slight warming and the solution allowed to stand overnight in the cold. This gave semicrystalline PACM[2] (33%, mp 45–55°) which was filtered in a nitrogen atmosphere and dried. PACM[2] (25 g) was heated with hexane (200 ml) and the mixture cooled to give white crystals (64%, mp 65–8) of PACM[3].

The diamines were made volatile for gas chromatographic analysis by conversion to their *N,N'*-trifluoroacetyl derivatives. The samples were treated with trifluoroacetic anhydride overnight, and the reagent was removed under vacuum to give a residue which was analyzed without further purification.

Polyamides (I). These were prepared by melt condensation of equimolar mixtures of the PACM sample and diacid in sealed tubes, under vacuum, at 250°. The polymerizations were allowed to proceed until the polymer melt solidified. The solid was ground, washed with water and methanol, and dried overnight at 75° in a vacuum oven. Polymer yields were generally 85% of the theoretical.

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Polyacrylamide Derivatives of Amino Acid Acylase and Trypsin

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ABSTRACT: Water-insoluble acylase and trypsin polyacrylamide derivatives were prepared by coupling the enzymes through an amide bond by reaction with a water-insoluble polyazide derived from a copolymer of acrylamide, *N,N'*-methylenebisacrylamide, and methyl acrylate. Water-insoluble acylase derivatives showed hydrolytic activities per unit weight of bound protein corresponding to 50–100% of that of the original acylase against *N*-acetyl-DL-methionine, *N*-acetyl-DL-alanine, and *N*-acetyl-DL-norleucine. Water-insoluble acylase derivatives were much more stable to heat inactivation at neutral pH than was free acylase. Optically active amino acids were easily isolated in high yields from the reaction mixtures containing *N*-acetyl derivatives of the corresponding DL-amino acids and the acylase derivatives. Water-insoluble trypsin derivatives showed an esteratic activity, per unit weight of bound protein, 40% of that of crystalline trypsin when assayed with *N*-benzoyl-L-arginine ethyl ester as substrate. Activity of the trypsin derivatives was retained after repeated use at 25°. Trypsin heated in a pH 7 phosphate buffer at 100° for 10 min lost 87% of its initial esteratic activity, whereas the trypsin derivative heated in the same way lost only 23% of its initial activity.

Recent reviews[2] show how extensively the conversion of soluble enzymes into water-insoluble forms has been studied. Water-insoluble enzyme derivatives are of interest from a practical point of view in that they may be used repeatedly to induce specific chemical reactions in relatively large amounts of substrates either by batchwise or continuous column processing. Since water-insoluble enzyme derivatives can be readily filtered from reaction mixtures, such processes reduce the amount of enzyme required and keep reaction products free from contamination by enzyme protein.

A number of papers have appeared on enzyme carriers such as diazotized poly(*p*-aminostyrene),[3] diazobenzylcellulose,[4] carboxymethylcellulose azide,[5,6] and nitrated copolymer of methacrylic acid and methacrylic acid *m*-fluoroanilide.[7] However, none of the enzymes coupled to these carriers was treated with large amounts of substrates because of either low activity or poor stability.

Diazotized copolymers of *p*-amino-DL-phenylalanine and L-leucine were also employed for coupling of various enzymes.[8–11] Among these enzyme derivatives, papain,[2a] urease,[10] and polytyrosyl trypsin[12] derivatives were used for the preparation of enzyme columns.

A proteolytic enzyme, trypsin, has been used frequently in such studies.[2a,5,6] The well-defined water-insoluble trypsin derivatives provide good model systems for comparison of the effects of various carriers on coupled trypsin.

Amino acid acylase was used in an attempt to couple it to poly(methacrylic acid).[13] The coupled product was reported unstable on storage. Apart from the method of covalent binding to water-insoluble carriers, insolubilization of acylase by its copolymerization with *N*-carboxy-γ-methyl-L-glutamate anhydride has been reported as another method for the preparation of water-insoluble acylase derivatives.[14]

Acylase was chosen because its derivative might be useful for the resolution of racemic amino acids.[15] To characterize the new water-insoluble acylase derivatives, their properties

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such as enzymatic activity, substrate specificity, and stability are described.

