Preparation of Pyridoxal 5'-Phosphate-bound Sepharose and Its Use for Immobilization of Tryptophanase

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Pyridoxal 5'-phosphate-bound Sepharose (SP) was prepared by coupling pyridoxal 5'-phosphate (PLP) to diazotized p-aminobenzamidohexyl-Sepharose. A derivative of pyridoxine having an absorption maximum at ca. 316 nm (possibly, 6-amino-pyridoxine 5'-phosphate) was liberated from SP by treatment with 0.1 M sodium dithionite at pH 9.0. SP catalyzed the cleavage of tryptophan in the presence of Cu^{2+}, a typical non-enzymatic model of tryptophanase reaction. From the spectrophotometric data and catalytic activity, it was estimated that SP contained about 1.5 μmoles of bound PLP per gram of Sepharose. Tetrameric apotryptophanase was immobilized by incubation with SP, followed by reduction with $NaBH_4$. The resulting immobilized tryptophanase retained ca. 60 % of the catalytic activity of free tryptophanase used. This method was much superior to other methods used commonly for preparation of immobilized enzymes.

A water-insoluble derivative of enzyme in which the protein is bound to an insoluble carrier is of practical as well as theoretical interest. A number of papers have dealt with the preparation of insoluble enzyme derivatives (1). However, as for the immobilization of enzymes requiring coenzymes, only few reports have been published. On the other hand, affinity chromatography based on the interaction between an immobilized cofactor and apoprotein is now widely used for purification of various enzymes (2). For example, pyridoxamine 5'-phosphate bound to Sepharose through a side arm has been successfully used for the purification of tyrosine aminotransferase (3) and aspartate aminotransferase (4). However, this insolubilized coenzyme would be useful mainly for the purification of aminotransferases.

This paper describes the preparation of Sepharose-bound pyridoxal 5'-

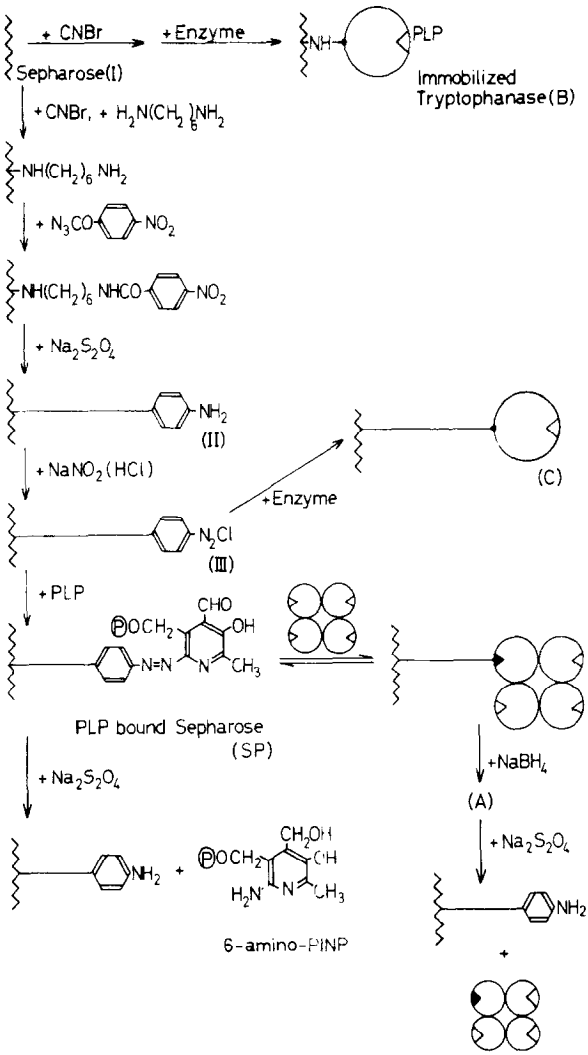
phosphate (SP) and its application to immobilization of tryptophanase. The insolubilized pyridoxal 5'-phosphate (PLP) retained functional groups for binding to apoproteins of B_6 enzymes. Hence, SP would be useful for both immobilization and affinity chromatography of various B_6 enzymes.

MATERIALS AND METHODS

Tryptophanase was prepared according to the method of Newton et al. (5) from E. coli B/1t 7-A. This strain was kindly gifted from Dr. H. Wada and Dr. Y. Morino, Osaka University School of Medicine. The specific activity of the enzyme used was 4-10 enzyme units/mg-protein. Apo- and holo-tryptophanase were prepared by the procedure of Morino and Snell (6). The activity of tryptophanase was assayed as described by Newton et al. (5). In the case of immobilized enzyme, the enzymatic reaction was carried out under gentle agitation in a glass funnel equipped with a sintered glass-filter. After the reaction was over, the reaction mixture was filtered and the amount of indole in the filtrate was determined. The specific activity of free and immobilized tryptophanase were compared using the amount corresponding to an equal amount of enzyme protein (1.0 μg). Protein concentrations were determined by the method of Lowry et al. (7). The amount of enzyme bound to Sepharose was determined by the difference between the initial amount of enzyme and that of free enzyme remaining in the supernatant after the immobilization process.

