Preparation and Properties of Water-insoluble Derivatives of Trypsin*

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The conversion of enzymes into water-insoluble products possessing specific catalytic activity is of interest, since such water-insoluble enzyme derivatives can readily be removed from the reaction mixture and can be used for the preparation of columns with enzymatic activity. When stable, they can be employed repeatedly to induce specific chemical changes in relatively large amounts of substrate. Incubation of substrate with a water-insoluble enzyme for a short period may facilitate the detection and isolation of intermediates formed at the early stages of an enzymatic reaction. Finally, it might be expected that the study of the properties of water-insoluble enzymes might shed new light on the mode of action of enzymes present in cell membranes.

Adsorption techniques have been applied to combine enzymes with insoluble carriers (2, 3). These procedures led, however, to partial denaturation and resulted in preparations from which the reversibly adsorbed enzyme was liberated while in contact with substrate. In order to obtain irreversible binding between enzyme and carrier, attachment by covalent bonds seems necessary. Such binding should obviously be carried out via functional groups not essential for enzymatic activity.

Some antigens and antibodies have been attached by chemical bonds to water-insoluble high molecular weight compounds (4–8). The immunologically active columns of this kind were used in the isolation of both antibodies and antigens. Few papers have been published on the chemical bonding of enzymes to water-insoluble polymers. The coupling of pepsin and carboxypeptidase with a diazotized poly-p-aminostyrene was reported by Grubhofer and Schleith (9). A low enzymatic activity was recorded for the water-insoluble enzymes obtained, which were poorly characterized. The synthesis of diazobenzyl cellulose and carboxymethyl cellulose derivatives of trypsin and chymotrypsin was described by Mitz and Summaria (10). 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 Anfossen (11) succeeded recently in reversibly reducing the disulphide bonds of trypsin bound to carboxymethyl cellulose. Free trypsin could not be used, as its reduction was accompanied by precipitation and irreversible inactivation. Manecke (12) reported the synthesis of enzyme resins by allowing papain, diastase, or β-fructosidase to react with a nitrated copolymer of methacrylic acid and methacrylamid m-fluoranoalide. The resin resin was the most stable preparation obtained. The preparation of a highly active water-insoluble papain derivative by direct coupling of papain with a diazotized copolymer of p-aminophenylalanine and leucine has recently been described by Cebra et al. (13). With the aid of the water-insoluble enzyme it was possible to digest partially γ-globulin, antibodies, and other proteins (13).

In this article the preparation, properties, and applications of some water-insoluble trypsin derivatives are described. The most active insoluble trypsin preparations were obtained by coupling polytyrosyl trypsin (14) with a diazotized copolymer of p-amino-phenylalanine and l-leucine. The water-insoluble polytyrosyl trypsins prepared are considerably more stable than trypsin in the pH range from 7.0 to 9.0. It was thus possible to prepare a trypsin column with high and enduring tryptic activity. The action of the water-insoluble polytyrosyl trypsins on substrates of low and high molecular weight was studied, and their inhibition with pancreatic and soybean trypsin inhibitors was tested.

EXPERIMENTAL PROCEDURE

Materials Twice crystallized trypsin (50% MgSO₄), acetyltrypsin, protamine sulfate, and “vitamin-free” casein were purchased from Nutritional Biochemicals Corporation. Pancreatic and soybean trypsin inhibitors were obtained from Worthington Biochemical Corporation.

L-Arginine methyl ester (15), benzoyl-L-arginine methyl ester (16), benzoyl-L-arginine ethyl ester (17), N-carboxy-L-lysine anhydride (18), p-aminoo-L-phenylalanine (19), poly-p-amino- L-phenylalanine (number average degree of polymerization, 12) (20), and poly-L-lysine hydrobromide (21) were prepared according to the literature. Geon (426, B. F. Goodrich) was obtained from the Goodrich Chemical Company.

Polytyrosyl Trypsin—Polytyrosyl trypsin was prepared from trypsin and N-carboxy-L-tyrosine anhydride according to the procedure of Glazer, Bar-Eli, and Katchalski (14). Three polytyrosyl trypsin preparations were obtained. Determination of their enrichment in tyrosine by the method described previously (14) yielded the following values: 6.2% for PT1, 13.7% for PT2, and 9.6% for PT3. PT1, PT2, and PT3 thus

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1 The abbreviations used are: PTT, polytyrosyl trypsin; IPTT, water-insoluble polytyrosyl trypsin; IT, water-insoluble trypsin; TatT, water-insoluble acetyltrypsin.
contained 9.2, 20.2, and 11.1 moles of added tyrosine per mole of trypsin, respectively. An assay of the esteratic and proteolytic activities (see below) revealed that the modified trypsins retained 70 to 75% of the enzymatic activity of the original trypsin.