In this article, a description is given of the preparation and properties of water-insoluble tryspin and acylase derivatives in which cross-linked copolymers of acrylamide and methyl acrylate are utilized as carrier polymers. Our interest in the copolymers stems from the successful use of cross-linked polyacrylamide for the fractionation of active enzymes. The hydrophilic nature of acrylamide residues in the copolymers was an aid in retaining the native structure and activity of the coupled enzymes. Control of the particle size of the copolymers is essential for their complete removal from reaction mixtures and was possible by an emulsion polymerization method. A thesis described our early studies on water-insoluble polymer derivatives of enzymes and antigens.[16] The use of similar cross-linked polyacrylamide derivatives, but prepared from 4-nitroacrylanilide or *tert*-butyl 3-acryloylcarbazate as water-insoluble carriers of amylolytic enzymes, has recently been reported.[17]

Experimental Section

Materials. Acrylamide, methyl acrylate, and *N,N'*-methylenebisacrylamide (BIS)[18] were purchased from Borden Chemical Co. "Tween 85" (polyoxyethylene sorbitan trioleate), and "Span 80" (sorbitan monooleate) were obtained from Atlas Chemical Industries.

Trypsin (bovine pancreatin, twice crystallized) and amino acid acylase (hog kidney) were purchased from Pentex Inc. and Calbiochem, respectively. *N*-α-Benzoyl-L-arginine ethyl ester–HCl (BAEE)[18] (mp 131°), *N*-acetyl-DL-alanine (mp 135°), and *N*-acetyl-DL-methionine (mp 112°) were products of Nutritional Biochemicals Corp. *N*-Acetyl-DL-norleucine (mp 105°) was prepared from DL-norleucine according to the literature.[15]

Preparation of Cross-Linked Copolymer of Acrylamide–Methyl Acrylate. Emulsion polymerization of acrylamide and methyl acrylate (distilled before use) in the presence of a cross-linking reagent (BIS) was carried out in a water–oil emulsion system. A 1-ml sample of an emulsifier, "Span 80," was mixed with an oil phase consisting of 156 ml of mineral oil (American white oil, $d_{25} = 0.80$) and 44 ml of carbon tetrachloride in a 1-l., three-necked flask fitted with a reflux condenser and a mechanical stirrer. An aqueous phase was prepared by dissolving 4.8 g of acrylamide, 0.4 g of BIS, and 4.8 g of methyl acrylate in 20 ml of a water–methanol (1:1) mixture. Nitrogen was bubbled for 10 min through the monomer solution cooled at 4°. Immediately after addition of 100 mg of ammonium persulfate and 10 mg of sodium bisulfite, the aqueous monomer solution was poured in small portions into the oil phase during vigorous agitation. A slow stream of nitrogen was passed over the surface of the polymerization mixture while its temperature was raised to 50°. Once the polymerization reaction started, the temperature reached 70°, at which the mixture was maintained for the following 3 hr. The copolymer which formed as fine particles was collected by filtration over suction, washed thoroughly with carbon tetrachloride followed by ethanol, and dried. The size of water-insoluble copolymer particles was determined by sieving on standard screens. The size of particles obtained ranged from 150 to 300 μ in diameter. The yield was 7.7 g.

Estimation of the Monomer Composition of Copolymer. The copolymer was ground in a mortar and cast into KBr disks. Infrared adsorption spectra measured with a Beckman IR-5 spectrophotometer showed characteristic bands for the methyl group of methyl acrylate at 2950 cm^{-1} and for the amide group of acrylamide at 3320 cm^{-1}. The ratio of the absorbancy of mixtures of polymethyl acrylate and polyacrylamide at these bands increased with the increase in the weight fraction of methyl acrylate residue and a standard curve was prepared. The absorbancy ratio (0.635) of the copoly(acrylamide–methyl acrylate), when compared with this standard curve, showed the weight fraction of methyl acrylate which was incorporated into the copolymer to be 0.48. This value agreed with the initial composition of the monomer solution.

