Preparation and Properties of Water-insoluble Derivatives of Urease*

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Water-insoluble enzyme derivatives possessing specific catalytic activity may provide a useful tool in biochemical studies, since they can readily be removed from the reaction mixture and can be used for the preparation of columns with enzymatic activity. The conversion of several enzymes into the corresponding water-insoluble derivatives by their binding via covalent bonds to insoluble carriers has been recently reported. The coupling of papain and carboxypeptidase with a diazotized poly-p-aminostyrene was described by Grubhofer and Schleith (1). A low enzymatic activity was recorded for the water-insoluble enzymes obtained. The synthesis of azobenzyl cellulose and carboxymethyl cellulose derivatives of trypsin and chymotrypsin was reported by Mits and Summaria (2). Carboxymethyl cellulose azide was used in the synthesis of the carboxymethyl cellulose derivatives. The water-insoluble enzyme preparations were found to be more stable than the corresponding water-soluble enzymes. Epstein and Anfinsen (3) used a preparation of trypsin bound to carboxymethyl cellulose in their experiments on the reversible reduction of the disulfide bonds of this enzyme. Manecke (4) reported the synthesis of enzyme resins by allowing carbosymethyl cellulose derivatives of trypsin and chymotrypsin to react with a nitroso derivative of p-aminophenylalanine and leucine as insoluble carrier (5, 6). The coupling of pepsin and carboxypeptidase with a diazotized poly-p-aminostyrene was described by Grubhofer and Schleith (1). A low enzymatic activity was recorded for the water-insoluble enzymes obtained.

The synthesis of azobenzyl cellulose and carboxymethyl cellulose derivatives of trypsin and chymotrypsin was reported by Mits and Summaria (2). Carboxymethyl cellulose azide was used in the synthesis of the carboxymethyl cellulose derivatives. The water-insoluble enzyme preparations were found to be more stable than the corresponding water-soluble enzymes. Epstein and Anfinsen (3) used a preparation of trypsin bound to carboxymethyl cellulose in their experiments on the reversible reduction of the disulfide bonds of this enzyme. Manecke (4) reported the synthesis of enzyme resins by allowing carbosymethyl cellulose derivatives of trypsin and chymotrypsin to react with a diazotized copolymer of p-aminophenylaniline and leucine as insoluble carrier (5, 6).

The water-insoluble papain derivative, possessing high proteolytic activity, was obtained by direct coupling of papain with the diazotized copolymer (7). It was used in the partial digestion of γ-globulin, antibodies, and other proteins (7, 8). The water-insoluble trypsin (9, 10) was obtained by coupling polytyrosyl trypsin (11) with the diazotized copolymer of leucine and p-aminophenylanilamine. It was more stable than native trypsin in the alkaline pH region, and was used in the preparation of a trypsin column with high and enduring trypsic activity.

In this paper the preparation, properties, and some applications of water-insoluble urease derivatives are described. The most active and stable water-insoluble urease derivatives were obtained by binding urease, previously treated with p-chloromercuribenzoate, with the diazotized copolymer of p-aminomethylphenylanilamine and L-leucine. A urease column was prepared and used in the assay of urea in body fluids as well as for the quantitative removal of urea from aqueous solution.

In 1925, Sumner and Graham (12, 13) obtained an enzymatically active water-insoluble urease preparation on adding small amounts of sodium chloride to neutral 30% alcohol urease and allowing the solution to stand in the cold for 1 or 2 days. The water-insoluble product, which most likely consists of intermolecularly disulfide cross-linked urease molecules, was only partially characterized, and its possible use as a heterogeneous catalyst was not investigated.

EXPERIMENTAL PROCEDURE

Materials

Urease with a specific activity of 4150 Sumner units (12) per g was obtained from Sigma Chemical Company, Lot 72B-704-1. This urease sample was used in the preparation of the water-insoluble urease derivatives described below because of the greater stability of crude urease preparations as compared with that of pure urease (14, 15) and the considerable difficulty encountered in the preparation of highly purified crystalline urease (16, 17).

Copolymer of p-aminomethylphenylanilamine and L-Leucine of Molar Residue Ratio 1:2.5 (I)—This compound was prepared according to the procedure of Bar Eli and Katchalski (10).