**Copolymer of p-Amino-DL-phenylalanine and L-Leucine (I)**—

p-N-Carbobenzoxyamino-$\alpha$-N-carboxy-DL-phenylalanine anhydride (20) (3.5 g) and N-carboxy-$\alpha$-leucine anhydride (29) (1.5 g) were copolymerized in anhydrous dioxane (100 ml) with triethylamine (0.1 ml) as initiator. The reaction mixture was magnetically stirred for 3 days at room temperature, and finally refluxed for 1 hour. The copolymer formed precipitated out on the addition of water (300 ml) to the cooled solution. Decarboxylation was effected by treatment of the dried copolymer with ether (500 ml). It was repeatedly washed with ice-cold 0.1 N hydrochloric acid (30 ml) for 1 hour at room temperature (23). The desired benzoxylation was effected by treatment of the dried copolymer with an ice-cold suspension of polydiazonium Salt II (100 mg) in 10% sodium acetate solution (6 ml). The pH of the mixture was brought to 7.4 with 0.1 N NaOH; the suspension was stirred magnetically and the coupling reaction was allowed to proceed for 1$\frac{1}{2}$ hours at 4°. The pH was readjusted to pH 6.8, and the reaction mixture was left for another 20 hours at 4°. The light brown water-insoluble trypsin obtained was separated by centrifugation, and the excess free diazonium groups were blocked by treatment with $\beta$-naphthol (4 ml of a 0.01% solution in 10% acetate, pH 4). The brown water-insoluble trypsin obtained was separated by centrifugation and washed with cold 10% sodium acetate (15 ml) and with 0.1 N phosphate buffer, pH 7.6 (15 ml). Final purification was attained by washing with 0.0025 N HCl until the washings showed no color.

**Water-insoluble Trypsin Derivatives**—(a) Obtained by coupling trypsin with polydiazonium Salt II. A trypsin solution containing 7.5 mg of enzyme per ml of 0.0025 N HCl (11.9 to 58.7 ml, corresponding to 89 to 440 mg of enzyme, see Table I) was mixed with an ice-cooled suspension of polydiazonium Salt II (100 mg) in 10% sodium acetate solution (6 ml). The pH of the mixture was brought to 7.4 with 0.1 N NaOH; the suspension was stirred magnetically and the coupling reaction was allowed to proceed for 1$\frac{1}{2}$ hours at 4°. The pH was readjusted to pH 6.8, and the reaction mixture was left for another 20 hours at 4°. The light brown water-insoluble trypsin obtained was separated by centrifugation, and the excess free diazonium groups were blocked by treatment with $\beta$-naphthol (4 ml of a 0.01% solution in 10% sodium acetate) for 10 minutes at 4°. The dark red water-insoluble trypsin derivative was separated by centrifugation and washed with cold 10% sodium acetate (15 ml) and with 0.1 N phosphate buffer, pH 7.6 (15 ml). Final purification was attained by washing with 0.0025 N HCl until the washings showed no color.

**Table I**

<table>
<thead>
<tr>
<th>Water-insoluble Trypsin derivative</th>
<th>Trypsin, acetyl-trypsin, or poly-tyrosyl trypsin in coupling mixture</th>
<th>Protein content per 100 mg of water-insoluble enzyme</th>
<th>Esteratic activity of bound protein</th>
<th>Amount of water-insoluble trypsin having the esteratic activity of 1 mg of soluble trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/100 mg carrier</td>
<td>mg</td>
<td>%</td>
<td>mg</td>
<td>mg</td>
</tr>
<tr>
<td>IT$_1$</td>
<td>89</td>
<td>17</td>
<td>5.9</td>
<td>100</td>
</tr>
<tr>
<td>IT$_2$</td>
<td>130</td>
<td>26</td>
<td>2.0</td>
<td>200</td>
</tr>
<tr>
<td>IT$_3$</td>
<td>440</td>
<td>29</td>
<td>0.7</td>
<td>500</td>
</tr>
<tr>
<td>IT$_4$</td>
<td>43</td>
<td>3</td>
<td>0.66</td>
<td>5000</td>
</tr>
<tr>
<td>IT$_5$</td>
<td>18</td>
<td>12</td>
<td>2.6</td>
<td>324</td>
</tr>
<tr>
<td>IAeT$_3$</td>
<td>29</td>
<td>28</td>
<td>6.0</td>
<td>59</td>
</tr>
<tr>
<td>IPTT$_1$</td>
<td>26</td>
<td>21</td>
<td>20.0</td>
<td>22</td>
</tr>
<tr>
<td>IPTT$_2$</td>
<td>22</td>
<td>20</td>
<td>15.0</td>
<td>34</td>
</tr>
<tr>
<td>IPTT$_3$</td>
<td>21</td>
<td>21</td>
<td>28.0: 9: 2</td>
<td>17</td>
</tr>
</tbody>
</table>

* Expressed as per cent of the activity of trypsin equal in amount to that of bound protein.

$^{a}$ Preparied by coupling trypsin with polydiazonium Salt II. The latter was obtained by diazotization of a copolymer of p-amino-DL-phenylalanine and L-leucine (1:2).

$^{b}$ Esteratic activity was determined after lyophilization.

$^{c}$ Prepared by coupling trypsin with the diazotization product of poly-p-amino-DL-phenylalanine.

$^{d}$ Water-insoluble acetyl-trypsin. Prepared by coupling acetyl trypsin with polydiazonium Salt II.

$^{e}$ IPTT$_1$, IPTT$_2$, and IPTT$_3$ were obtained by coupling the polytyrosyl trypsins PTT$_1$, PTT$_2$, and PTT$_3$, respectively, with polydiazonium Salt II.
no proteolytic activity toward casein. The different water-insoluble trypsin derivatives obtained by the above procedure (IT1, IT2, IT3, and IT4) are listed in Table I. The table also gives the protein content and enzymatic activity of the different enzyme preparations specified. All preparations were stored in 0.0025 N HCl at 4°C.