Preparation of the Hydrazide of Cross-Linked Copolymer of Acrylamide–Methyl Acrylate. A 7-g sample of the cross-linked copoly(acrylamide–methyl acrylate) was treated with 50 ml of 95% hydrazine at 70°. After 9 hr of reaction, the insoluble product was filtered and washed with three 25 ml-portions of absolute ethanol. A 6-g sample of water-soluble hydrazide of copoly(acrylamide–methyl acrylate) was obtained after drying.

Preparation of a Cross-Linked Polymer of Methyl Acrylate. A 0.5-g sample of BIS was dissolved in 9.5 g of methyl acrylate. The mixture was emulsified in 90 ml of deionized water using 1.0 ml of "Tween 85." After the emulsion had been saturated with nitrogen gas, 10 ml of an aqueous solution containing 100 mg of ammonium persulfate and 10 mg of sodium bisulfite was added with vigorous stirring. The polymerization reaction was allowed to proceed by raising the temperature of the emulsion to 70°. After 3 hr, 20 ml of a saturated calcium chloride solution was added slowly to the polymerization mixture. The mixture was then cooled until a powdery polymer began to precipitate. The polymer that precipitated was collected and washed with hot water to remove salts. The polymer was produced in 84% yield and was insoluble in benzene.

Preparation of the Hydrazide of Cross-Linked Polymer of Methyl Acrylate. An 8-g sample of cross-linked poly(methyl acrylate) was converted to its hydrazide derivative under the same conditions as described for copoly(acrylamide–methyl acrylate). This hydrazinolysis reaction gave 7.6 g of the hydrazide of cross-linked poly(methyl acrylate). The extent of conversion of methyl ester to hydrazide was not determined. The conversion, however, appeared to be almost quantitative because hydrazinolysis under the same conditions made poly(methyl acrylate) completely water soluble.

Coupling of Trypsin or Acylase to Copolymers. A 1.2-g portion of the hydrazide of cross-linked copoly(acrylamide–methyl acrylate) or the hydrazide of cross-linked poly(methyl acrylate) was suspended in 60 ml of deionized water. To this aqueous suspension, 6 ml of 36.5% hydrochloric acid was added, followed by a slow addition of 30 ml of 4% sodium nitrite solution. The reaction temperature was kept below 0° for 1 hr with stirring. The azide was isolated by filtration without delay and washed with ice cold water until the filtrate became neutral. The water-insoluble azide was transferred directly into 120 ml of 0.1 *M* potassium phosphate buffer (pH 7.5) or 0.5 *M* sodium bicarbonate solution (pH 8.2) in which 120 mg of trypsin (0.5 *M* sodium bicarbonate solution, pH 8.2, for acylase) had been dissolved. The mixture was magnetically stirred in a cold room for 24 hr. The reaction mixture was then centrifuged at 6000*g* for 10 min. The water-insoluble product was suspended in 100 ml of 0.02 *M* potassium phosphate buffer (pH 7.0) and spun down at the same speed. This procedure was repeated another four times. No residual enzyme could be detected in the last supernatant by either a Folin test or an enzyme activity assay. The water-insoluble enzyme derivative was suspended in an appropriate buffer (0.1 *M* potassium phosphate buffer of pH 7.5 for trypsin derivative, 0.05 *M* sodium barbital–HCl buffer of pH 7.0 for acylase derivative) and stored at 4° for assay.

Determination of the Amount of Coupled Enzyme. The protein content of the insoluble enzyme derivatives free of soluble enzyme was calculated from the total amount of amino acids released after exhaustive acid hydrolysis with 6 *N* HCl for 30 hr at 110° in sealed tubes. Amino acid analysis was performed with a Spinco amino acid analyzer.[19] The total amount of amino acids released under the same conditions from the soluble enzyme was used as a standard.