Preparation of pyridoxal 5'-phosphate-bound Sepharose (SP)

p-Aminobenzamidohexyl-Sepharose (Scheme 1, II) was prepared from Sepharose 4B as described by Cuatrecasas (8). This Sepharose derivative was diazotized by treatment with sodium nitrite (0.1 M) in 0.5 N HCl for 7 min. at 4° C. The resulting diazonium salt of Sepharose derivative (Scheme 1, III) (10 ml) was added to 10 ml of 0.2 M borate buffer (pH 8.0) containing 40 mg PLP. The coupling reaction was allowed to proceed for about 8 hrs. at 4° C. The PLP-bound Sepharose thus obtained was throughly washed with water and then heated up to ca. 60° C for 30 min. to destroy unreacted diazonium derivative.



Scheme I

Immobilization of tryptophanase using PLP-bound Sepharose (SP)

Immobilization of apotryptophanase on SP was carried out as follows: wet SP (0.5 g) was mixed with 1.0 ml of 0.1 M K-phosphate buffer (pH 7.0) containing 0.50 mg of apotryptophanase. The mixture was incubated for 20 min. at 37° C. The resulting SP-apotryptophanase complex was reduced with $NaBH_4$ and then washed throughly with an eluting buffer (Buffer A) consisting of 0.1 M K-phosphate buffer (pH 6.0), 5 mM PLP and 11.4 % ammonium sulphate (Scheme 1, (A)).

Immobilization of tryptophanase on CNBr-activated Sepharose

Tryptophanase was immobilized on Sepharose 4B in a similar way to that described by Axen and Ernbach (9): activated Sepharose 4B (0.5 g) by treatment with CNBr was mixed with 0.20 mg of apo- or holotryptophanase dissolved in 1.0 ml of 0.1 M K-phosphate buffer (pH 7.0). In a glass funnel equipped with a sintered glass-filter, the mixture was gently agitated for 24 hrs. at 4° C. The resulting insolubilized tryptophanase was throughly washed with 0.1 M K-phosphate buffer (pH 7.0) (Scheme 1, (B)).

Immobilization of tryptophanase using diazotized p-aminobenzamidohexyl derivative of Sepharose

Diazotized p-aminobenzamidohexyl-Sepharose (0.5 g) was mixed with 1.0 ml of 0.1 M K-phosphate buffer (pH 8.0) containing 1.0 mg of apo- or holotryptophanase. The coupling reaction was allowed to proceed for 15 min. at 4° C. The immobilized tryptophanase thus obtained was throughly washed with 0.1 M K-phosphate buffer (pH 8.0) (Scheme 1, (C)).

Table I Model reaction of tryptophan cleavage catalyzed by Sepharose-bound pyridoxal 5'-phosphate (SP)

Expt. No.	Pyridoxal derivative used		Indole formed (μmole)
1	Pyridoxal	(0.30 μmole/ml)	0.053
2	SP	(20 mg/ml)	0.011
3	SP	(200 mg/ml)	0.098
4	——	——	0.000

The reaction mixture contained 0.15 M formate buffer (pH 4.0), $CuSO_4$ (20 mM), tryptophan (12.5 μM) and pyridoxal derivative cited in the table. The reaction mixture was heated for 50 min. at 15 lbs. The indole formed was determined colorimetrically using p-dimethylaminobenzaldehyde as reagent.

RESULTS AND DISCUSSION

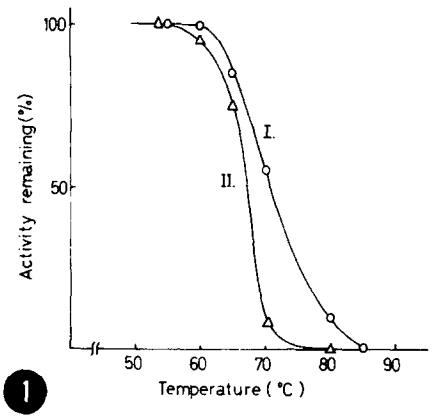
Non-enzymatic cleavage of tryptophan by the use of Sepharose-bound pyridoxal 5'-phosphate (SP)

