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Water-Insoluble Enzymes. Synthesis of a New Carrier and Its Utilization for Preparation of Insoluble Derivatives of Papain, Trypsin, and Subtilopeptidase A*

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ABSTRACT: A new type of highly insoluble polyfunctional, diazotizable resins, (dialdehyde)–starch–methylenedianiline (S-MDA), could be prepared by the condensation of dialdehyde starch (a commercially available periodate oxidation product of starch) with *p,p'*-diaminodiphenylmethane (MDA) and the subsequent reduction of the Schiff's base-type polymeric product. The S-MDA resins following diazotization were coupled with papain and mercuripapain, subtilopeptidase A (subtilisin Carlsberg), and polytyrosyl trypsin, giving highly active water-insoluble derivatives of these enzymes. The S-MDA resins had diazotization capacities of 0.26–0.33 mequiv/g and a protein binding capacity of about 100 mg of protein/g. The water-insoluble S-MDA–protein conjugates had particulate form, were easily filtered and could be conveniently used in columns.

Amino acid analysis of acid hydrolysates of S-MDA derivatives of papain, subtilopeptidase A, and polytyrosyl trypsin showed that the tyrosine, arginine, and lysine contents of the insoluble derivatives were considerably lower than the respective values obtained for the native enzymes. It was assumed that the missing amino acids represented the amino acid residues through which the covalent bonds between the protein and the diazotized carrier were formed. The pH–activity profiles of S-MDA–papain, S-MDA–subtilopeptidase A, and S-MDA–polytyrosyl trypsin, acting on benzoyl-glycine ethyl ester, acetyl-L-tyrosine ethyl ester, and benzoyl-L-arginine ethyl ester, respectively, were displaced toward more alkaline pH values by one to two pH units, as compared with the native enzymes. This effect was found to be essentially independent of the ionic strength of the medium.

Enzyme derivatives in which the biologically active protein is covalently bound to a water-insoluble polymeric carrier may serve as easily removable reagents of considerably improved shelf-stability. Immobilized enzyme derivatives are well suited for repeated or continuous use. Immobilized derivatives of proteolytic enzymes have been successfully applied in the limited digestion of proteins (Goldstein and Katchalski, 1968), and in column form for the isolation and purification of specific enzyme inhibitors (Fritz *et al.*, 1968). The methods which have been utilized for the immobilization of proteins have been recently summarized in several reviews (Silman and Katchalski, 1966; Goldstein and Katchalski, 1968; Goldstein, 1969).

A method that has been widely used for both the preparation of water-insoluble enzyme derivatives and in the preparation of immunoadsorbents utilized the coupling reaction between a protein and the polydiazonium salt derived from a water-insoluble resin such as poly-*p*-aminostyrene (Grubhofer and Schleith, 1954), *p*-aminobenzylcellulose (Campbell *et al.*, 1951), and more recently a *p*-amino-DL-phenylalanine-L-leucine copolymer (Bar Eli and Katchalski, 1963). The recoveries of enzymic activity in the insoluble derivatives when diazotized poly-*p*-aminostyrene or *p*-aminobenzylcellulose were used as carriers were in general rather low. The diazotized *p*-amino-DL-phenylalanine–L-leucine copolymer has been successfully used for the preparation of highly active water-insoluble derivatives of polytyrosyl trypsin (Bar Eli and Katchalski, 1960, 1963), papain (Cebra *et al.*, 1961; Silman *et al.*, 1966), and urease (Riesel and Katchalski, 1964). The rather elaborate procedures involved in the preparation of the *p*-amino-DL-phenylalanine–L-leucine copolymer, however, precluded its wider application, despite its superior properties as a carrier.