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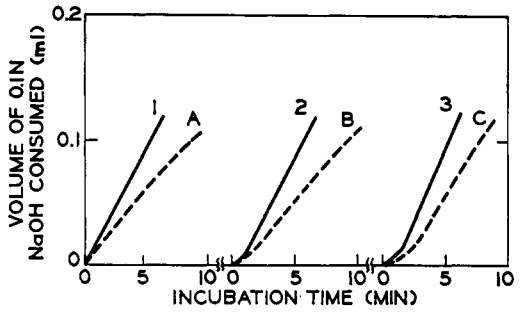

Figure 1. Linearity and reproducibility of standard trypsin assay. Trypsin (80 μg) dissolved in 0.1 *M* potassium phosphate buffer (pH 7.5) or the insoluble trypsin derivative (115 μg of coupled protein) suspended in the same buffer was incubated with a *N*-benzoyl-L-arginine ethyl ester solution at 25°. The well-stirred reaction mixture contained 0.05 *M* potassium phosphate buffer, 2.92×10^{-3} *M* substrate, in 5.0 ml. The alkali consumption at pH 7.5 by the liberated acid was followed by maintaining the pH constant with an automatic titrater. For details, see the Experimental Section. Curves designated 1, 2, and 3 represent recorder tracing of triplicate determinations on the fresh preparation of free trypsin. Curves designated A, B, and C represent the tracing of triplicate determinations on the insoluble trypsin derivative.

Determination of Enzymatic Activity of Trypsin Derivatives. The esteractic activity of trypsin and trypsin derivatives was determined from the initial rate of hydrolysis of BAEE. A 2.5-ml sample of a BAEE solution in distilled water was added to the enzyme solution or the enzyme derivative suspension prepared in 2.5 ml of 0.1 *M* potassium phosphate buffer (pH 7.5). The final reaction mixture (5.0 ml) was 0.05 *M* in potassium phosphate buffer and 2.92×10^{-3} *M* in substrate. The enzyme reaction was initiated by the addition of substrate. The reaction mixture in a water-jacketed round-bottom cell (3 cm in diameter) maintained at 25° was stirred with a 1-cm Teflon-coated magnetic bar at speed setting 9 (*ca.* 600 rpm) of the TRI-R magnetic stirrer, Model MS (TRI-R Instruments, N. Y.). Since subsequent studies showed that rate of hydrolysis by water-insoluble trypsin derivatives was maximum at this stirring rate, the rheostat was held at the constant maximum point (setting 9) through all the assays. The rate of hydrolysis was measured by holding pH constant at 7.5 by addition of 0.1 *N* NaOH using an automatic titrator, Model TTT 1c, and titrigraph Type SBR 2c (Radiometer, Copenhagen) according to the pH-stat method.[20,21] Under these assay conditions, the volume of 0.1 *N* NaOH added to hold pH constant increased linearly with incubation time for at least 5 min and initial rate of hydrolysis was reproducible as illustrated in Figure 1.

Determination of Enzymatic Activity of Acylase Derivatives. The activity of soluble acylase and of acylase derivatives in suspension was estimated from the initial rate of hydrolysis of *N*-acetyl-DL-amino acids. An aqueous solution of *N*-acetyl-DL-amino acid adjusted to pH 7.0 was added to the enzyme solution or to the enzyme derivative suspension in 0.05 *M* sodium barbital–HCl buffer (pH 7.0). The final reaction conditions were 0.033 *M* sodium barbital, 0.017 *M* *N*-acetyl-DL-amino acid, and 15.0 ml total volume. The enzyme reaction was initiated by addition of substrate. The reaction mixture was incubated at 37° under maximal stirring (*ca.* 760 rpm, 1-cm Teflon-coated magnetic bar) with a magnetic stirrer (MAG-MIX, Precision Scientific Co., Chicago). Slower stirring did not alter the reaction rate. Nevertheless, the highest, practically reproducible stirring rate was used. At zero time and at 10-min intervals thereafter, 3.0 ml of the reaction mixture was taken out and filtered through a 5-ml fine sintered glass filter over suction. To the filtrate, 0.1 ml of glacial acetic acid was added to stop the enzyme reaction. After the addition of 2.0 ml of the ninhydrin solution used for the Spinco amino acid analyzer,[19] the mixture was immersed in boiling water for 20 min. After cooling, the mixture was diluted by addition of a mixture of water and *n*-propyl alcohol (1:1 v/v ratio) if necessary. The concentration of amino acid in 3 ml of the reaction mixture was determined from the absorbancy at 570 mμ using the corresponding amino acid as a standard.

