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ADSORPTIVE IMMOBILIZATION OF MICROSOMAL MEMBRANES*

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Abstract – In this article, the immobilization of microsomal membranes on Fractosil and hexadecyl Fractosil by hydrophobic adsorption is reported. Microsomes were prepared from rat brain and the catalytic activity of antimycin A insensitive NADH-cytochrome *c* reductase (NCCR), one of the membrane bound enzymes in the microsomal electron transport chain, was chosen as a representative of the microsomal membrane enzymes. The effect of pH on the enzyme activity and the effect of membrane concentration on adsorption was explored. Physical adsorption on Fractosil and hexadecyl Fractosil caused stabilization when the catalytic potential of the enzyme was followed in a continuous operation. The presence of hydrophobic ligand on Fractosil caused higher stabilization of the immobilized enzyme at 25ºC and 4ºC, making it more useful for continuous operations. It is suggested that using supports with appropriate hydrophobic groups are useful for the immobilization of biologic membranes.

Keywords – Immobilization, hydrophobic adsorption, microsomal membrane, fractosil, fractosil lipid, continuous catalytic operation

1. INTRODUCTION

The importance of immobilizing membrane enzymes, and in particular, the immobilization of submitochondrial particles by hydrophobic adsorption on Fractosil (a porous form of silica) and a thorough literature review on the subject were previously reported by our group [1].

Subcellular organelles such as mitochondria, chloroplasts and microsomes carry out essential functions. They are very labile and stabilization of their biological functions in catalyzing various bioconversions can be of practical significance. Studying the immobilized form of an organelle helps to simulate the invivo situation and predict its performance during continuous use. Some reviews on immobilization of microsomes [2], mitochondria and other organelles demonstrate the potential usefulness of such preparations [3, 4].

Fractosil and hexadecyl Fractosil have been used in our laboratory for adsorptive immobilization of enzymes [5, 6] and membrane particles [1, 7, 8]. The results demonstrated the stabilization of microsomal membranes upon adsorption on such solid supports and provided clear evidence for the potential usefulness of such preparations in continuous catalytic operations. It was also shown that such preparations could be used in diagnostic kits or employed for the construction of biosensors.

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Since endoplasmic reticulum has many important metabolic enzymes participating in carbohydrate metabolism, drug detoxification, lipid synthesis, and microsomal electron transport chain, the purpose of present communication was to use Fractosil and hexadecyl Fractosil as supports to immobilize microsomal membranes and to investigate the catalytic potential of NADH-cytochrome *c* reductase (NCCR) as a model of a multienzyme system.

2. MATERIALS AND METHODS

a) Materials

Fractosil-1000 (Art 9384) was purchased from MERCK (Darmstadt, Germany). Hexadecyl substituted Fractosil was synthesized at the Institute of Biochemistry and Biophysics of Tehran University [9]. All biochemicals were obtained from Sigma (St. Louis, Mo, USA). Chemicals were of analytical grade. The medium used was 0.32 M sucrose, 0.1 mM EDTA and 1mM sodium dihydrogen phosphate, pH 7.5, and henceforth referred to as SES medium

b) Preparation of Microsomes

2-3 month old rats, bred in a colony at the University of Tehran, were decapitated and the brains quickly removed, frozen and kept in liquid air until used. Approximately 4.7g of frozen brain tissue were washed and homogenized in 60 ml of SES medium at 4ºC.The Microsomes were prepared according to the procedure of Gurd et al. [10]. The resultant pellet (microsomes) was suspended in 2.5 ml of SES medium and the microsomal protein concentration was adjusted to about 5.5 mg/ml. Protein estimation was done by the Markwell method [11].

c) Assay of Antimycin A Insensitive NADH-Cytochrome c Reductase (NCCR)

NCCR was assayed according to the procedure of Tolbert [12] using a Shimadzu UV-160 double beam spectrophotometer. The incubation mixture in a final volume of 0.27 ml consisting of 0.1 ml of 0.2M phosphate buffer (pH 7.0), 50 µl of oxidized cytochrome *c* (5 mg/ml), 5 µl of 10 mM KCN, 2 µl of antimycin A (2 mg/ml ethanol) and 63 µl microsomal suspension. After a pre-incubation period of 5 min at 25ºC, the reaction was initiated with 50 µl of NADH (3 mg/ml). Based on an extinction coefficient of 21.1 mM^{-1} cm^{-1} for cytochrome *c*, a change of one absorbance unit at 550 nm was considered to be equivalent to 12.8 nmoles. The effect of pH on the activity of this membrane enzyme was determined using MPTG buffer (Mes, Pipes, Trizma and Glycine-each at the concentration of 20 mM) at pH values ranging from 6-9.

d) Immobilization of Microsomes on the Adsorbent

50 mg of Fractosil- 1000 was washed twice, each time with 1000 µl of 10 mM phosphate buffer pH 7.0. The required amount of microsomal suspension at a protein concentration of 5.5 mg/ml was diluted to the desired concentration in 10 mM phosphate buffer pH 7.0, and 300 µl aliquots were added to the matrix. The resulting suspension was then mixed by rotation for 2 hrs at 4° C. At the end of this period, the immobilized product settled and the supernatant was collected for the assay of the enzyme [1].

