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IMMOBILIZATION OF PROTOCATECHUATE 3,4-DIOXYGENASE WITH ACTIVATED AGAROSE

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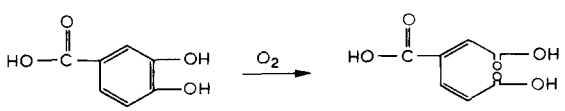
SUMMARY

Protocatechuate 3,4-dioxygenase (protocatechuate:O_2 3,4-oxidoreductase, EC 1.13.1.3) was immobilized with CNBr-activated agarose (Sepharose 6B). An examination of the immobilized and soluble enzymes revealed that the water-insoluble derivative (1) possesses good activity (42 and 68%); (2) exhibits a pH–activity profile shift of approx. 0.5 pH units toward more acid pH; (3) shows a slight increase in thermal stability; and (4) shows a substantially enhanced stability toward high concentrations of urea relative to the native protein. The immobilization of protocatechuate 3,4-dioxygenase with Sepharose represents the first reported example of the preparation of a water-insoluble derivative of a dioxygenase.

INTRODUCTION

Protocatechuate 3,4-dioxygenase (protocatechuate:O_2 3,4-oxidoreductase, EC 1.13.1.3) is a non-heme trivalent iron-containing enzyme that catalyzes the transformation of protocatechuic acid to β-carboxy-*cis*, *cis*-muconic acid. The enzyme has been obtained from several sources, but the particular enzyme isolated from *Pseudomonas aeruginosa* has been purified, crystallized, and characterized most extensively[1–5].



The enzyme has an estimated molecular weight of 700 000, consists of approx. 8 subunits each containing a mole of trivalent iron, and is relatively stable under normal conditions. It also forms, anaerobically, an easily detectable enzyme–substrate complex[2] and, aerobically, a transient ternary complex composed of enzyme, substrate, and oxygen[5]. The latter complex, observed through stopped-flow spectrophotometry, has been reported to be an obligatory intermediate in the transformation of substrate

to product and was the first example of such an enzymatically active ternary oxygen complex observed in a non-heme iron-containing oxygenase.

These characteristics of the enzyme and the relative ease of obtaining large quantities of it prompted us also to investigate this particular enzyme as a suitable model enzyme for oxygenase reactions. One of our aims was to successfully immobilize this enzyme on a water-insoluble support in order to investigate the characteristics of such a dioxygenase system. To date, there has been no report of a successful or even attempted immobilization of a specific member of this class of enzymes.

Our primary choice of support for immobilizing protocatechuate 3,4-dioxygenase was CNBr-activated agarose (Sepharose). General considerations which influenced this choice were: (1) Sepharose is a relatively neutral carrier even after CNBr activation, (2) conditions for successful activation of polysaccharides with CNBr and covalent coupling of enzymes have been adequately described[6–13], (3) the procedure is simple, and (4) relatively high protein loading and good enzymatic activity of the derivatives can usually be achieved. Another reason for selecting Sepharose was the substantial molecular weight (size) of the octameric protocatechuate 3,4-dioxygenase. Sepharose, not being crosslinked, has a relatively porous structure and thus allows entry of large molecules such as protocatechuate 3,4-dioxygenase. In addition, there is a considerable operational advantage gained by using a well described carrier for a not-too-well described enzyme. Even the amino acid residues essential for catalysis in protocatechuate 3,4-dioxygenase have to our knowledge not yet been determined.

This paper describes the immobilization of protocatechuate 3,4-dioxygenase on Sepharose 6B and a comparison of some of the properties of the derivatized enzyme with the soluble unmodified protein. We also achieved immobilization on other water-insoluble supports, *e.g.* Enzacryl AH (ref. 14), but no extensive characterization of these derivatives was undertaken.