Stability Test of Water-Insoluble Acylase Derivative. The stability of the acylase derivative at a high temperature was examined by heating it in a test tube containing 0.05 *M* sodium barbital buffer (pH 7.0) at 100° for 20 min. After cooling, the suspension of the acylase derivative was assayed for its enzymatic activity.

Isolation and Check of Optical Purity of Products Formed by Acylase Derivatives. Isolation of products (amino acids) from reaction mixtures was made according to the directions of Fieser.[22] In our case, no additional step for protein removal was needed because the reaction mixtures were filtered through a sintered glass filter to remove the water-insoluble acylase derivative. The crude precipitated product was recrystallized once from an ethanol–water mixture. The optical rotation of the product obtained was measured in solution with a Cary Model 60 recording spectropolarimeter at 25°.

Results

Preparation of Cross-Linked Copolymer of Acrylamide and Methyl Acrylate. After more than 20 experiments using different conditions for the emulsion polymerization, a procedure for the production of uniformly sized, spherical acrylic copolymer particles was established as described in the Experimental Section.

Preparation and Properties of Water-Insoluble Trypsin Derivatives. Crystalline trypsin was coupled to the azide derivative formed from the hydrazide of cross-linked copoly(acrylamide–methyl acrylate). The water-insoluble product contained 0.46 mg of protein per 100 mg of derivative on the basis of the amount of amino acids liberated on acid hydrolysis. Its esteratic activity per milligram of coupled protein is compared with that of free trypsin in Table I. The trypsin derivative showed an esteratic activity, per milligram of coupled protein, which was 40% of that of crystalline trypsin under the same assay conditions. However, it should be noted that crystalline trypsin was inactivated to 43% of its original activity when exposed to the same conditions used in coupling reaction without the azide derivative (Table I).

Trypsin lost practically all of its activity within 1 month on storage at pH 7.5 and 4°; in contrast, the trypsin derivative lost only 13% of its initial activity under the same conditions (Table I). The thermal stability of the trypsin derivative was also superior to that of the free trypsin, because, after heating, the former lost only 23% of its initial esteratic activity, whereas free trypsin lost 87% of its initial activity (Table I). No change in the esteratic activity of the trypsin derivative was observed after washing five times with 0.1 *M* potassium phosphate buffer (pH 7.5).

Preparation of Water-Insoluble Acylase Derivatives. Crude amino acid acylase was coupled to the cross-linked acrylic copolymers. The results in Table II show that the acylase derivatives obtained starting from copoly(acrylamide–methyl acrylate) contain 0.5–0.8 wt % of coupled protein on the basis of the amount of amino acids released on acid hydrolysis. Cross-linked poly(methyl acrylate) gave a high protein content of 4.3–5.3 wt %.

Stability of Water-Insoluble Acylase Derivatives. The stability of the acylase derivative was tested with respect

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TABLE I
COMPARISON OF THE ACTIVITY OF TRYPSIN WITH THAT OF A WATER-INSOLUBLE TRYPSIN DERIVATIVE

Sample	Esteratic activity,[a] μmol/(min mg) of protein	Per cent activity[b]
	Free Trypsin	
Fresh[c]	27.5	100
Treated[d]	11.7	43
Heated[e]	3.5	13
Stored[f]	1.6	6
	Trypsin Derivative	
Treated[d]	11.0	40
Heated[e]	8.6	31
Stored[f]	9.5	35