Copolymer of p-Amino-DL-phenylanilamine and Glycine of Molar Residue Ratio 1:1 (II)—This copolymer was prepared from p-N-carboxybenzoylamino-α-N-carboxy-DL-phenylanilic anhydride (18) and N-carboxyglycine anhydride (19) by a procedure similar to that used for the preparation of Copolymer I.

Copolymer of p-Amino-DL-phenylanilamine and L-Alanine of Molar Residue Ratio 1:1 (III)—This compound was prepared from p-N-carboxybenzoxoamino-α-N-carboxy-DL-phenylanilic anhydride and N-carboxy-L-alanine anhydride (20) by a procedure similar to that used for the preparation of Copolymer I.

Water-insoluble Urease Derivatives—(a) Obtained by coupling urease with the polydiazonium salts of Copolymers 1, 11, or 111: The copolymer was diazotized according to the procedure of Bar Eli and Katchalski (10). The light brown polydiazonium salt which separated out of the solution was centrifuged down and washed several times with cold 0.1 M Tris-sulfate buffer, pH 7.5. A urease solution in the Tris-sulfate buffer (8 to 60 ml), containing 2.5 mg of enzyme per ml, was added to a suspension of the polydiazonium salt (100 mg in 4 ml of the same buffer), and the coupling was allowed to proceed for 20 hours at 4°C with magnetic stirring. The precipitate was centrifuged down, washed with 10-ml portions of the Tris-sulfate buffer until the washings were free of protein, and stored under the buffer at 4°C. The enzymatic activity and protein content of the supernatant and subsequent washings were determined by the methods.
Table I
Composition and activity of water-insoluble urease preparations

The water-insoluble urease preparations listed were obtained as described in "Experimental Procedure." The coupling mixtures contained, per 100 mg of water-insoluble polydiamzonium salt, the amount of urease (1450 Sumner units per g) given in Column 2. The protein content of the water-insoluble preparations was calculated from the difference between the amount of protein originally added to the reaction mixture and that found in the supernatant after the coupling reaction. Urease activity was determined by a modified Sumner method (see "Experimental Procedure").

<table>
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<th>3. Protein content per 100 mg of water-insoluble enzyme</th>
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<td>13</td>
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<td>5</td>
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<td>IU3</td>
<td>108</td>
<td>51</td>
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<td>2.5</td>
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<tr>
<td>IU4</td>
<td>145</td>
<td>58</td>
<td>71</td>
<td>2.5</td>
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<td>IU7</td>
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<td>IU8</td>
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<td>IU9</td>
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<td>46</td>
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<tr>
<td>IU10</td>
<td>24</td>
<td>16</td>
<td>49</td>
<td>13</td>
</tr>
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</table>

a Expressed as a percentage of the activity of the urease preparation used, equal in amount to that of bound protein.

IU1 prepared by coupling urease with the polydiamzonium salt of Copolymer I, consisting of p-amino-L-phenylalanine and L-leucine (1:2.5).

IU2 prepared by coupling urease with the polydiamzonium salt of Copolymer II, consisting of p-amino-DL-phenylalanine and glycine (1:1).

IU3 prepared by coupling urease with the polydiamzonium salt of Copolymer III, consisting of p-amino-DL-phenylalanine and L-alanine (1:1).

Urease was incubated with p-chloromercuribenzoate (0.025 ml of 0.01 M p-chloromercuribenzoate per 10 mg of urease) for 1 hour at room temperature (25°), and the inactivated enzyme was coupled with the polydiamzonium salt of Copolymer I. The water-insoluble derivative (1.5 mg) was activated by treatment with 0.001 N cysteine solution (0.1 ml) prior to determination of activity.

Most of the urease which did not participate in the coupling reaction with the diazotized copolymer and remained in the supernatant displayed enzymatic activity when activated by cysteine.

Urease was incubated with p-chloromercuribenzoate (0.15 ml of 0.01 M p-chloromercuribenzoate solution per 10 mg of urease) for 1 hour at room temperature, and the inactivated enzyme was coupled with the polydiamzonium salt of Copolymer I. The water-insoluble derivative was activated with cysteine solution as described above before measurement of activity.