In the present article, a new, readily synthesized, diazotizable resin, S-MDA,[1] is described. The S-MDA resins were prepared by the condensation of dialdehyde–starch, DAS[2] (a commercially available periodate-oxidation product of starch) with *p,p'*-diaminodiphenylmethane (bismethylenedianiline, MDA) and the subsequent reduction of the Schiff's base polymeric product (Scheme I). Diazotized S-MDA resins were employed for the preparation of water-insoluble derivatives of papain, trypsin, and subtilopeptidase A (subtilisin Carlsberg) of high enzymic activity. Preparations containing up to 10%

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[1] The name of the resin, S-MDA, is derived from the names of its chemical components: (dialdehyde)–starch–methylenedianiline (see Scheme I).

[2] Abbreviations used are: DAS, dialdehyde starch; MDA, *p,p'*-diaminodiphenylmethane (methylenedianiline); PAB-cellulose, *p*-aminobenzylcellulose; EMA, ethylene–maleic acid copolymer.

by weight of bound protein could be prepared using the S-MDA resins as carriers. The water-insoluble S-MDA–protein conjugates had particulate form, were easily filtered, and could be conveniently used in columns.

Materials and Methods

Trypsin, twice crystallized, salt free, and lyophilized, and papain, twice crystallized in suspension in 0.05 M acetate buffer, pH 4.5, were purchased from Worthington Biochemical Corp., Freehold, N. J. Subtilopeptidase A (subtilisin Carlsberg), crystalline, was a gift from Novo Industries, Copenhagen, Denmark. Dialdehyde starch, Sumstar 190, the periodate-oxidation product of starch (90% oxidized), was obtained from Miles Laboratories Inc., Elkhart, Ind. *p*-Aminobenzylcellulose (Cellex PAB) was obtained from Bio-Rad Laboratories, Richmond, Calif. The exchange capacity of the resin given by the manufacturer was 0.35 mequiv/g. *N*-Carboxy-L-tyrosine anhydride was prepared by the method of Berger *et al.* (1958). Bz-L-ArgOEt and *N*-Ac-L-TyrOEt were purchased from Miles Laboratories Inc., Elkhart, Ind. Casein, Hammersten quality, was obtained from Nutritional Biochemical Corp. Cleveland, Ohio. MDA, sodium borohydride, and all other materials were of the best grade available.

Polytyrosyl trypsin was prepared by the procedure of Glazer *et al.* (1962). The enrichment in tyrosine was determined by amino acid analysis (see below). The polytyrosyl sample used in this study contained about 20 additional tyrosyl residues per mole of trypsin (see Table V). The specific activity of polytyrosyl trypsin sample was 20 esterase units per mg (the specific activity of the starting material, crystalline trypsin, was 35 esterase units per mg, *vide infra*).

Mercuripapain was prepared by a modification of the procedure of Kimmel and Smith (1958). A suspension of crystalline papain (1 ml; 20–30 mg/ml; 13–18 esterase units per mg) was added to a solution 0.005 M in cysteine–0.002 M in EDTA, pH 7 (20 ml). The mixture was incubated at 37° for 15 min. The solution of activated papain was brought to pH 5.5 with 0.1 N HCl and a solution of mercuric chloride (33 mg of $HgCl_2$, dissolved in 1 ml of distilled water and adjusted to pH 5.5) was added dropwise with stirring. The reaction mixture was stirred for a few minutes, transferred to a dialysis tube (Visking Cellophane Tubing, size 23/32), and dialyzed exhaustively against 0.01 M acetate buffer, pH 5.5 (5 l.), at 4°. In the course of the dialysis varying amounts of insoluble material of very low enzymic activity separated out. The dialyzed mercuripapain solution was clarified by centrifugation and used directly in the binding experiments. The protein content of the mercuripapain solution was estimated from its absorbance at 280 mμ using an $E_{280}^{1\%}$ value of 24.6 (Whitaker and Bender, 1965). The recovery of protein varied with the papain sample used and was in the range of 60–85%. The recovery of enzymic activity was 70–95%. The specific activity of the mercuripapain preparations was 18–24 esterase units per mg of protein.