[a] The esteratic activity was determined at pH 7.0 with *N*-benzoyl-L-arginine ethyl ester as a substrate and is expressed as micromoles of substrate hydrolyzed per minute per milligram of protein. [b] Defined as per cent activity compared to fresh trypsin equal in amount to that of the free trypsin or the coupled trypsin derivative. [c] Crystalline trypsin was dissolved in 0.0001 *N* HCl (pH 4.0). An 0.08-ml portion of the 1 mg/ml of trypsin solution was made up to a volume of 2.5 ml with 0.1 *M* potassium phosphate buffer (pH 7.5) immediately before assay. [d] Crystalline trypsin was treated under the same conditions used for its coupling to the polymer (dissolved in 0.1 *M* potassium phosphate buffer (pH 7.5) and stirred at 4° for 24 hr). The derivative was prepared by a reaction of trypsin with the azide derivative from the hydrazide of copoly(acrylamide–methyl acrylate) under the same conditions. The trypsin derivative was separated from free trypsin by thorough washing and suspended in 0.1 *M* potassium phosphate buffer (pH 7.5). [e] Crystalline trypsin and the trypsin derivative were dissolved or suspended in 0.1 *M* potassium phosphate buffer (pH 7.5) and heated at 100° for 10 min, respectively. [f] Crystalline trypsin and the trypsin derivative were dissolved or suspended in 0.1 *M* potassium phosphate buffer (pH 7.5) and stored at 4° for 30 days.

TABLE II
PROTEIN CONTENT OF WATER-INSOLUBLE ACYLASE DERIVATIVES[a]

Acylase derivative	Starting polymer	pH of coupling mixture	Acylase in coupling mixture[e]	Protein content[b,f]
A-48-1	Copoly(Am–MA)[c]	7.5	10	0.46
A-48-2	Copoly(AAm–MA)[c]	8.2	10	0.76
A-48-3	Copoly(AAm–MA)[c]	7.5	10	0.47
A-95-1	Poly(MA)[d]	7.5	20	4.34
A-95-2	Poly(MA)[d]	8.2	20	5.32

[a] For the preparative conditions, see the Experimental Section. [b] The protein content was calculated from the total amount of amino acids released on acid hydrolysis. [c] Copoly(4.8, 0.4, 4.8)-(acrylamide–*N*,*N*′-methylenebisacrylamide–methyl acrylate). [d] Copoly(0.5, 9.5)(*N*,*N*′-methylenebisacrylamide–methyl acrylate). [e] mg/100 mg of polymer. [f] mg per 100 mg of derivative.

to thermal inactivation. The acylase derivative (A-48-2) which was heated in 0.1 *M* potassium phosphate buffer (pH 7.0) at 100° for 20 min hydrolyzed 0.10 μmol of *N*-acetyl-L-alanine/min/mg of coupled protein, whereas the free acylase heated under the same conditions hydrolyzed 0.06 μmol of substrate/min/mg of protein. The activities corresponded to 20 and 12% of the activity of free acylase unheated.

Enzymatic Activity of Water-Insoluble Acylase Derivatives. The water-insoluble acylase derivatives give fine suspensions in water and can be readily removed from reaction mixtures by filtration through a fine sintered-glass filter. The enzymatic activity of the acylase derivatives was measured when the insoluble derivatives were in suspension.

TABLE III
THE RATES OF HYDROLYSIS OF THREE ACETYL AMINO ACIDS AT 37° BY WATER-INSOLUBLE ACYLASE DERIVATIVES

Acylase derivative	Starting polymer	Rates of hydrolysis[a] — Substrate: *N*-Ac-DL-Ala	*N*-Ac-DL-Met	*N*-Ac-DL-Leu
A-48-2	Copoly(AAm–MA)[b]	0.5	1.2	
A-95-2	Poly(MA)[c]	0.3	0.7	0.6
Acylase	None	0.5	1.4	1.3

[a] The rates of hydrolysis were calculated from initial rates of formation of amino acids estimated by reaction with ninhydrin. The rates are expressed as micromoles of substrate hydrolyzed per minute per milligram of protein. The amino acid release was linear over the time period used (Figure 2). Duplicate measurements for the free acylase and for the acylase derivatives varied by not more than 10%. [b] Copoly(4.8, 0.4, 4.8)(acrylamide–*N*,*N*′-methylenebisacrylamide–methyl acrylate). [c] Copoly(0.5, 9.5)-(*N*,*N*′-methylenebisacrylamide–methyl acrylate).