(b) Obtained by coupling polytyrosyl trypsin with poly-diazonium Salt II: The procedure adopted for coupling polytyrosyl trypsin with II was similar to that given for the preparation of the corresponding water-insoluble trypsin derivatives (see preceding section). The 10% sodium acetate solution used in the above procedure was substituted by 0.1 M phosphate buffer, pH 7.8. The polytyrosyl trypsin solution added contained 3.0 mg of modified enzyme per ml of 0.0025 N HCl. The treatment of the water-insoluble polytyrosyl trypsin with β-naphthol to block the excess unreacted diazonium groups was found unnecessary, since decomposition of these groups occurred within 4 to 6 days under the standard conditions of storage (suspension in 0.0025 N HCl; 4°C). The composition and enzymatic activities of the different water-insoluble polytyrosyl trypsins prepared are summarized in Table I.

(c) Obtained by coupling trypsin with poly-dL-phenylalanine diazonium salt: Poly-dL-phenylalanine diazonium salt (26) was obtained from poly-p-amino-dL-phenylalanine (20) by a procedure similar to that given for the preparation of II from the copolymer of p-amino-dL-phenylalanine and L-leucine. It was coupled with trypsin under the conditions given in (a) for the preparation of IT1. Two water-insoluble trypsin preparations, IT4 and IT5, were obtained by this procedure. Their composition and enzymatic activity are given in Table I.

**Determination of Bound Protein in Various Water-insoluble Trypsin Preparations**—The protein content of the various water-insoluble trypsin preparations was calculated from the amount of valine liberated on acid hydrolysis, assuming a valine content of 6.2% for crystalline trypsin (this value was obtained when trypsin was substituted for water-insoluble trypsin in the procedure given below). Hydrolysis of each of the water-insoluble trypsin derivatives (10 mg) was effected by heating in 6 N HCl (3 ml) for 48 hours at 115°C in a sealed tube. The acid was removed under reduced pressure and the residue was dissolved in water (2 ml). An aliquot of 20 to 50 μl was withdrawn and subjected to paper chromatography with n-butanol-acetic acid-water (25:6:25 by volume). The paper was sprayed with ninhydrin, and the spot of valine was located and assayed quantitatively by the method of Kay et al. (24). An independent estimate of the amount of bound protein in the water-soluble trypsin preparations IT1, IT2, IT3, IT4, and IT5 (Table I) could also be made by determining the decrease in protein content of the supernatant, after coupling trypsin with the corresponding water-insoluble carriers. Protein concentration was calculated from ultraviolet absorption at 280 μm, assuming for trypsin a specific extinction coefficient of ε280 = 14.4 (27). Fair agreement was found between the values obtained by both methods.

**Determination of Enzyme Activity by pH-Stat Method**—Rates of hydrolysis of benzoyl-L-arginine methyl ester and of L-arginine methyl ester were determined by the pH-Stat method (28) with an automatic titrator. The assay mixture was placed in a jacketed cell maintained at 25°C. The specific activity of trypsin; the polytyrosyl trypsins PT1, PT2, and PT3; the water-insoluble trypsins IT1, IT2, IT3, IT4, IT5; the water-insoluble acetyltrypsin IAcT; and the water-insoluble polytyrosyl trypsins IPT1, IPT2, and IPT3, were calculated from the initial rates of hydrolysis.

In experiments in which benzoyl-L-arginine methyl ester was used as substrate, the rate of hydrolysis was determined at pH 7.8, with 0.1 N NaOH as titrant. The reaction mixture (2.5 ml) was 0.01 M in phosphate buffer, and 0.02 M in substrate. In experiments in which L-arginine methyl ester was used as substrate, the rate of hydrolysis was followed at pH 6.1. The reaction mixture (3 ml) was 0.015 M in substrate. Trypsin at a concentration of 5 to 30 μg per ml of reaction mixture gave a specific activity of 7.8 X 10⁻² and 5.3 X 10⁻² M per minute per mg of enzyme per ml toward benzoyl-L-arginine methyl ester and L-arginine methyl ester, respectively. The determination of the rate of substrate hydrolysis by the polytyrosyl trypsins or by the water-insoluble trypsin preparations permitted the calculation of the corresponding equivalent amounts of intact trypsin possessing the same enzymatic activity. Practically the same results were obtained when either benzoyl-L-arginine methyl ester or L-arginine methyl ester was used as substrate.

**Activity Determination by Casein Digestion Method**—This was carried out by the method of Northrop, Kunitz, and Herriott (29). The concentration of products soluble in trichloroacetic acid was determined by measuring the optical density at 280 μm. In experiments in which the activity of water-insoluble trypsins was determined, the reaction mixture was stirred magnetically during proteolysis at 37°C. The water-insoluble enzyme was filtered off before addition of trichloroacetic acid.

**RESULTS**

**Coupling of Trypsin with Low Molecular Weight Diazonium Salts**—To test the possible binding of trypsin to a water-insoluble carrier via azo links, the enzyme was coupled with low molecular weight diazonium salts and the proteolytic activity of the modified enzymes obtained was determined by the casein digestion method (29). The results obtained (Table II) show that trypsin loses practically all of its activity on coupling with a large excess of diazonium salts derived from p-aminobenzoic acid, p-aminophenylalanine, or p-aminophenylacetic acid. On the other hand, most of the enzymatic activity is retained when the coupling is carried out at a relatively low molar ratio of diazonium salt to enzyme (24:1). These results suggested that an active water-insoluble trypsin derivative might be obtained by binding trypsin to a water-insoluble macromolecular diazonium salt under the proper conditions.