Preparation of S-MDA Resins. Dialdehyde starch, Sumstar-190 (10 g), was suspended in water (200 ml) and stirred at room temperature for 10–15 min to obtain a fine slurry and 2 M carbonate buffer, pH 10.5 (40 ml), was then added. The suspension was then poured slowly into a vigorously stirred 10% solution of MDA in methanol (300–500 ml). The reaction mixture was stirred at room temperature for 2–3 days. The insoluble, polymeric Schiff's base thus obtained was separated on a Büchner funnel, washed with methanol, and then suspended in water and reduced with sodium borohydride (40 g) with stirring at room temperature for 18 hr. The reaction mixture was brought to neutral pH with acetic acid. The resin was separated on a funnel, washed with water and methanol, and then refluxed with methanol (3–4 changes of solvent, 300 ml each) to remove methanol-soluble aromatic amines. The material was filtered and air dried. The total weight of dry resin was 14–15 g. The nitrogen content of the S-MDA resins was 6.5–6.9% (see Table I).

Diazotization of the S-MDA Resin. S-MDA resin (100 mg) was suspended in 50% acetic acid (8 ml) and stirred for 1 hr at 4°. An aqueous solution of sodium nitrite (20 mg in 1 ml) was then added dropwise to the chilled suspension. The diazotization mixture was stirred for 1 hr over ice and then brought to pH 8.5 by the dropwise addition of 5 N NaOH, crushed ice being added as needed to keep the temperature down. The polydiazonium salt separated as a dark brown, lumpy precipitate. The precipitate was separated on a Büchner funnel and washed with cold 0.2 M phosphate buffer, pH 7.8. The wet polydiazonium salt was suspended in the same buffer and used directly in the coupling experiments (see below).

Determination of the Diazotization Capacity of the S-MDA Resins. The diazotization capacity of the S-MDA resins was estimated by coupling their polydiazonium salt with *p*-bromophenol and determination of the nitrogen and bromine content of the reaction product; S-MDA resin (50 mg) was diazotized as described above. The washed polydiazonium salt precipitate was suspended in cold 0.2 M phosphate buffer, pH 7.8 (5 ml). An aqueous solution of *p*-bromophenol (50 mg, dissolved by the dropwise addition of 2 N NaOH) was then added with stirring to the chilled suspension. The reaction mixture was stirred overnight at 4°. The precipitate was separated on a funnel, washed with 0.1 M carbonate buffer, pH 10.5, water, and then methanol, and dried *in vacuo* over phosphorus pentoxide. The nitrogen and bromine contents of the dry material were determined by the Dumas and Schöniger combustion methods (Steyermark, 1961), respectively. From these two values and a knowledge of the nitrogen content of the S-MDA resin the diazotization capacity of the various resins could be estimated (see Table I).

Determination of the Protein Capacity of the S-MDA Resins. The maximal binding capacity of the S-MDA resins for a specific protein was estimated from the binding curves obtained by coupling the polydiazonium salt derived from the S-MDA resin (50 mg) with varying amounts of the protein (1–20 mg). (For the procedures employed with specific proteins, see below.) The enzymic activity of the insoluble enzyme derivatives was determined. The amount of bound, enzymically active protein was calculated from these values. The data were plotted as amount of active bound protein *vs.* amount of protein in the coupling mixture. The maximal binding capacity of the S-MDA resin for a specific protein was estimated from the region where the binding curve leveled off (see Figure 1 and Table II).

The maximal binding capacities of the S-MDA resins estimated from the binding curves were confirmed by amino acid analysis (see below) of acid hydrolysates of the appropriate saturated S-MDA–protein sample (see Table II).