It should be mentioned that despite various trials, a direct assay of the immobilized enzyme was not successful.

e) Effect of Microsome Concentration on Immobilization

To obtain the optimum membrane concentration for immobilization, 0.25, 0.5, 1, 1.5, 2, 2.5, and 3 mg/ml membrane particles were prepared in triplicate. Each concentration of microsome was immobilized on Fractosil according to the above procedure and the optimum concentration for immobilization was estimated using an indirect assay method.

f) Effect of the Duration of the Immobilization Procedure

In order to determine the effect of the duration of the immobilization procedure on enzyme activity, two immobilized products were prepared as above. The incubation time for the first preparation was 24 hours, and for the second, preparation was 15 min. The activity of the immobilized enzyme was then estimated in a 4 hr continuous operation and compared.

g) Adsorption of Microsomes on Fractosil and Hexadecyl Fractosil

200 mg Fractosil – 1000 or hexadecyl Fractosil-1000 was washed twice, each time with 2 ml of 10 mM phosphate buffer, pH 7. Using 1 ml phosphate buffer, the adsorbent was then transferred to a jacketed glass column with an internal diameter of 0.7 cm. To the packed gel, 300µl of microsomal suspension containing 1.5 mg of membrane protein was added. A peristaltic pump with a downward flow rate of 0.15 ml/min was used to introduce the microsomes from the membrane suspension into the adsorbent layer. As soon as the first drop of membrane suspension reached the bottom of the column, the pump was disconnected and the column containing the adsorbent, and the microsomal suspension was incubated for 24 hrs at 4ºC. The column was then washed with 6 ml of 10 mM phosphate buffer (pH 7.0) prior to its use in the continuous catalytic operation.

h) Continuous Catalytic Operation with Immobilized Microsome

To the column containing the bed with immobilized microsomes, 500 µl of a 5 mg/ml solution of cytochrome *c* was added to saturate the adsorbent. After washing the excess cytochrome *c* from the bed, the assay mixture containing 13 ml of 10 mM phosphate buffer (pH 7.0), 3.25 ml of 5 mg/ml cytochrome *c*, 0.455 ml of 10 mM KCN, 195 µl of 2 mg/ml antimycin A, and 3.25 ml of 3 mg/ml NADH, were passed through the column at a flow rate of 9.5 ml hr^{-1} at 4ºC. One and a half milliliter fractions were collected and the absorbances were immediately read at 550 nm. The difference between the absorbances of the initial mixture and each fraction was used to evaluate the catalytic activity. Due to the instability of the assay mixture and changes in the oxidation-reduction properties of cytochrome *c* in the incubation mixture upon long-term use, the assay mixture had to be freshly prepared every 2 hrs, and immediately used in continuous catalytic operations. A stop of about 12 hrs in the operation during the night was also unavoidable.

Continuous catalytic operations were carried out at 25ºC for membranes immobilized on Fractosil and hexadecyl Fractosil and compared with the initial activity. A comparison was also made with the activity of the free membrane enzyme conserved under similar conditions.

3. RESULTS AND DISCUSSION

a) Effect of pH on the Activity of Enzyme in Unbound Microsomes

The effect of pH on the activity of NCCR in unbound microsomes is summarized in Fig. 1. As observed, the maximum specific activity of 28.7 nmole/min/mg protein was obtained at an optimum pH value around 7.0. There was no significant difference in the specific activity of the enzyme between pH values of 6.5-7.