MATERIALS AND METHODS

Protocatechuate 3,4-dioxygenase was isolated and crystallized from *p*-hydroxybenzoic acid-grown *Pseudomonas aeruginosa* cells. The specific activity of the crystalline fraction was approx. 62 units/mg protein, and the enzyme had identical spectral properties as those previously reported[1,2,5]. Reagents employed for immobilization and further characterization were of commercial grade and these were used without further purification. Urea and guanidine·HCl were Schwarz-Mann grade. Sepharose 6B was purchased from Pharmacia Fine Chemicals.

Immobilization of protocatechuate 3,4-dioxygenase

Immobilization of the enzyme on Sepharose 6B was accomplished by the CNBr-activation procedure described by Axen and co-workers[6,7].

Preparation I. To approx. 8.3 g moist Sepharose 6B (previously washed with water to remove any bacteriostatic agent and corresponding to about 1 g dry agarose) suspended in 50 ml water at room temperature was added 100 ml CNBr solution (2.5 g per 100 ml water). *Operations involving CNBr were done in a well-ventilated hood.* The suspension was stirred magnetically, the pH was adjusted to 11.0 with 5 M NaOH, and base was added until the pH remained constant. The activated Se-

pharose was transferred to a Millipore filtration unit (47 mm, 0.45 μm HAWP filter) and washed quickly with 800–1000 ml cold 50 mM phosphate buffer (pH 8.5).

The moist Sepharose was transferred quickly to a 100-ml beaker containing 20 ml of the same phosphate buffer and then 4.5 ml of a brightly red-colored protocatechuate 3,4-dioxygenase solution (10.2 mg protein/ml 50 mM phosphate buffer, pH 8.0) was added. The pink suspension was stirred at 5 °C for 75 h.

The cold suspension was filtered once again with a Millipore filtration unit (47 mm, 0.45 μm HAWP filter), and the material was washed with 50 ml additional cold phosphate buffer. Some enzymatic activity was detected in the original filtrate. The pink-colored Sepharose was quickly transferred to a small column with more cold buffer and was then further washed at 5 °C with phosphate buffer using a precision metering pump at 30 ml/h; total wash volume was 650 ml. No enzymatic activity was detected in the wash liquid at the end of this washing period. The phosphate buffer of the immobilized enzyme preparation was exchanged for 50 mM Tris–HCl buffer (pH 8.4) and washing was continued until 700 ml of eluant was collected.

In order to assure that no physically adsorbed enzyme was still present, a portion of the immobilized protocatechuate 3,4-dioxygenase was washed with 500 ml 10 mM protocatechuic acid solution (50 mM Tris–HCl buffer, pH 8.4) at 5 °C in a column using a precision metering pump set at a rate of 30 ml/h. No change in color intensity or activity (after appropriate wash with 50 mM Tris–HCl buffer, pH 8.4) was observed in the immobilized enzyme conjugate; also, no activity was detected in the substrate wash solution. In addition, the enzyme–Sepharose conjugate was washed with 350 ml 1 M NaCl solution (50 mM Tris–HCl buffer, pH 8.5) at a rate of 20 ml/h. No change in activity was observed with the immobilized enzyme, and the wash solution again exhibited no enzymic activity. A small sample of the enzyme–Sepharose conjugate was also stirred in a 5 M NaCl solution (50 mM Tris–HCl buffer, pH 8.5) for 24 h. Even after this treatment, no enzymic activity was detected in the wash solution.

The Sepharose-bound protocatechuate 3,4-dioxygenase was normally stored at 5 °C in 50 mM phosphate or Tris–HCl buffers (pH 8.3–8.5).

Preparation II. Immobilization was achieved by the same procedure described above except that the following different conditions were used: 500 mg dry Sepharose, 0.52 g CNBr, 50 mM phosphate buffer (pH 8.2) as the coupling buffer, 5 ml protocatechuate 3,4-dioxygenase solution (10.2 mg protein/ml 50 mM phosphate buffer, pH 8.0), and an 18 h reaction time. The immobilized preparation was washed in a column with 500 ml 50 mM phosphate buffer (pH 8.2) at 30 ml/h. No enzymatic activity was detected in the wash solution.