Preparation of Water-Insoluble S-MDA–Protein Conjugates. (*a*) *Water-Insoluble S-MDA–Subtilopeptidase A Derivative.*

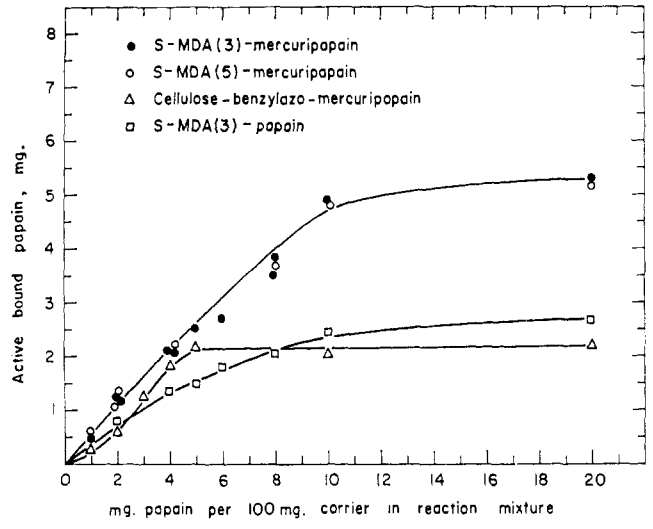


FIGURE 1: Binding curves of papain and mercuripapain with S-MDA (3), S-MDA (5), and PAB-cellulose. The polydiazonium salt derived from 50 mg of the appropriate resin was coupled with varying amounts of papain or mercuripapain (1–20 mg). The amounts of bound, enzymically active protein were estimated from the esterase activities of the water-insoluble enzyme preparations obtained (for details, see Experimental Section).

S-MDA resin (100 mg) was diazotized as described. The wet polydiazonium salt precipitate was suspended in cold 0.2 M phosphate buffer, pH 7.8 (4 ml). A solution of subtilopeptidase A (15 mg) in the same buffer (3 ml) was slowly added. The reaction mixture was stirred overnight at 4°. The water-insoluble S-MDA–subtilopeptidase A derivative was separated on a funnel, washed with 1 M KCl (200 ml), and then with deionized water (100 ml) and resuspended in water or 0.1 M phosphate buffer, pH 7.5.

The recovery of enzymic activity (tested with *N*-Ac-L-TyrOEt in the water-insoluble subtilopeptidase A derivative was 15–20%.

(*b*) *Water-Insoluble S-MDA–Papain Conjugates.* The S-MDA resin (100 mg) was diazotized as described. The wet polydiazonium salt precipitate was suspended in cold 0.2 M phosphate buffer, pH 7.8 (6 ml). A solution (about 10 ml) of mercuripapain or papain (8–10 mg) was added slowly. The reaction mixture was stirred overnight at 4°. The water-insoluble S-MDA–papain conjugate was separated on a funnel, washed with 1 M KCl (200 ml), deionized water (100 ml), and resuspended in water or 0.1 M phosphate buffer, pH 7.0.

The recovery of enzymic activity (tested with Bz-L-ArgOEt) in the water-insoluble derivative was about 60% when mercuripapain was used. With crystalline papain the recovery of enzymic activity was only 20–30%.

(*c*) *Water-Insoluble S-MDA–Polytyrosyl Trypsin Conjugate.* The S-MDA resin (100 mg) was diazotized as described. The washed polydiazonium salt precipitate was suspended in cold 0.2 M phosphate buffer, pH 7.8 (10 ml). A solution of polytyrosyl trypsin (30 mg) in 0.001 N HCl (8 ml) was then slowly added to the chilled suspension and the reaction mixture was stirred overnight at 4°. The water-insoluble S-MDA–polytyrosyl trypsin conjugate was separated on a funnel, washed with 1 M KCl (200 ml), with deionized water (100 ml), and resuspended in water or in 0.1 M phosphate buffer, pH 7.0.

The recovery of enzymic activity (tested with Bz-L-ArgOEt) in the water-insoluble derivative was about 40%.