**Enzymatic Activity of Various Water-insoluble Trypsin Preparations**—The water-insoluble trypsin derivatives obtained are listed in Table I. They give a fine suspension in water, and can readily be removed from a reaction mixture by centrifugation at 5000 X g for 5 to 10 minutes or by filtration through Whatman No. 1 filter paper. No active enzyme could be detected in solution after incubation of the various water-insoluble trypsin preparations with 0.0025 N HCl, 0.1 M phosphate buffer, pH 7.6, 1% casein solution, or 0.02 M benzoyl-L-arginine methyl ester for 10 to 20 minutes at 30°C. It is thus plausible to assume that the insoluble preparations do not contain any reversibly adsorbed enzyme, and that all of the detectable tryptic activity resides in...
The corresponding diazonium readings (280 nm). The data presented show that trypsin and polytyrosyl trypsin salt solutions were used as blanks in the spectrophotometric assay of the enzymatic activity of an aliquot (0.1 ml) was assayed by the method (29). The corresponding diazonium salt solutions were used as blanks in the spectrophotometric readings (280 nm).

<table>
<thead>
<tr>
<th>Molar ratio of diazonium salt to trypsin</th>
<th>Proteolytic activity left after coupling with diazonium salts derived from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-Aminobenzoic acid; coupling carried out in: p-Aminobenzoic acid; coupling carried out in:</td>
</tr>
<tr>
<td>0.125 M KCl</td>
<td>Δaqueous 10% sodium acetate solution</td>
</tr>
<tr>
<td>12:1</td>
<td>%</td>
</tr>
<tr>
<td>24:1</td>
<td>100</td>
</tr>
<tr>
<td>48:1</td>
<td>64</td>
</tr>
<tr>
<td>120:1</td>
<td>45</td>
</tr>
<tr>
<td>400:1</td>
<td>5</td>
</tr>
</tbody>
</table>

* The molecular weight assumed for trypsin was 24,000 (30).

The same value was obtained after dialysis against 0.0025 N HCl for 5 days at 4°C.

The coupling products of trypsin with diazotized poly-p-aminophenylalanine (IT4 and IT5, see Table I) possessed low esteratic activity (0.5 to 2% of the activity of free trypsin equal in weight to that of bound protein). The marked inactivation of bound trypsin in these cases might be attributed to the coupling of enzyme with water-insoluble macromolecules possessing a high local concentration of diazonium groups.

To reduce the molecular concentration of diazonium groups on the carrier, copolymers of p-amino-LL-phenylalanine and L-leucine in molar residue ratios of 1:2; 1:6, and 1:13 were prepared. Coupling of trypsin with the diazotized copolymers 1.0 and 1.13, containing a relatively high amount of leucine, led to hydrophobic, enzymatically inactive preparations. On the other hand, the water-insoluble product IT1, derived from copolymer 1:2, possessed an esteratic activity somewhat higher than that of IT4 and IT5. The amount of bound protein in the coupling product of acetyl trypsin with copolymer 1:2 (IAcT) was greater than in IT1. The same esteratic activity, however, was recorded for both water-insoluble preparations when calculated per unit weight of bound protein (see values given for IAcT and IT1 in the fourth column of Table I).

Water-insoluble trypsin derivatives IPTT1, IPTT2, and IPTT3, with a relatively high enzymatic activity (15 to 30%) of the activity of free trypsin equal in weight to that of bound protein) were obtained by coupling polytyrosyl trypsin with a diazotized copolymer of p-amino-LL-phenylalanine and leucine (1:2) (II). The high activities recorded are undoubtedly due to the decrease in the number of bonds between the carrier and the tyrosyl residues of trypsin, and to the formation of azo links with the enzymatically nonessential polytyrosyl side chains of the modified enzyme. All of the experiments to be described below were carried out with the water-insoluble polytyrosyl trypsin derivatives because of their marked trypsic activity.

Properties of Water-insoluble Polytyrosyl Trypsins—(a) Stability as a function of pH. The enzymatic activity of the water-insoluble trypsin preparation IPTT2, after incubation at 25°C for 25 hours at different pH values, is recorded in Fig. 1. For comparison the activities of trypsin and polytyrosyl trypsin (IPTT2), incubated under similar conditions, are also included. The data presented show that trypsin and polytyrosyl trypsin are somewhat more stable than IPTT2 in the pH range from 2.0 to 4.0. The water-insoluble enzyme (IPTT2), however, exhibits a remarkably high stability in the alkaline pH range from 7.0 to 9.0. Thus, whereas IPTT2 retains 80% of its initial enzymatic activity after incubation for 25 hours at pH 8.0 or pH 9.0, trypsin and polytyrosyl trypsin lose all or most of their enzymatic activity on similar treatment.

(b) Decrease in activity on prolonged storage at 4°C in 0.0025 N HCl: The drop in enzymatic activity of IPTT1 on prolonged storage at 4°C in 0.0025 N HCl is given in Fig. 2. The water-insoluble enzyme lost, under the conditions specified, 50% of its initial activity within 4.5 months, and 70% of its activity within 12 months. IPTT3 lost only 35% of its initial activity within 5 months. No decrease in protein content of both water-insoluble polytyrosyl trypsin preparations could be detected during the above time intervals.
freshly prepared rabbit myosin (31) with IPTT1 (0.2 mg per ml) assigned to the water-insoluble enzyme. Thus, incubation of
shown to possess an esteratic activity corresponding to that of
benzoyl-n-arginine methyl ester. From the rate of digestion of
1.0 mg of trypsin (see Table I). The proteolytic activity of the
fore about one-sixth that derived from the rate of hydrolysis of
trypsin. On the other hand, 22.2 mg of IPTT1 have been
assayed by the pH-Stat method with benzoyl-n-arginine methyl
ester as substrate (see "Experimental Procedure"). The enzyme
activity is expressed as per cent of initial activity.