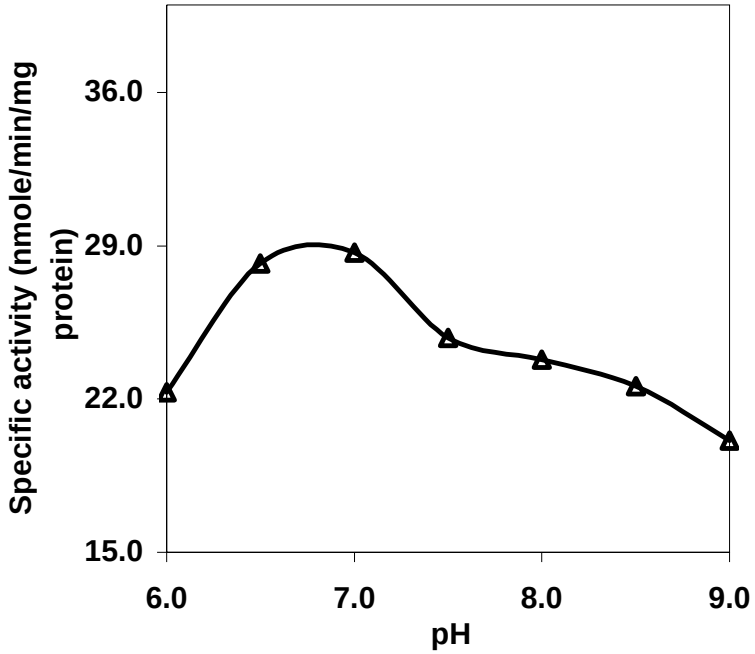

Fig. 1. Activity of antimycin A insensitive NADH-cytochrome *c* reductase of free microsomes in 20 mM MPTG buffer at different pH values

b) Effect of Microsome concentration on Immobilization

The extent of binding when using different concentrations of microsomal membranes with a constant amount of Fractosil in the form of suspension is shown in Fig. 2. As indicated, the highest proportion of membrane adsorbed was found to be at a concentration of 0.5 mg/ml of microsomal suspension per 50 mg of the matrix. There was a good agreement between the results of the enzyme assay and the protein measurements (Fig. 2). As observed, there was no constant ratio between the amount of the membrane adsorbed and the activity of the enzyme at higher membrane concentrations. This is probably due to the aggregation of microsomal particles resulting in the masking of their active sites.

c) Effect of Duration of Immobilization Procedure

Figure 3 presents the effect of two different durations of the immobilization procedure on the enzyme activity in a 4 hr continuous catalytic operation. The 24 hr duration had 84% activity after the 4 hr operation, compared with 61% for the system with a 15 min duration. It seems that a prolonged time of incubation has an important role in the homogeneous adsorption of the enzyme system by the matrix.

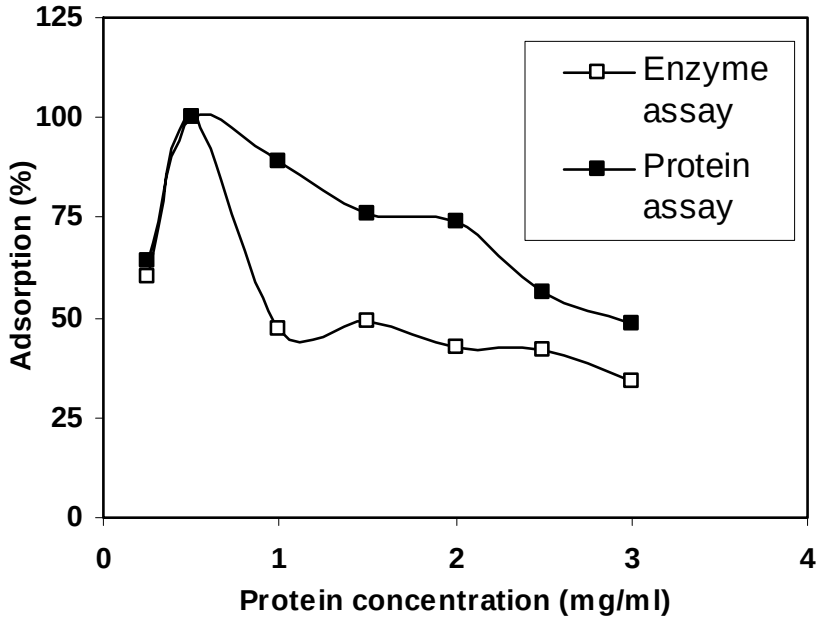

Fig. 2. Adsorption of microsomes on Fractosil as a function of microsome concentration, 50 mg of Fractosil was incubated with 300 μl of the sample, containing different concentrations of microsomes in 10 mM phosphate buffer, pH 7.0. After mixing by rotation for 2 hrs at 4 °C, the pellets were washed twice using 50 mM phosphate buffer and the optimum concentration for adsorption was estimated by the indirect assay