Determination of the Protein Content of the S-MDA Conjugates of the Various Enzymes. The S-MDA–protein conjugate (10–12 mg) was hydrolyzed in an evacuated, sealed tube using 6 N HCl (24 hr, 110°). Phenol (5 μl) was added to each tube to prevent halogenation of tyrosine. The acid was evaporated and the residue suspended in 0.2 M citrate buffer, pH 2.2 (4 ml). Insoluble, colored material was removed by strong centrifugation (Sorval, 12,000 rpm) and amino acid analysis was carried out employing an automatic amino acid analyzer (Spackman, 1967). The amount of protein was calculated from the amounts of alanine, leucine, glycine, and valine (Dayhoff, 1969).

After the analysis, the amino acid column was regenerated with five times the volume of 0.2 N NaOH normally employed in order to remove colored diazotization products adsorbed on the column. After analysis of mercuripapain derivatives, the amino acid analyzer column was also washed with 50 ml of a 1% cysteine solution to remove mercuric salts adsorbed on the column.

Preparation of Cellulose–Benzylazoprotein Conjugates. Water-insoluble protein derivatives, prepared by coupling the protein to the polydiazonium salt derived from commercial PAB-cellulose, were found to liberate color into aqueous buffers. This deficiency of PAB-cellulose could be partly overcome by refluxing the resin with methanol for about 30 min and air drying prior to diazotization.

The diazotization of PAB-cellulose and the coupling of its polydiazonium salt to various proteins were carried out as described for the S-MDA resins. The following amounts of protein were coupled to the polydiazonium salt derived from 100 mg of PAB-cellulose: papain or mercuripapain, 4–5 mg; polytyrosyl trypsin, 10 mg; subtilopeptidase A, 4–5 mg. The recoveries of enzymic activity in the insoluble precipitates were as follows: cellulose-benzylazomercuripapain, 50%; cellulose-benzylazopapain, about 15%; cellulose-benzylazopolytyrosyl trypsin, about 50%; cellulose-benzylazosubtilopeptidase A, less than 10%.

The diazotization and protein binding capacities of PAB-cellulose were determined as described for the S-MDA resins.

Determination of Enzyme Activities. The activities of the various enzymes and of their water-insoluble S-MDA and cellulose-benzylazo derivatives were determined at 25° by the pH-Stat method (Jacobsen *et al.*, 1957), using the appropriate ester substrates (*vide infra*). The pH was controlled with an automatic titration assembly (SBR-2c titrigraph and TTT-1c titrator, Radiometer, Copenhagen). NaOH (0.1 N) was used as titrant. The activities, calculated from the initial rates of substrate hydrolysis, were expressed in esterase units. One unit of esterase activity was defined as that amount of enzyme which catalyzed the hydrolysis of 1 μmole of substrate/min under the specified assay conditions.

Papain and mercuripapain were assayed at pH 6.3 using Bz-L-ArgOEt as substrate (5 ml; 0.05 M Bz-L-ArgOEt–0.005 M cysteine–0.002 M EDTA) (Smith and Parker, 1958). The specific activity of the papain samples used was 13–16 esterase units/mg.

Subtilopeptidase A was assayed at pH 8.6 using Ac-L-TyrOEt as substrate (5 ml; 0.018 M *N*-Ac-L-TyrOEt, 0.01 M in KCl) (Glazer, 1967). The specific activity of the subtilopeptidase A sample used was 800 esterase units/mg.

TABLE I: Composition and Properties of S-MDA Resins Prepared under Different Conditions.