An assay of the tryptic activity of IPTT1 by Kunitz's method
resembles that of trypsin. The water-insoluble enzyme, how-
for trypsin. The activity of IPTT2 as a function of pH also
show that the pH-activity curve for PTT2 resembles closely that
insoluble polytyrosyl trypsin IPTT2, for polytyrosyl trypsin
ester as substrate (see “Experimental Procedure”). The enzyme
drops of 0.0025 N HCl, and its activity at the time intervals specified was
recorded are given as per cent of activity at pH 7.7.

![Figure 2. Decrease of activity of water-insoluble polytyrosyl trypsin (IPTT1) on storage. IPTT1 was stored at 4° under
0.0025 N HCl, and its activity at the time intervals specified was
assayed by the pH-Stat method with benzoyl-l-arginine methyl
ester as substrate (see “Experimental Procedure”). The enzyme
activity is expressed as per cent of initial activity.]

![Figure 3. pH-activity curves for trypsin (O O O), polytyrosyl trypsin (PTT2, Q Q Q), and water-insoluble polytyrosyl trypsin
(IPTT6, △ △ △). The esteratic activity of the enzyme prepara-
tions used was assayed, at the pH values specified, by the pH-
Stat method. The assay mixtures (5 ml) were 0.005 M in benzoyl-
l-arginine ethyl ester and contained trypsin (0 μg per ml), PTT2
(0 μg per ml), or IPTT2 (0.2 mg per ml, corresponding in activity
at pH 7.7, to 6 μg of trypsin per ml). The esteratic activities
recorded are given as per cent of activity at pH 7.7.]

(c) pH-activity curve: The pH-activity curves for the water-
insoluble polytyrosyl trypsin IPTT2, for polytyrosyl trypsin
PTT2, and trypsin are given in Fig. 3. The data presented
show that the pH-activity curve for PTT2 resembles closely that
for trypsin. The activity of IPTT2 as a function of pH also
resembles that of trypsin. The water-insoluble enzyme, how-
ever, possesses at alkaline pH values (pH 8.6 to 9.5) a somewhat
higher activity than trypsin.

(d) Enzymatic cleavage of high molecular weight substrates:
An assay of the tryptic activity of IPTT1 by Kunitz's method
(29), with casein as substrate, showed that 130 mg of water-
insoluble enzyme possess the activity of 1.0 mg of crystalline
trypsin. On the other hand, 22.2 mg of IPTT1 have been
shown to possess an esteratic activity corresponding to that of
1.0 mg of trypsin (see Table I). The proteolytic activity of the
water insoluble enzyme, determined by the casein assay, is there-
fore about one-sixth that derived from the rate of hydrolysis of
benzoyl-l-arginine methyl ester. From the rate of digestion of
native myosin by IPTT1, an even lower tryptic activity had to be
assigned to the water-insoluble enzyme. Thus, incubation of
freshly prepared rabbit myosin (31) with IPTT1 (0.2 mg per ml)
for 160 minutes at 29°, under the conditions specified by Lowey
and Holtzer (32), yielded light and heavy meromyosin (sepa-
rated by ultracentrifugation) in amounts similar to those ob-
tained on incubation of the myosin solution with trypsin (11.4
μg per ml) for 7 minutes. The tryptic activity of 400 mg of
IPTT1 corresponds in this case to that of about 1.0 mg of crystall-
line trypsin; i.e. it is one-eighth the activity derived from the
rate of hydrolysis of benzoyl-l-arginine methyl ester.

(c) Inhibition by pancreatic trypsin inhibitor: The inhibition
of the polytyrosyl trypsin PTT3, and of the water-insoluble polytyrosyl trypsin IPTT3, by pancreatic trypsin inhibitor was
tested under conditions similar to those given by Green (33). An
aqueous solution of PTT3 (46 μg in 2 ml), 0.013 M in KCl and
0.026 M in CaCl2, was mixed with L-arginine methyl ester (8.9 mg)
in water (1 ml), and the rate of hydrolysis of the substrate, at
pH 6.0, was followed by the pH-Stat method. Pancreatic
trypsin inhibitor (0.15 mg) in 0.0025 N HCl (0.15 ml) was added
after 5 minutes of ester hydrolysis, whereupon complete inhibi-
tion of the modified enzyme occurred. Trypsin was similarly
inhibited by the pancreatic trypsin inhibitor.

When the above procedure was applied to the water-insoluble
enzyme derivative IPTT3 (1 mg of a 3-month-old preparation, corresponding in esteratic activity to 47 μg of trypsin), no inhibi-
tion could be detected. The inhibition of IPTT3 by prior incu-
bation with the pancreatic trypsin inhibitor, therefore was
attempted. A suspension of IPTT3 (1 mg) in an aqueous solu-
tion could be detected. The inhibition of IPTT3 by prior incu-
bation with the pancreatic trypsin inhibitor occurred on
incubation with 0.001 N HCl at 24° for 60 minutes. Practically no reacti-
vation of the inhibited water-insoluble enzyme occurred on
incubation with 0.001 N HCl at 24° for 30 minutes.