Immobilization of protocatechuate 3,4-dioxygenase was also achieved on Enzacryl AH *via* the azide derivative. An active insolubilized preparation was obtained. It was not further characterized because it was more difficult to handle than the Sepharose-bound enzyme conjugates.

Protein determinations

The protein content of the protocatechuate 3,4-dioxygenase–Sepharose enzyme conjugates was determined by amino acid analysis (after appropriate 6 M HCl hydrolysis at 110 °C for 20 h, evacuated sample), using a modification of the gas–liquid chromatographic procedure described by Gehrke and co-workers[15–17]. No interference

was encountered in the preparation or gas–liquid chromatographic analysis of the *N*-trifluoroacetyl *n*-butyl ester derivatives of amino acids of interest (alanine, valine or glutamic acid). Normally, the protein content of the immobilized enzyme was determined from the amount of alanine experimentally found and by assuming that the molecular weight of the enzyme is 700 000 and that there are 477 alanine residues per molecule[1].

Soluble protein was determined spectrophotometrically at 280 nm using the reported extinction coefficient, $E^{1\%}_{280\,nm} = 13.2$, in 50 mM Tris-HCl buffer (pH 8.5)[1,2].

Assay of soluble and immobilized protocatechuate 3,4-dioxygenase

The spectrophotometric assay procedure of Fujisawa and Hayaishi[1] and Fujisawa[2] was used for both soluble and immobilized enzyme derivatives employing either a Beckman Kintrac VII A or Acta V spectrophotometer. These instruments are equipped with a built-in magnetic stirring system (Teflon-coated stirring bar inside the cuvette) and therefore are quite suitable for monitoring the rate of the reaction of water-insoluble enzyme derivatives, provided, of course, that the spectral interference due to the support is tolerable. The typical assay entailed adding 5–50 μl of enzyme solution or suspension to a previously equilibrated solution (at 25 °C) of 2.90 ml 50 mM Tris–HCl buffer (pH 7.5) and 0.10 ml 12 mM protocatechuic acid (in water or 50 mM Tris buffer, pH 7.5) and monitoring the absorbance decrease at 290 nm with time. Usual enzyme concentrations were 0.5–2 μg/3 ml; the substrate concentration was $4 \cdot 10^{-4}$ M. One unit of enzyme activity was defined as the amount of enzyme that oxidizes one μmole of protocatechuic acid per min at 25 °C under the above specified conditions. A stirring speed well above the minimum required for obtaining constant specific activities of the immobilized enzyme conjugates was used throughout these studies.

Thermal inactivation of soluble and immobilized protocatechuate 3,4-dioxygenase

The following procedure was employed for determining the thermal inactivation of soluble and Sepharose-bound protocatechuate 3,4-dioxygenase. A 1–2-ml sample of either soluble (containing approx. 0.10 mg protein/ml solution) or immobilized (containing approx. 10 mg immobilized enzyme conjugate/ml suspension) enzyme in 50 mM Tris–HCl buffer (pH 8.5) was placed in a small test tube having a sealable standard ground-glass joint. The closed test tube containing the suspension or solution was then placed into a constant-temperature water bath of the desired temperature and samples were removed periodically and tested for enzymatic activity. Before removing a sample of immobilized enzyme, the suspension was vigorously shaken with a Vortex mixer to ensure homogeneity of the sample. Duplicate assays were performed at each time interval. The relative activities reported were determined using the time at zero (no heating) as 100% activity for both the soluble and immobilized enzymes. All assays were conducted at 25 °C.

pH–activity profile determination

The pH–activity profiles of soluble and immobilized protocatechuate 3,4-dioxygenase were determined spectrophotometrically at either 290 or 272 nm using the appropriate and experimentally determined extinction coefficients for substrate and product at the specific pH. Relative activities were calculated by comparing the

specific activity at a particular pH with the maximum specific activity obtained (pH approx. 8.4 and 7.9 for soluble and Sepharose-bound enzymes, respectively). Buffers used were 50 mM in acetate (pH 4.97 and 6.01), Tris–HCl (pH 7.10, 7.53, 8.21 and 8.41) or glycinate (pH 9.15 and 10.10).