Sample	Weight Ratio of Dialdehyde Starch to MDA in Reaction Mixture	Yield of S-MDA Resin per Gram of Dialdehyde Starch in Reaction Mixture	S-MDA Resin			
			Nitrogen Content[a] (%)	MDA Content[b] (moles/g)	Diazotization Capacity[c] (equiv/g)	Fraction of MDA in Resin Available for Diazotization (%)
S-MDA 1	1:1	0.8	5.2	1.86×10^{-3}	0.125×10^{-3}	6.7
S-MDA 2	1:2	1.18	6.4	2.3	0.180	7.8
S-MDA 3	1:3	1.4	6.8	2.43	0.26	10.8
S-MDA 4	1:4	1.4	6.5	2.32	0.29	12.5
S-MDA 5	1:5	1.4	6.9	2.45	0.33	13.5
PAB-cellulose			1.51		0.088×10^{-3}	

[a] Determined by the Dumas combustion method (see Experimental Section). [b] Calculated from the nitrogen content of the resin. [c] Estimated by coupling the diazotized resin with *p*-bromophenol and determining the nitrogen and bromine contents of the reaction product (see Experimental Section).

Trypsin and polytyrosyl trypsin were assayed at pH 8 using Bz-L-ArgOEt as substrate (5 ml; 1.16×10^{-2} M Bz-L-ArgOEt, 0.01 M in KCl) (Laskowski, 1955). The specific activity of the trypsin samples used was 35 esterase units/mg.

S-MDA–papain and cellulose–benzylazopapain were assayed as described for papain.

S-MDA–subtilopeptidase A and cellulose–benzylazosubtilopeptidase A were assayed as described for subtilopeptidase A, at pH 9.4 (the optimal pH of these derivatives, *vide infra*).

S-MDA–polytyrosyl trypsin and cellulose–benzylazopolytyrosyl trypsin were assayed as described for trypsin, at pH 10 (the optimal pH of these insoluble polytyrosyl trypsin derivatives, *vide infra*).

The protease activities of trypsin, polytyrosyl trypsin, papain, subtilopeptidase A, and of the water-insoluble S-MDA and cellulose–benzylazo derivatives of these enzymes, were determined at pH 7.5, 37°, by the casein digestion method (Kunitz, 1947; Laskowski, 1955). The amounts of enzyme or insoluble enzyme derivative added to the digestion mixture were expressed in units of esterase activity. The reaction mixtures containing water-insoluble enzyme derivatives were stirred magnetically to ensure effective mixing of the reagents.

Results

The Preparation and Properties of the S-MDA Resins. The conditions under which S-MDA resins of maximal capacity could be obtained were determined by preparing a series of S-MDA samples at weight ratios of dialdehyde starch to MDA in the reaction mixture, varying from 1:1 to 1:5. The yields, nitrogen contents, and diazotization capacities of the materials were determined as described in the experimental section. The data are presented in Table I. For comparison, the nitrogen content and diazotization capacity of *p*-aminobenzylcellulose are also given. The data in Table I show that the MDA content of the S-MDA resins, calculated from nitrogen analysis, increased with increasing MDA to DAS ratio in the reaction mixture and reached its limiting value (~2.5 mmoles of MDA/g of resin) at a ratio of MDA:DAS of 3:1. The diazotization capacity of the S-MDA resins also increased with increasing MDA to dialdehyde starch ratio, asymptoting to a limiting diazotization capacity of about 0.4 mequiv/g of resin (see Table I). The MDA content of the limiting-capacity samples [S-MDA (3), S-MDA (4), and S-MDA (5)] was about 48% by weight. This finding was in reasonable agreement with the total yields of resin reported in Table I (1.4 g of resin/g of dialdehyde starch in the reaction mixture). From the MDA content of the S-MDA resins, it could be estimated that about one MDA molecule was incorporated in the resin per hexose residue.

In the studies reported below only the limiting-capacity resins [S-MDA (3) to S-MDA (5)] (Table I) were investigated.

It should be noted that the experimentally determined diazotization capacity of the PAB-cellulose batch employed in this investigation (0.088 mequiv/g, Table I) was only about 25–30% of that of the limiting-capacity S-MDA samples [S-MDA (3) to S-MDA (5)]; moreover, it was considerably lower than the value for the exchange capacity of PAB-cellulose given by the manufacturer (0.35 mequiv/g).