(f) Inhibition with soybean trypsin inhibitor: In our previous
article (14) it was demonstrated that the esteratic and proteo-
lytic activities of polytyrosyl trypsin are completely inhibited
by soybean trypsin inhibitor at a weight ratio of inhibitor to
modified enzyme of about 1:1. It seemed desirable, therefore,
to investigate also the effect of the high molecular weight inhibi-
tor on water-insoluble polytyrosyl trypsin preparations. The
inhibitory effect of soybean trypsin inhibitor on the water-in-
soluble trypsin derivative IPTT2, at different weight ratios of
inhibitor to insoluble trypsin, is given in Fig. 4. The data pre-
sented show that even with a large excess of inhibitor, only
partial inhibition of enzyme takes place after incubation at 25°
for ½ hour. Thus, the activity of the water-insoluble enzyme
decreased by only 60% even when the weight ratio of inhibitor
to bound protein was 100:1 or 200:1.

To test the decrease in activity of water-insoluble polytyrosyl trypsin on incubation with inhibitor as a function of time of
incubation, the following experiment was carried out. IPTT3
(10 mg, containing 2 mg of bound protein, corresponding in
esteratic activity to 0.47 mg of trypsin) was mixed in 15 ml of
water with soybean trypsin inhibitor (12 mg) dissolved in 0.1 M
phosphate buffer, pH 7.6 (3.0 ml). The pH was adjusted to 7.8
and the mixture was incubated at 25° with stirring. At differ-
ent time intervals 1.5-ml aliquots were withdrawn and assayed
for esteratic activity by the pH-Stat method, with benzoyl-L-arginine methyl ester as substrate. The initial enzyme activity decreased by 60% after only 15 minutes of incubation. No increase in the extent of inhibition was observed after a further incubation period of 22 hours.

There was no reactivation of IPTT3 inhibited by soybean trypsin inhibitor when a suspension of the inhibited enzyme was kept in 0.001 N HCl for 18 hours at 4°. Complete reactivation of soluble trypsin, inactivated by soybean inhibitor, occurs under similar conditions (34).

Trypsin Column—(a) Preparation and general properties: A trypsin column 0.6 cm in diameter and 3 cm in height was prepared by mixing the water-insoluble trypsin derivative IPTT3 (6 mg of a 4-month-old preparation, corresponding in esteratic activity to 0.25 mg of crystalline trypsin) with an inert polyvinyl resin, Geon 426 (0.5 g), in 0.0025 N HCl (10 ml) and by pouring the mixture into a glass tube. All experiments recorded below were performed at 25°. Before use the column was washed with the suitable buffer. It was flushed with 0.0025 N HCl at the end of each experiment and kept in the cold (4°) until further use. No tryptic activity could be detected in the effluent when 0.1 M phthalate buffer, pH 6.1, or 0.1 M phosphate buffer, pH 7.6, was passed through the column. The enzymatic activity of the column did not decrease even after the hydrolysis of approximately 2.0 g of L-arginine methyl ester, which were passed in solution (0.01 M ester in 0.1 M phthalate buffer, pH 6.1) through the column within 1 week at a rate of flow of 0.2 ml per minute.

The enzyme column permits the regulation of the extent of hydrolysis of a given substrate by varying parameters such as rate of flow of substrate through the column, concentrations of substrate and enzyme, and height of column. In the following section the effect of concentration and rate of flow of substrate (L-arginine methyl ester) on the extent of hydrolysis are given.

(b) Effect of rate of flow and concentration of substrate on extent of hydrolysis: To test the effect of the rate of flow on the extent of hydrolysis, in the presence of a relatively large excess of substrate, a solution of L-arginine methyl ester (3.5 mg per ml of 0.1 M phthalate buffer, pH 6.1) was passed through the trypsin column at different rates, and the concentration of remaining intact ester was determined by the hydroxamic acid assay (35). The different flow rates recorded in Fig. 5 were obtained by varying the hydrostatic pressure on the solution investigated. The data given show that the fraction of substrate hydrolyzed is inversely proportional to the rate of flow of substrate through the column.

(c) Use of column in the digestion of poly-L-lysine, protamine, and oxidized insulin: A solution of poly-L-lysine hydrobromide (10 mg per ml of 0.05 M phosphate buffer, pH 7.5) was passed through the trypsin column, and the effluent was chromatographed on paper according to the method of Waley and Watson (36). The concentrations of lysine, di-lysine (Lys2), trilysine (Lys3), and tetralysine (Lys4) found in an effluent derived from the polylysine solution, passed at a rate of flow of 1 ml per 26 minutes, were 0.1, 1.87, 3.07, and 1.9 mg per ml, respectively. The concentrations of the lysine oligopeptides for a rate of flow

![Fig. 4. Inhibition of water-insoluble polytyrosyl trypsin (IPTT2) with soybean trypsin inhibitor. The incubation mixtures contained IPTT2 (0.8 mg containing 0.16 mg of bound protein with esteratic activity corresponding to 24 μg of trypsin) and soybean trypsin inhibitor (0.16 to 32 mg) in 0.0125 M phosphate buffer, pH 7.8 (4 ml). Each of the mixtures was incubated for 4 hours at 25° with stirring, and the esteratic activity was assayed by the pH-Stat method after addition of 0.025 M benzoyl-L-arginine ethyl ester in water (1 ml, brought to pH 7.8).](image-url)
of 1 ml per 120 minutes were: Lys, 0.31; LysZ, 2.75; Lysa, 3.27; and Lysr, 0.44 mg per ml.