Described below. The supernatant of the coupling mixture contained a considerable fraction of the enzyme protein when a relatively large amount of enzyme was used. However, no enzymatic activity could be detected in the supernatants of any of the reaction mixtures. The various urease derivatives obtained by the above procedure (IU1, IU2, IU3, IU4, IU5, IU6) are listed in Table I, which also gives the protein content and the enzymatic activity of these preparations.

(b) Obtained by coupling urease, inactivated with p-chloromercuribenzoate, with the polydiamzonium salt of Copolymer I: A urease solution in 0.1 M Tris-sulfate buffer, pH 7.5 (8 to 60 ml), containing 2.5 mg of enzyme per ml, was mixed with 0.01 M p-chloromercuribenzoate solution (0.025 to 0.15 ml per 4 ml of enzyme solution), and the mixture was incubated for 1 hour at room temperature. The inactivated enzyme was then coupled with the polydiamzonium salt of Copolymer I (100 mg), suspended in the Tris buffer (4 ml) by the procedure described in the preceding section. The water-insoluble preparations obtained were stored under Tris-sulfate buffer and before use were activated by cysteine as described below. The enzymatic activity and protein content of the various derivatives obtained by the above procedure (IU7, IU8, IU9, IU10) are given in Table I. It is of interest to note that the enzymatic activity of the inhibited urease left in the supernatants of the reaction mixtures in the preparation of IU8, IU9, and IU10 could be fully recovered on treatment with cysteine.

Methods
Determination of Urease Activity—A modification of Sumner’s method (21) was used. The enzyme activity was measured at 25° with a 3% urea solution in 0.1 M Tris-sulfate buffer, pH 7.5, as substrate.

The enzymatic activity of the water-insoluble urease derivatives IU1, IU2, IU3, IU4, IU5, and IU6 was determined as follows. An aliquot of the water-insoluble urease suspension (0.4 to 0.6 Sumner unit) in 0.1 M Tris-sulfate buffer, pH 7.5, was diluted with the buffer to a volume of 1 ml. Then 1 ml of a 3% aqueous urea solution was added, and the reaction mixture was incubated with shaking at 25°. The reaction was stopped after 5 minutes by the addition of 1 ml of 1 N HCl, and the water-insoluble enzyme was removed by centrifugation. The ammonia formed was estimated colorimetrically after nesslerization of a 0.75-ml aliquot of the supernatant according to Sumner (21).

The enzymatic activity of the water-insoluble urease derivatives IU7, IU8, IU9, and IU10, derived from urease inactivated with p-chloromercuribenzoate, was assayed after reactivation with cysteine. An aliquot of the water-insoluble urease suspension (0.4 to 0.6 Sumner unit) in 0.1 M Tris-sulfate buffer, pH 7.5, to a volume of 0.9 ml, 0.1 ml of 0.001 N cysteine in the same buffer was added, and the mixture was incubated for 2 minutes at 25°. The enzymatic activity was then determined as above.

Determination of Bound Protein in Various Water-insoluble Urease Preparations—The protein content of the various water-insoluble urease derivatives was calculated from the decrease in protein content of the supernatant after coupling urease with the corresponding water-insoluble carriers. The method of Lowry et al. (22) was used for protein assay.
after incubation of the various water-insoluble urease derivatives with 0.1 M Tris-sulfate buffer, pH 7.5, or 3% urea solution in the same buffer, for 20 minutes at 25°, and removal of the insoluble enzyme by centrifugation. Nor is any protein removed by treatment with 0.0025 N HCl or 0.0025 N NaOH for 1/2 hour at room temperature. It may, therefore, reasonably be assumed that urease is covalently bound to the insoluble carrier, and that the water-insoluble preparations obtained do not contain reversibly adsorbed urease.