The substrate specificity of the two types of acylase derivatives in suspension is summarized in Table III. The rates of hydrolysis by the coupled acylase decrease in the following order of substrates: *N*-acetyl-DL-methionine > *N*-acetyl-DL-norleucine > *N*-acetyl-DL-alanine. This order is identical with that of free acylase. The acylase derivative from cross-linked copoly(acrylamide–methyl acrylate) showed higher specific activities both with *N*-acetyl-DL-methionine

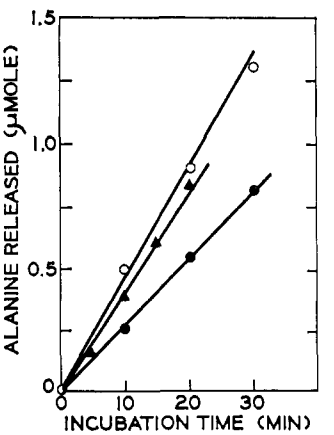

Figure 2. Linearity of standard acylase assay. Acylase (100 μg) dissolved in 0.05 *M* sodium barbital buffer (pH 7.0), acylase derivative A-48-2 (80 μg of coupled protein), or acylase derivative A-95-2 (100 μg of coupled protein) suspended in the same buffer was incubated with a *N*-acetyl-DL-alanine solution at 37°. The final reaction solutions contained 0.033 *M* sodium barbital (pH 7.0), 0.017 *M* *N*-acetyl-DL-alanine, in 15.0 ml. The reaction mixture was stirred with a magnetic stirrer. At intervals, 3.0 ml of the reaction mixture was taken out. The reaction was stopped by removal of water-insoluble acylase derivative by filtration and by addition of 0.1 ml of glacial acetic acid to the filtrate. The amount of alanine formed was determined by reaction with ninhydrin using alanine as a standard. For details, see the Experimental Section. The ordinate shows the micromoles of alanine released in the total reaction mixture. Three straight lines were obtained from the assays using different amount of acylase (–O–), acylase derivative A-48-2 (–×–), and acylase derivative A-95-2 (–●–)

and *N*-acetyl-DL-alanine than the acylase derivative from cross-linked poly(methyl acrylate).

Optical Purity of Products Isolated from Reaction Mixtures with Water-Insoluble Acylase Derivatives. A 2-g sample of an acylase derivative (A-95-1, total protein content 86.8 mg) was incubated with 200 ml of 0.05 *M* *N*-acetyl-DL-methionine solution (pH was adjusted to 7.0 with lithium hydroxide) at 37° under stirring. After 20 hr, the ninhydrin test of the incubation mixture showed that the reaction had reached maximum conversion. The product (methionine) was isolated from the clear filtrate of the reaction mixture according to the method described in the Experimental Section. Crystalline platelets were obtained in 63.7% yield (475 mg). The molecular rotation of the product was $[M]^{25}D$ +34.3° (*c* 2%, 5 *N* HCl). The literature value for L-methionine is $[M]^{25}D$ +34.6° under the same conditions.[15]

Treatment of 200 ml of 0.05 *M* *N*-acetyl-DL-norleucine solution (pH 7.0) with 2.0 g of the acylase derivative (A-95-1) for 20 hr yielded shiny crystalline leaflets in 64.8% yield (425 mg) after crystallization, $[M]^{25}D$ 31.6 (*c* 2%, 5 *N* HCl) The literature value for L-norleucine is $[M]^{25}D$ +32.1°.[15] Similarly, 200 ml of 0.05 *M* *N*-acetyl-DL-alanine solution (pH 7.0) gave crystalline needles in 67.6% yield (301 mg) with $[M]^{25}D$ +12.9° (*c* 5%, 5 *N* HCl) after 60 hr of incubation with 2.0 g of an acylase derivative (A-95-2). The literature value for L-alanine is $[M]^{25}D$ +13.0.[15]

Discussion

Polyacrylamide derivatives of trypsin and acylase, in which the enzymes are covalently coupled to the surface of the polymer particles, have been prepared by the use of new cross-linked copolymers of acrylamide and methyl acrylate. Since the enzyme derivatives were obtained as water-insoluble particles, they were easily recovered by filtration from reaction mixtures and packed directly in columns possessing enzymatic activities.