A preliminary experiment was performed to test the possible digestion of protamine on the trypsin column. A solution of protamine sulfate (10 mg per ml of 0.1 M phosphate buffer, pH 7.4) was passed through the column at a rate of 1 ml per hour, and the effluent was analyzed electrophoretically on paper (0.05 M phthalate buffer, pH 5.9) at 10 volts per cm in a Consden instrument (37). Four different, ill defined spots were obtained, after 2 hours, on spraying with ninhydrin or with a Sakaguchi reagent (38). A similar electrophoretogram was obtained when the protamine sulfate solution was digested with water-soluble trypsin (0.3 mg per ml) for 1 hour at 25° and the digest was analyzed as above.

The B chain of insulin has a COOH-terminal lysylalanine sequence (39). An attempt was made, therefore, to determine the amount of alanine liberated on the digestion of oxidized insulin by the trypsin column at different flow rates. Oxidized insulin (40) was dissolved in 0.1 M phosphate buffer, pH 7.6, and the solution (5 mg of insulin per ml of buffer) was passed through the trypsin column at different rates of flow. The effluent was chromatographed on paper with n-butanol-acetic acid-water (25:6:25 by volume) as developer, and the alanine was detected by spraying with ninhydrin and assayed quantitatively (24). Alanine in 85% of the theoretical yield was found in an effluent of the oxidized insulin solution passed through the column at a flow rate of 1 ml per 170 minutes. When the rate of flow was increased to 1 ml per 70 minutes, the yield of alanine dropped to 51%.

**DISCUSSION**

The water-insoluble trypsin derivatives described in this paper were obtained by coupling trypsin or polytyrosyl trypsin, via azo links, to a suitable water-insoluble polymer. The azo linkage was chosen since many proteins retain their biological activity after coupling with low or high molecular weight diazonium salts (4, 6, 41, 42). The following functional groups of proteins have been shown to react with diazonium salts: phenol groups of tyrosine, imidazole groups of histidine, and NH₂-terminal α-amino groups and ε-amino groups of lysine (43). Tyrosine residues seem to be the most reactive ones (44), and it might be expected that they will be the first to react with a diazonium salt. The role of the various functional groups in determining the proteolytic activity of trypsin has not as yet been fully clarified (45, 46). No prediction, therefore, could be made as to the extent of inactivation of trypsin by coupling with diazonium salts. Partial inactivation by coupling with p-diazo-benzenesulfonic acid was reported by Fraenkel-Conrat, Bean, and Lineweaver (47) and by Mouuter et al. (48). Our findings show that the enzymatic activity of the coupling product of trypsin and diazotized p-aminophenyalanine or p-aminobenzoic acid is determined, under the experimental conditions used, by the molar ratio of enzyme to diazonium salt. The modified protein retains 30 to 50% of the initial activity of trypsin when the molar ratio of diazonium salt to enzyme is smaller than 120:1.

At a greater excess of diazonium salt, a further loss in tryptic activity was observed. Practically complete inactivation occurred at a molar ratio of diazonium salt to trypsin of 400:1. The mechanism by which trypsin is inactivated in the above reaction is unknown. However, since trypsin is inactivated by low molecular weight diazonium salts only to a small extent, at relatively low concentrations of the latter, the preparation of an active water-insoluble trypsin by direct coupling of enzyme with a water-insoluble polydiazonium salt seemed plausible. The water-insoluble trypsin derivatives IT1 and IT2 (see Table I), obtained by direct coupling of trypsin with polyphenylalanine diazonium salt, possessed an esteratic activity which corresponded only to 0.5 to 2% of the activity of trypsin equal in weight to the amount of bound protein. The relatively high local concentration of diazonium groups in the polymer chains of the water-insoluble carrier seems, therefore, to affect markedly the catalytic activity of the bound enzyme molecules. In this connection it is pertinent to note that bovine serum albumin and ovalbumin, which were coupled with diazotized poly p-aminoanisyl, were found to possess a low binding capacity for their homologous antibodies (7). A more efficient water-insoluble antigen was obtained when bovine serum albumin was coupled with a polytyrosine derivative in which the concentration of diazonium groups along the polymer chains was markedly reduced (7).

Water-insoluble trypsin derivatives (IT1, IT9, and IT123) with low enzymatic activity were also obtained when trypsin was coupled with a diazotized copolymer of p-amino-α-phenylalanine and ε-leucine (molar residue ratio, 1:2), in which the "local" concentration of diazonium groups is considerably smaller than that in the polydiazonium salt derived from poly-p-aminophenylalanine. Dilution of the diazonium groups along the polymer chains in this case, therefore, had little effect on the preservation of the tryptic activity of the bound enzyme. It is possible that the hydrophobic leucine residues of the copolymer exhibit a denaturing effect on the closely bound protein molecules. No attempt was made to prepare copolymers of p-aminophenylalanine and amino acids possessing hydrophobic side chains, since coupling of trypsin with the diazotization derivatives of such copolymers might lead to water-soluble trypsin derivatives.