The water-insoluble preparations IU1, IU2, IU3, and IU4, obtained by coupling active urease with the diazotized copolymer of p-amino-m-phenylalanine and L-leucine (Copolymer I; molar residue ratio, 1:2.5), showed high enzymatic activity (35 to 80% of the activity of free urease equal in weight to that of bound protein) when assayed within 1 or 2 days after their preparation (see Table I). However, their enzymatic activity dropped markedly on storage (see below). The water-insoluble urease derivative IU6, obtained by coupling active urease with the diazotized copolymer of L-alanine and p-amino-m-phenylalanine (Copolymer III), also possessed high initial enzymatic activity. The activity decreased markedly on storage, however, as in the previous cases. The water-insoluble urease derivative IU5, obtained by coupling active urease with the diazotized copolymer of glycine and p-amino-m-phenylalanine (Copolymer II), showed low enzymatic activity immediately after preparation. The use of Copolymer II as insoluble carrier, therefore, was abandoned in further studies.

The protein thiol groups of urease are essential for enzymatic activity (23, 24). It seemed therefore advisable to block the —SH groups of urease reversibly before coupling the enzyme with the water-insoluble carrier, in order to prevent their participation in any unfavorable reaction. Urease was thus reversibly blocked with p-chloromercuribenzoate (24), and the inactive enzyme obtained was coupled with diazotized Copolymer I. The water-insoluble preparations IU7, IU8, IU9, and IU10 (see Table I) thus obtained showed high enzymatic activity when assayed, after reactivation with cysteine, a short while after their preparation. The enzymatic activity of IU7 derived from urease, inhibited with the minimal amount of p-chloromercuribenzoate, was retained to a considerable extent when stored for several months in inactive form (see below).

Stability of Water-insoluble Urease Preparations on Prolonged Storage—The drop in enzymatic activity of some water-insoluble urease derivatives (IU1, IU7, IU10) on prolonged storage at 4° in 0.1 M Tris-sulfate buffer, pH 7.5, is given in Fig. 1. The water-insoluble derivative IU7, prepared from Copolymer I and urease inactivated with the minimal amount of p-chloromercuribenzoate, lost only 35% of its initial activity within 5 months under the conditions specified. On the other hand, Preparation IU10, obtained by coupling the insoluble carrier with urease inactivated with excess p-chloromercuribenzoate, lost 80% of its initial activity within a fortnight on storage at 4° in 0.1 M Tris-sulfate buffer, pH 7.5. Preparation IU1, obtained by coupling active urease with the diazotization product of Copolymer I, lost similarly most of its activity within several days. The stability of IU2, IU3, IU4, and IU6 on storage at 4° under 0.1 M Tris-sulfate buffer, pH 7.5, closely resembled that of IU1. The inactivation of these preparations on storage in the cold could not be prevented by the addition of protecting agents such as gum arabic (25), ovalbumin (26), and glycercol (27), or mild-reducing agents such as cysteine (28) or sulfite-bisulfite mixtures (29). No increase in stability could also be detected when the excess of the diazonium groups left in the various water-insoluble preparations after coupling of enzyme with carrier was blocked with β-naphthol.

It is well known that solutions of purified crystalline urease are inactivated on storage. Sumner, Granén and Eriksson-Quensel (29) suggested that the deactivation is caused by some oxidation reaction of sulphydryl groups, since urease is more stable when stored in a mixed sulfite-bisulfite solution. Creeth and Nichol (28), who studied the effect of sulfite-bisulfite on urease solutions in the ultracentrifuge, suggested that the inactivation reaction is brought about by the formation of intermolecular disulfide crosslinkages, which give rise to the formation of urease dimers, trimers, etc., with specific activities lower than that of the monomer. Our findings that the water-insoluble urease IU7, which contains bound urease inactivated by p-chloromercuribenzoate, is considerably more stable on storage than the water-insoluble urease preparation IU1, which contains bound urease with free —SH groups, suggest that the —SH groups of the bound protein participate in the chemical reactions leading to the inactivation of the water-insoluble enzyme preparations. Since cysteine and sulfite-bisulfite solutions did not stabilize Preparation IU1, it is plausible to assume that the water-insoluble enzyme is inactivated by a mechanism different from that of the soluble enzyme, or that the reduction of the bound protein by low molecular weight reducing agents is considerably more difficult than that of urease in solution.