The loss in esteratic activity of trypsin under the same conditions used for the trypsin coupling (Table I) suggests that free trypsin is partly inactivated at pH 7.5 even at 4°. Since both inactive and active forms of trypsin are coupled to the azide derivative, most of the coupled trypsin can be considered to maintain its original activity after binding to the polymer particles.

The relatively high stability of the trypsin derivatives (Table I) can be attributed to the prevention of autodigestion and thermal denaturation as a result of the fixation of trypsin molecules on the separate sites of the copolymer. The covalent bonding between trypsin molecules and the polymer chains is demonstrated by the fact that there was no appreciable change of the esteratic activity of the trypsin derivative after successive washings. If trypsin molecules are merely adsorbed in the polymer matrix, a marked drop in activity could be expected after each washing.

Levin, *et al.*,[21] have reported that trypsin coupled to a copolymer of maleic anhydride and ethylene showed 42–77% of the esteratic activity of crystalline trypsin on the basis of coupled protein. Table I shows that the activity of trypsin coupled to the copolymer of acrylamide and methyl acrylate and of free trypsin exposed to the coupling conditions without polymer was about 40% of that of crystalline trypsin under the assay conditions used.

The higher amount of acylase coupled to poly(methyl acrylate) (Table II) appears to be due to the increase of coupling sites (azide groups from methyl acrylate hydrazide residues) on the polymer chains. Since the azide derivatives decompose gradually in water, a high concentration of acylase in an initial coupling mixture favors a high yield of coupling. The higher pH value in the coupling mixture seems to increase the amount of coupled protein (Table II, A-48-1 and -3 *vs.* A-48-2). But an appropriate pH must be chosen from considerations of the rate of the coupling reaction of the azide derivative with the enzyme and the stability of the enzyme in the coupling mixture.

Comparison of the effect of heat inactivation between free acylase and the acylase derivative indicates that the acylase coupled to the copolymer is more resistant to thermal denaturation than free acylase. In this connection, an acylase which was insolubilized by a copolymerization with *N*-carboxy-γ-methylglutamate anhydride was reported to be inactivated by heating at 100°.[14]

The substrate specificity of the acylase coupled to the copolymers seems not to have changed significantly (Table III). The lower activity of the acylase coupled to the poly(methyl acrylate) (Table III) may be due to the replacement of the acrylamide residues which have a hydrophilic nature with methyl acrylate residues. The rate of hydrolysis by the coupled acylase was reduced when the reaction mixtures were stirred very slowly. This observation suggests that the reaction of water-insoluble acylase derivative is diffusion controlled by the accessibility of substrate to the coupled enzyme. Therefore, a stirred-batch reaction would be more efficient than a column.

The isolation of L-amino acids in high yield demonstrates that the optical specificity of the coupled acylase was not impaired by coupling to the copolymers and that *N*-acetyl-L-amino acids were selectively hydrolyzed by the insoluble acylase derivatives. Use of highly purified acylase should permit the preparation of more active acylase derivatives.

From the above, it can be concluded that hydrophilic acrylamide residues in the carrier polymers have a stabilizing effect on coupled trypsin and acylase. Since this effect of acrylamide is expected to be true for many other enzymes, our method could be applicable for preparations of other active enzyme derivatives. The preparation of the acrylamide carrier by emulsion polymerization of acrylamide and methyl acrylate followed by reaction with hydrazine and nitrous acid is a simple, inexpensive, direct method to prepare small water-insoluble particles which readily couple to proteins to give active water-insoluble polyacrylamide–enzyme derivatives.

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