Polytyrosyl trypsin (14), obtained by initiating the polymerization of N-carboxytyrosine anhydride with trypsin, was found to possess proteolytic activity equivalent to that of trypsin. An attempt was made, therefore, to prepare a water-insoluble trypsin preparation by binding polytyrosyl trypsin, via its tyrosine side chains, to a diazotized copolymer of p-aminophenylalanine and leucine. By this procedure, binding of carrier to enzymatically nonessential tyrosine side chains could be effected, and close contact between enzyme and hydrophobic carrier could be prevented. Furthermore, it was expected that the polytyrosyl bridges would give more freedom to the bound enzyme molecules, and thus facilitate their reaction with substrate.

The water-insoluble polytyrosyl trypsins obtained (ITPT1, ITPT2, and ITPT3) possess an esteratic activity corresponding to 15 to 30% of that of trypsin equal in weight to the amount of bound protein. The enzymatic specificity and the pH-activity curve of the water-insoluble polytyrosyl trypsins are similar in those of trypsin and polytyrosyl trypsin. The stability of the water-insoluble polytyrosyl trypsin (ITPT2), however, particularly in the alkaline pH range (pH 7 to 9.0), was markedly greater than that of trypsin or of polytyrosyl trypsin. Thus, whereas at pH 8.0 to 9.0 trypsin loses practically all of its activity, and polytyrosyl trypsin loses approximately 70 to 80% of its activity on incubation for 24 hours at 25°, the water-insoluble polytyrosyl trypsin (ITPT2) loses only 20% of its original activity after a similar treatment (see Fig. 1). The relatively high enzymatic stability of the water-insoluble polytyrosyl trypsin...
might be attributed mainly to the prevention of autodigestion as a result of the fixation of enzyme molecules on the water-insoluble carrier. As fixed enzyme molecules can hardly come in contact with each other, the probability of trypptic digestion of active enzyme molecules is greatly reduced. The substitution of the e-amino groups of trypsin by polytyrosyl side chains converts lysyl peptide bonds, originally susceptible to trypptic digestion, to bonds not affected by trypsin. This stability of the water-insoluble polytyrosyl trypsin is thus further enhanced.

The high stability of the water-insoluble polytyrosyl trypsin preparations permitted their repeated use in the digestion of various substrates. The water-insoluble enzyme could be readily removed from the reaction mixture, without loss in activity, by centrifugation or by filtration. An enzyme column prepared from water-insoluble polytyrosyl trypsin retained full activity even after digestion of relatively large amounts of substrate.

In assaying the activity of the water-insoluble polytyrosyl trypsin derivatives, benzoyl-l-arginine methyl ester was the substrate mostly used. With the aid of this assay it was found, as mentioned above, that the bound protein exhibits 15 to 30% of its original esteratic activity. However, when the proteolytic activity of the water-insoluble trypsin preparations was tested on high molecular weight substrates, it was found to be considerably lower than that to be expected from their esteratic activities. Thus when IPTT1 and trypsin, in amounts possessing equal esteratic activities, were tested for proteolytic activity on casein, the water-insoluble enzyme exhibited only one-sixth of the activity of trypsin. The relative proteolytic activity of IPTT1 towards myosin was even lower than that towards casein, reaching only one-eighth of the value predicted from its esteratic activity. The relatively low activity of the water-insoluble trypsin toward proteins is most likely due to the shielding effect of the carrier, which prevents ready contact between the active sites of the bound inhibitor and prolonged times of incubation (see also (14)). The water-insoluble trypsin preparations permitted their repeated use in the digestion of various substrates. The water-insoluble enzyme could be readily removed from the reaction mixture, without loss in activity, by centrifugation or by filtration. An enzyme column prepared from water-insoluble polytyrosyl trypsin retained full activity even after digestion of relatively large amounts of substrate.

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The techniques described in this paper for the preparation of water-insoluble proteolytic enzymes illustrate some of the possible methods for the synthesis of water-insoluble enzymes in general. The principles used, particularly that of attachment of the enzyme to the water-insoluble carrier via a linear polymeric chain, might be used for the preparation of other water-insoluble proteins with biological activity. In such cases it will be of primary importance to determine the functional groups which are non-essential for the activity and to find the suitable chemical reactions for forming the desired biologically active water-insoluble products.
water-insoluble polytyrosyl trypsin preparations contained about
20 mg of protein covalently bound per 100 mg of insoluble en-
zyme, and showed an esteratic activity per unit weight of bound
protein, as assayed with benzoyl L-arginine methyl ester as sub-
strate, corresponding to 15 to 30% of that of crystalline trypsin.
Lower enzymatic activities were recorded for the water-insoluble
trypsin derivatives obtained by coupling unmodified trypsin
with the water-insoluble carrier.

The water-insoluble enzyme preparations were considerably
more stable at the alkaline pH range, pH 7.0 to 9.0, than either
tryptsin or polytyrosyl trypsin. The enzymatic activity of the
water-insoluble polytyrosyl trypsin preparation IPTT1 (Table I)
decreased, on storage for 12 months at 4°C in 0.0025 N HCl, to
30% of its initial activity. The proteolytic activity of the water-
insoluble polytyrosyl trypsin toward low and high molecular weight substrates.
Only partial inhibition of the water-insoluble trypsin prepara-
tions could be effected on incubation with large excess of soybean trypsin
inhibitor.

A column possessing tryptic activity was prepared from water-
insoluble polytyrosyl trypsin by use of an inert polyvinyl resin
as filler. The hydrolysis of the following substrates by the
tryptin column was investigated: arginine methyl ester, prote-
dase, and casein as filler. The hydrolysis of the following substrates by the
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