Determination of activity in urea and guanidine solutions

The effect of the two denaturants on enzymatic activity was determined by the standard spectrophotometric assay at pH 7.5. An appropriate amount of 6 M urea or guanidine·HCl solution, pH 7.5 (previously adjusted to that value with NaOH) was added to 0.10 ml 12 mM substrate and sufficient 50 mM Tris–HCl buffer (pH 7.5) to give a total volume of 3.0 ml. After equilibration, the soluble or immobilized enzyme was added. Activities are relative to the specific activity obtained in the absence of urea or guanidine.

TABLE I

IMMOBILIZATION OF PROTOCATECHUATE 3,4-DIOXYGENASE ON SEPHAROSE 6B, COUPLING CONDITIONS AND RESULTS

Prepn I and II refer to the protocatechuate 3,4-dioxygenase–Sepharose preparations (see text).

	Prepn	
	I	*II*
Coupling pH	8.5	8.2
Coupling time (h)	75	18
mg CNBr/mg "dry" Sepharose used in coupling step	2.5/1	1/1
mg protein/mg immobilized enzyme conjugate*	$2.8 \cdot 10^{-2}$	$1.7 \cdot 10^{-2}$
Spec. act. (units/mg protein)**	26	42
Relative activity (%)***	42	68

* Average values of at least two independent determinations.
** Spec. act. of soluble unmodified enzyme was 62 units/mg protein.
*** Compared to soluble enzyme.

RESULTS AND DISCUSSION

Table I lists the conditions used for coupling protocatechuate 3,4-dioxygenase to CNBr-activated Sepharose. It also gives the protein binding and the specific as well as the relative activities obtained in the two preparations. Immobilization of protocatechuate 3,4-dioxygenase by this procedure was simple and gave pink-colored Sepharose–enzyme conjugates of relatively high activity. The relative activities of 42 and 68% obtained were quite good considering the complexities of the enzyme system and the fact that no optimization of the factors affecting activation of the Sepharose or coupling of the protein was pursued. Protein binding was 28 and 17 mg protein/g of immobilized enzyme conjugate for Preparations I and II, respectively. Although higher binding capacities have been reported for enzymes immobilized on agarose[6], the degree of binding obtained with protocatechuate 3,4-dioxygenase is reasonable considering the substantial size of the molecule. The differences in protein binding and specific activities observed between Preparations I

and II were likewise anticipated from the experimental conditions used and from previous reported data on other immobilized enzymes[6–13]. As expected, greater coupling of protein to activated Sepharose or other polysaccharides is favored at (1) higher coupling pH values (2) longer reaction times, and (3) for more highly activated agarose. However, as protein coupling increases, the relative activity of an immobilized enzyme can be lowered. Similar results have been obtained with other Sepharose-immobilized enzymes[6]. The mode of binding of protocatechuate 3,4-dioxygenase to the activated Sepharose is assumed to occur through covalent bond formation between primary amino groups of the enzyme and the proposed cyclic imidocarbonate groups of activated Sepharose[6]. Sepharose and the CNBr-activated material are essentially neutral carriers, and little, if any, immobilization should occur by physical adsorption. Although it has not yet been established firmly that immobilization of an enzyme with activated agarose indeed involves covalent bond formation in the above-mentioned manner, this mode of binding is strongly suggested from studies of model compounds[6]. Furthermore, if physical adsorption were responsible for the immobilization of protocatechuate 3,4-dioxygenase, the washing procedure employed by us would have eluted any adsorbed enzyme from the support. Extensive washing of the immobilized enzyme with buffers, substrate solution, and solutions of high salt concentrations did not eluate the bound enzyme. Some initial elution of unbound, presumably non-covalently bonded, protocatechuate 3,4-dioxygenase was, however, observed before the extensive washings with substrate and salt solutions were conducted.