Urease Column—(a) Preparation and operation: A urease
column 0.6 cm in diameter and 2 cm in height was prepared by pouring the water-insoluble urease derivative, IU7 (52.8 mg, corresponding in activity to 18.5 Sumner units), into a glass tube. Before use, the inert enzyme was activated by flushing with 5 ml of a cysteine solution (0.001 N cysteine in 0.1 M Tris-sulfate buffer, pH 7), and the column was washed with 20 ml of Tris-sulfate buffer. When urea solutions of different concentrations in 0.1 M Tris-sulfate buffer, pH 7, were passed through the column at room temperature, at a rate of 0.2 ml per minute, it was found that quantitative hydrolysis of the substrate occurred up to a concentration of 2 mg of urea per ml. None of the ammonia liberated in the reaction was retained on the column.

(b) Use of column in analysis of urea in body fluids: Serum containing 1.5 to 2.2 mg of urea per ml, or urine (19.5 to 28 mg of urea per ml), was diluted with 0.1 M Tris-sulfate buffer, pH 7, to a concentration of about 1.0 mg of urea per ml. Aliquots of 0.2 ml of the diluted sample were applied to a urease column 0.6 cm in diameter and 1.0 cm high, containing a total activity of 10 Sumner units. Elution was carried out with 14 ml of the Tris-sulfate buffer, and the effluent was collected in a 25-ml volumetric flask and nesslerized. Blanks on both the reagents and the diluted serum or urine were performed, since urine usually contains small quantities of preformed ammonia. The complete procedure required about 1 hour per sample. The results were in good agreement with those obtained by a routine clinical method (30). For example, a sample of serum which was found to contain 2.2 mg of urea per ml by the clinical method yielded 2.14 mg of urea per ml when assayed by our method. Similarly, a sample of urine which contained 27.7 mg of urea per ml when assayed by the clinical method proved to contain 27.8 mg of urea per ml by our method.

(c) Use of a urease column in removal of urea from an aqueous urea solution containing a low molecular weight peptide: A solution of 1% L-tyrosyl-L-phenylalanine, 3 M in urea and 0.1 M in Tris-sulfate buffer, pH 7, was prepared. It was diluted 0.9-fold with the Tris buffer, and an aliquot of 10 ml was applied to a urease column 1 cm in diameter and 5 cm high, containing a total activity of 255 Sumner units. The enzymatic hydrolysis was carried out at room temperature, at a rate of flow of 0.1 ml per minute. The column was then eluted with water, and 50 ml of effluent were collected. The effluent was evaporated to dryness, and the ammonium carbonate was removed quantitatively by sublimation on a rotatory evaporator. The residue, which was void of urea, was dissolved in a minimal quantity of 0.1 N NaOH, and its dipeptide content was determined by measuring the optical density at 293 µm. A quantitative recovery of the dipeptide was obtained.

The above experiments clearly demonstrate that urease columns may be of use in the quantitative removal of urea from biological fluids and from systems in which urea has been employed as a denaturing agent, as well as from mixtures containing other low or high molecular weight compounds in addition to urea. The possible application of the urease columns in urea assay has also been indicated.

SUMMARY

Water-insoluble urease preparations were obtained by coupling unmodified urease, or urease reversibly inactivated with p-chloromercuribenzoate, with a water-insoluble diazonium salt derived from a copolymer of p-amino-L-phenylalanine and L-leucine (molar residue ratio, 1:2.5). The enzymatic activity of the insoluble preparations derived from inactivated urease was determined after reactivation with cysteine. All of the insoluble derivatives obtained, which contained 15 to 60 mg of protein per 100 mg of water-insoluble enzyme, displayed after preparation an enzymatic activity, per unit of weight of bound protein, corresponding to about half of that of the original urease preparation used in the coupling reaction. The water-insoluble urease derivative IU7, derived from urease inactivated by the minimal amount of p-chloromercuribenzoate, retained most of its activity even after 5 months of storage at 4° under 0.1 M Tris-sulfate buffer, pH 7.5. The water-insoluble urease preparations IU1, IU2, IU3, and IU4, derived from unmodified urease, on the other hand, lost most of their activity within 2 weeks when stored under similar conditions.

Columns possessing urease activity were prepared from the water-insoluble urease derivative IU7. These were found useful in the quantitative removal of urea from aqueous solution, and in its assay in urine and serum.

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