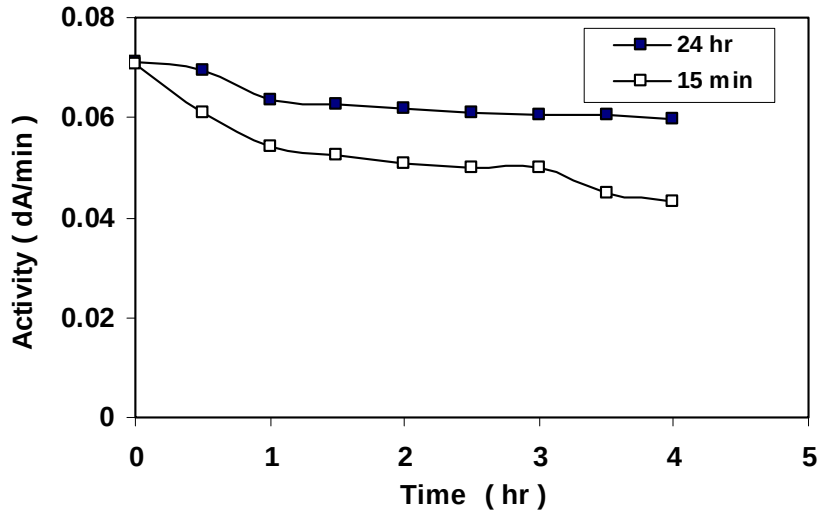

Fig. 3. Effect of duration of immobilization on the enzyme activity in a 4 hr continuous catalytic operation, Microsomes were immobilized for either 15 min or 24 hr on Fractosil as described in the text and then assayed for NCCR in a 4 hr continuous catalytic operation

d) Stability of Immobilized NCCR

The NCCR activity of microsomes immobilized on Fractosil and hexadecyl Fractosil was investigated following the continuous reduction of cytochrome *c* at 25ºC. Figure 4 shows the thermal stability of immobilized NCCR activity on Fractosil and the Fractosil lipid at 25ºC. The half life of the activity of NCCR immobilized on Fractosil at 25ºC was found to be 43 hrs and that of NCCR immobilized on the Fractosil lipid was 61 hrs. NCCR immobilized on Fractosil retained 33-40% of its activity during a 20-day storage at 4ºC, while the NCCR enzyme immobilized on hexadecyl Fractosil

stored at the same condition retained its catalytic activity to a much higher level. Dramatic inactivation was observed after several days of storage of the free form at 4ºC, due to the aggregation of the membranes. As these membranes aggregate quickly [13], it is suggested that their immobilization is a good and useful method to enhance their stability and use their catalytic potential in the long term. Results (not shown) also indicated that at 25ºC, the enzyme immobilized on Fractosil showed more initial activity, approximately 0.07 dA/min, compared to that immobilized on hexadecyl Fractosil (0.037). The longer half life of the immobilized enzyme on hexadecyl Fractosil, and the retention of much higher activity when stored at 4ºC for 20 days, are indications of a much higher stability of the immobilized enzyme on the derivatized adsorbent.

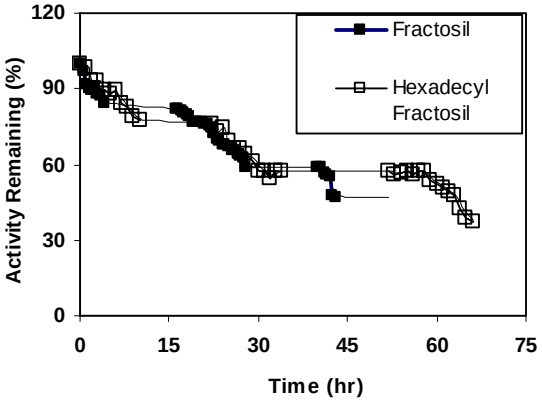

Fig. 4. Thermal stability of microsomal NCCR immobilized on Fractosil or hexadecyl Fractosil and used in continuous catalytic operation at 25°C. A total of 1.5 mg of membrane protein was applied to a column containing either 200 mg Fractosil or hexadecyl Fractosil. Percentage activity remaining was calculated based on using 0.07 dA/min as 100% activity for immobilized membranes on Fractosil and 0.037 for immobilized membranes on hexadecyl Fractosil

The higher half life and the higher storage stability of the immobilized enzyme on hexadecyl Fractosil is probably due to the entrance of alkyl groups of the derivatized Fractosil molecules into the lipid bilayer of microsomal membranes and their interaction with the hydrophobic components of the membrane. This will probably result in a higher adsorption of the membranes, and eventually, higher stability of the immobilized enzyme. Although the presence of such residues on the matrix may cause lower immobilization due to steric hindrance, they would induce more stability of the immobilized enzyme.

It is concluded that simple adsorption of microsomal membranes on Fractosil may provide a convenient method for their immobilization and their use in continuous catalytic transformations. The presence of alkyl residues on the inorganic support appears to introduce the possibility of additional non-ionic interactions, which may contribute toward stronger associations.

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