The activity of the immobilized enzymes was easily measured using the standard assay with the Beckman spectrophotometers. A sufficient stirring speed was employed to ensure good agitation. No apparent deviation from linearity of absorbance change per time (initial) was observed under normal conditions, and no problems with reproducibility were encountered. It is not known, of course, whether the observed activity of the immobilized enzyme is due to monomeric, dimeric, *etc.* or the native octomeric complex. High CNBr activation of the Sepharose was desired in this instance in order to ensure good binding of the enzyme. Thus in both Preparations I and II, multiple-site covalent bonding of enzyme to activated Sepharose is highly likely. To determine the possibility that the enzymatic activity of the immobilized enzyme is due to monomeric units, lower CNBr activation of the Sepharose would be desired in addition to knowing the exact conditions for subunit dissociation. At present, these conditions have not been determined by us nor have they been reported elsewhere.

Fig. 1 gives the pH–relative activity behavior of soluble and immobilized protocatechuate 3,4-dioxygenase determined by the spectrophotometric assay. The pH optimum of the soluble enzyme (approx. 8.3–8.4) compares quite favorably with that reported earlier (approx. 8.1–8.2) determined by an oxygen-electrode method[1]. As can be seen, a comparison of the pH–activity profile of the soluble with the immobilized enzyme reveals, however, that these two enzymes differ slightly in both the pH optimum and general shape of the curve around that optimum. The pH–activity profile of the Sepharose-immobilized protocatechuate 3,4-dioxygenase is broader at the optimal activity range and the optimum is slightly displaced (approx. 0.5 units) toward the acidic pH scale. The precise reason for this apparent pH shift toward the acidic side is difficult to give at the moment. It could be due to chemical modification

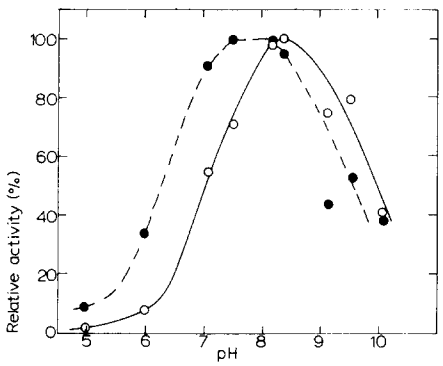


Fig. 1. pH–activity profiles of soluble and immobilized protocatechuate 3,4-dioxygenase. Activities of soluble (○—○) and immobilized (●—●) enzymes are relative to the maximum activities exhibited by the two enzymes. The pH optimum of the soluble and immobilized enzyme is approx. 8.4 and 7.9, respectively. Points presented on graph are average values from 2 to 10 assays.

of the enzyme *via* one or more of its subunits, the electrostatic character of the functionalized polymeric support, or to an enzymatically generated microenvironment caused by substrate to product conversion. Although there are no definitive data to favor or eliminate unequivocally any of these possibilities at the present time, the last possibility mentioned seems unlikely in the light of many well-characterized immobilized enzymes[6,7,9]. Because a relatively strong acid is produced in the oxidative cleavage of protocatechuic acid to β-carboxy-*cis*, *cis*-muconic acid, it is expected that an enzymatically generated pH-gradient would be maintained if diffusion of this product from the immobilized enzyme into the external phase of the solution is slow (relative to the enzymatic reaction). The pH of the internal phase containing the immobilized enzyme would be lower and consequently the pH–activity behavior profile would be shifted toward a more alkaline pH. This is exactly what was observed with Sepharose-immobilized α-chymotrypsin (EC 3.4.4.5)[6] and trypsin (EC 3.4.4.4)[6] acting on *N*-acetyl-L-tyrosine ethyl ester and *N*-tosyl-L-arginine methyl ester, respectively. These reactions are characterized by high activity causing a very high rate of production of H^+ in the gel and shifting the pH–activity profiles of these immobilized enzymes by about 2 units toward more alkaline optima. On the other hand, Sepharose-immobilized papain (EC 3.4.4.10)[6] acting on *N*-benzoyl-L-arginine ethyl ester (a reaction characterized by low activity) gave no significant profile displacement toward alkaline pH. Consequently, if this factor were important in our case then we would expect a similar pH–activity displacement toward more alkaline pH or, at the very least, no displacement at all.