Pyridoxal (PL) or its appropriate analogs are well known to catalyze non-enzymatic cleavage of tryptophan in the presence of Cu^{2+} (10). To confirm

whether SP possesses the functional groups necessary for catalysis, the catalytic activity in the model system was examined. As shown in Table I, SP catalyzed the cleavage of tryptophan similarly to PL. The catalytic activity of SP used in this experiment corresponded to ca. 1.5-3.0 μmoles of PLP per gram of Sepharose. Treatment of SP (250 mg) with 0.1 M sodium dithionite (2 ml) in 0.2 M sodium borate (pH 9.0) resulted in liberation of a pyridoxine-like substance having an absorption maximum at about 316 nm at pH 9.0 and at 282 nm at pH 3.0, respectively. Katritsky et al. (11) synthesized 6-amino-pyridoxine by reduction with sodium dithionite of 6-phenylazo-pyridoxine which was prepared by coupling diazotized aniline to pyridoxine. These facts strongly suggest that 6-amino-pyridoxine 5'-phosphate (6-amino-PINP) or its closely related compound was released from SP by the dithionite treatment. From the optical density of this pyridoxine derivative liberated, it was estimated that SP contained about 1.5 μmoles of PLP per gram of Sepharose, consistent with the above-mentioned catalytic activity in the model tryptophanase reaction.

Comparison of enzymatic activities of immobilized tryptophanase prepared by different methods

Table II shows the enzymatic activities of immobilized tryptophanase prepared by three different methods, (A), (B) and (C) in Scheme 1. Of the methods employed, the immobilization of apotryptophanase on SP was most effective. The efficiency of immobilization was about 80 % and the catalytic activity of the resulting insoluble enzyme was ca. 60 % of that of free tryptophanase when compared using the same protein concentration. The SP-apoenzyme complex, before reduced with $NaBH_4$, lost ca. 90 % of the activity when treated with Buffer A. Although the coenzyme moiety of the immobilized enzyme prepared by Method A was not released by the elution treatment, it lost about 80 % of the initial activity by treatment with 0.1 M sodium dithionite at pH 9.0. These results indicate that the apoenzyme is bound to SP through its PLP moiety. Since holotryptophanase was scarcely immobilized on SP, the interaction between apotryptophanase and SP appears to occur at the active site of one subunit of tetrameric apotryptophanase.



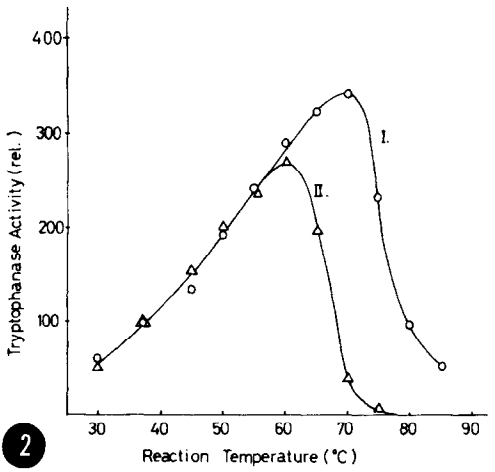


Fig. 1 Heat stability of the immobilized tryptophanase prepared by Method A. Activity was measured after heated for 10 min. at the indicated temperature in 0.1 M K-phosphate buffer (pH 7.0) containing 5 mM mercaptoethanol. Curve I, immobilized enzyme; Curve II, free enzyme. The protein concentration was 10 μg/ml.

Fig. 2 Temperature effect on the reaction rate catalyzed by the immobilized tryptophanase prepared by Method A. Reaction mixture was incubated for 10 min. at the indicated temperature and the amount of indole formed was determined. The activity was expressed as a relative value to the activity of immobilized or free tryptophanase at 37°C. Curve I, immobilized enzyme; Curve II, free enzyme.

Table II Comparison of enzyme activities of immobilized tryptophanase prepared by different methods

Expt. No.	Method	Sepharose deriv. used	(mg)	Tryptophanase used for immobilization (mg)	Efficiency of immobilization (%)	Relative activity (%)*
1	A	SP	500	0.50 (apo)	81	60
2	B	I	500	0.20 (apo)	73	34
3	B	I	500	0.20 (holo)	68	42
4	C	III	500	1.00 (apo)	45	3
5	C	III	500	1.00 (holo)	42	10

* Activity was measured in the presence of added pyridoxal 5'-phosphate and was expressed as relative value to the activity of free enzyme.

The activities of immobilized tryptophanase prepared using CNBr-activated Sepharose (Scheme 1, Method B) and diazotized p-aminobenzamidohexyl-Sepharose (Method C) are lower than that prepared by Method A (see, Table II).

Some properties of the immobilized tryptophanase prepared by Method A

The optimal pH range of the immobilized tryptophanase prepared by Method A was pH 8 to 10, somewhat shifted to an alkaline side as compared with free tryptophanase. Figures 1 and 2 show the heat stability and the temperature effect on the reaction rate of immobilized enzyme, respectively. These results indicate that the immobilized tryptophanase is appreciably stable and much useful at a high temperature (e. g., above 60°C), in comparison with free tryptophanase. The reverse reaction yielding tryptophan from indole, pyruvate and ammonia (12) was also catalyzed by the immobilized enzyme. The activities of the enzyme preparations obtained by the above-mentioned three methods were kept unchanged for at least 20 days at 4°C in a dry state. A more detailed study on the properties and more extensive application of the immobilized enzyme will be described in a further publication.

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