations of the membrane at 52°C while mixing, for 30 min. After placing the suspensions on ice, the pellets were washed twice using the buffer, and bound protein was obtained for each sample. Further details are described in Materials and Methods.

membrane concentrations. Hill plot analysis of the binding data (presented in Fig. 8) suggested positive cooperative interactions occurring at high membrane concentrations, similar to adsorption of membrane structures to other matrices *(8,9)*, and interaction of soluble proteins with the hydrophobic support used in the present investigation *(14)*.

The K_m of LPH in free IBBM vesicles was found to be 14.4 m*M*, very similar to the values (16 ± 2) reported earlier *(31)*. Because of the nature of the assay and the immobilized preparations, reproducible data could not be obtained, and, therefore, kinetic parameters related to the immobilized membrane are not presented. However, from the data obtained, it can be concluded that the specific activity of LPH in the free and adsorbed forms are very similar and that immobilization of IBBM occurs with retention of the catalytic potential of the glycohydrolase. By extrapolation of this observation, it may also be suspected that the overall biologic functions of the membrane are presumably retained on immobilization.

Experiments related to thermostability, pH stability, and pH profiles of free and immobilized IBBM vesicles were also performed. Regarding thermostability, results were not reproducible enough to merit presentation. However, they do indicate that immobilized IBBM prepared by the described procedure is at least as thermally stable as its free form. As for pH, it appears that the immobilized preparations experience higher stabilities in alkaline conditions (Fig. 9). The pH profile is clearly broader for the immobilized form compared with the free form (Fig. 10), indicating that the active-site microenvironment of LPH becomes less sensitive to bulk pH changes on formation of the immobilized network.

IBBM vesicles immobilized at 52°C following the procedure described in Materials and Methods were tested in continuous catalytic transforma-

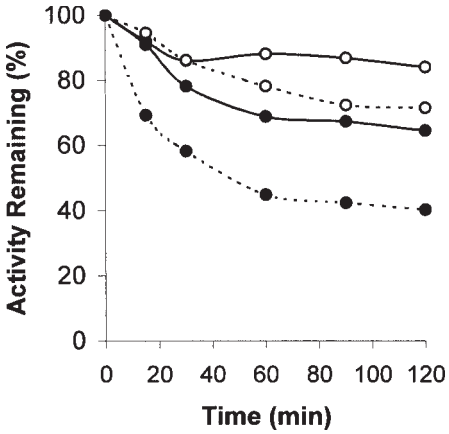

Fig. 9. Stability of LPH activity in free (– – – –) and immobilized (——) IBBM vesicles at pH 9.0 (○) and 11.0 (●). A citrate-glycine-phosphate buffer mixture, each at 33 m*M* concentration, was adjusted to the required pH. Samples were incubated at the desired pH for 2 h at room temperature, and the remaining activity of LPH was determined following the procedure described under Materials and Methods.

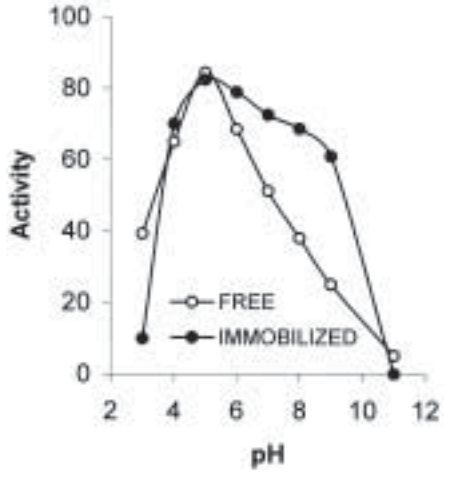

Fig. 10. Activity of free (○) and immobilized (●) IBBM vesicles at various pH values. Further details are described in Materials and Methods and the text.

tions, employing a stirred reactor (Fig. 1). As indicated, the preparations may be used successfully in continuous operations of LPH at 25°C. Similar results were obtained when the experiment was performed at 4 and 37°C.

The procedure described for immobilization of biologic membranes involves *denaturation* of their protein structures for exploiting their *natural* potentials. Interestingly, recent in vitro studies of some globular proteins have indicated that various *nonnative* protein states may be involved in *physiologic* processes such as protein penetration into biologic membranes *(32)* or ligand delivery to target cells via transport proteins *(33)*. In these

reports, the intermediates are referred to as molten globule states *(28)*, structures apparently similar to those formed in the present investigation.

In conclusion, we have described a simple and effective procedure involving limited denaturation of ectoproteins of IBBM vesicles for their adsorptive immobilization. We suggest that the strategy may be of general utility for immobilization of biologic membranes involving their extramembranous structures, with possible retention of their physiologic activities.

Acknowledgment

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