Preliminary determination of the apparent K_m of the protocatechuate 3,4-dioxygenase–Sepharose conjugate indicates that it is 15–20 times greater than the value of the soluble enzyme. Such increases in apparent K_m values of immobilized enzymes most often have been attributed to diffusion limitations.

The thermal inactivation of soluble and immobilized protocatechuate 3,4-dioxygenase was determined at 65, 60, and 55 °C. The results obtained at 60 °C are given in Fig. 2. At 65 °C, both the soluble and immobilized enzymes lose all activity after incubation for 15 min. At 60 °C, a comparison of relative activities with incubation time reveals that the immobilized enzyme has slightly enhanced thermal sta-

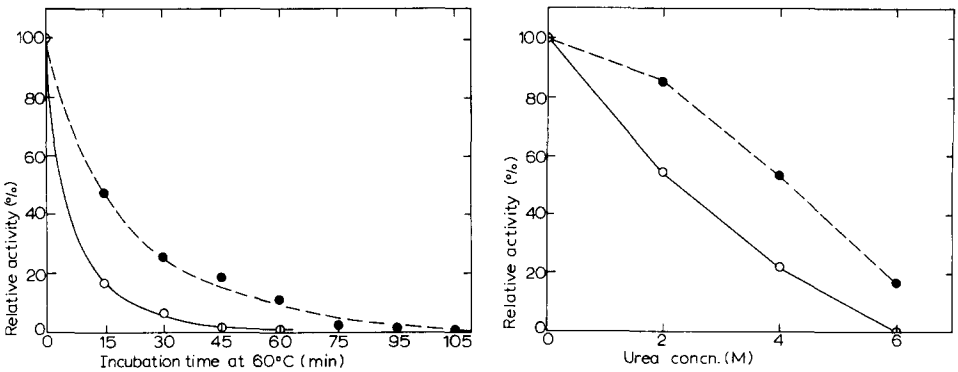


Fig. 2. Thermal inactivation of soluble and immobilized protocatechuate 3,4-dioxygenase at 60 °C. Relative activities of soluble (○—○) and immobilized (●—●) enzymes were determined at 25 °C employing the standard assay. Specific activity at time zero (no heating) was taken as 100% relative activity. Points presented on graph are average values of three independent studies.

Fig. 3. Effect of urea on activity of soluble and immobilized protocatechuate 3,4-dioxygenase. Activities of soluble (○—○) and immobilized (●—●) enzymes were determined in solutions of varying urea concentration (pH 7.5). Specific activity at zero urea concentration (only 50 mM Tris–HCl buffer, pH 7.5) was taken as 100% relative activity. Points presented on graph are average values of two independent studies.

bility. Although this thermal stabilization is not striking, it appears to be real. Inactivation of both soluble and immobilized enzymes is considerably slower at 55 °C (data not shown), but again a comparison of relative activities revealed no dramatic difference between the two. At the present time one cannot yet *a priori* predict the degree or outcome of the thermal stability of a particular immobilized enzyme. Enzymes immobilized through CNBr-activated Sepharose or Sephadex most often have essentially unchanged thermal stabilities in comparison with the soluble enzymes[9,11]. However, there are exceptions[8].

The storage stability of the immobilized enzyme also seems to be quite similar to its soluble counterpart. Although no long-term storage stability data are presently available, it will be interesting to see what these will be. The soluble enzyme has been reported to polymerize depending on the state of the sulfhydryl groups in the enzyme molecule.

Finally, the effect of high concentrations of urea and guanidine on the activity of soluble and immobilized protocatechuate 3,4-dioxygenase was examined in order to assess the relative stabilities of the enzymes in these two denaturants. Both soluble and immobilized enzymes have no activity in 2 M guanidine solutions (pH 7.5). However, both enzymes exhibit substantial activity in 2 M urea. Fig. 3 presents the relative activities of both enzymes in varying concentrations of urea and shows that the immobilized enzyme has substantially greater stability.

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