The protein binding capacities of the S-MDA resins and of PAB-cellulose were estimated by coupling the polydiazonium salt (see Experimental Section) derived from the appropriate resin with varying amounts of papain, mercuripapain, polytyrosyl trypsin, or subtilopeptidase A. The data were plotted as the amount of active bound protein (estimated by a rate assay) *vs.* the amount of protein in the coupling mixture (see Experimental Section). Such curves for S-MDA–papain, S-MDA–mercuripapain, and cellulose–benzylazomercuripapain are shown in Figure 1. The maximal binding capacity of a resin for a specific protein was estimated from the region where the binding curve leveled off.

The maximal binding capacities of the S-MDA resins cal-

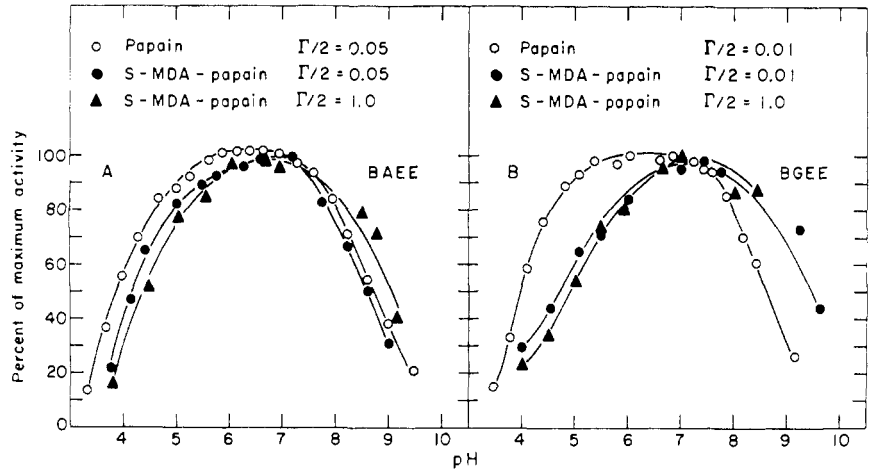


FIGURE 2: pH–activity curves at different ionic strengths (Γ/2) for papain and S-MDA–papain acting on: (A) benzoyl-L-arginine ethyl ester (BAEE) and (B) benzoylglycine ethyl ester (BGEE). The reaction mixtures (5 ml) were 0.05 M in BAEE (frame A) or 0.015 M in BGEE (frame B), and 0.005 M in cysteine and 0.002 M in EDTA. Amounts of papain or S-MDA–papain equivalent to 2 esterase units were used for each assay in A, and 4 esterase units for each assay in B. Substrate hydrolysis was determined potentiometrically using 0.1 N NaOH as titrant. The ionic strength was adjusted with KCl. The experimentally determined rates were not corrected for the incomplete ionization of the carboxyl group of the products at low pH values.

TABLE II: Binding Capacities of S-MDA Resins and PAB-Cellulose for Various Enzymes.

Water-Insoluble Enzyme Derivative	Protein Content: Calculated from Binding Curves[a] (mg/100 mg)	Protein Content: Calculated from Amino Acid Analysis[a] (mg/100 mg)	Active Bound Protein[b] (mg)	Active Bound Protein / Total Bound Protein × 100: Calculated from Binding Curves	Active Bound Protein / Total Bound Protein × 100: Calculated from Amino Acid Analysis
S-MDA (3) papain	10		2.5	25	
S-MDA (3) mercuripapain	10	9.7	5.3	53	55
S-MDA (5) mercuripapain	10	9.8	5.3	53	54
PAB-cellulose mercuripapain	4.5		2.1	47	
S-MDA (3) polytyrosyl trypsin	30	24.2	12	40	50
PAB-cellulose polytyrosyl trypsin	10		3.3	33	
S-MDA (3) subtilopeptidase A	8	5.7	2.4	30	42
PAB-cellulose subtilopeptidase A					

[a] Details described in Experimental Section. [b] Estimated from the esterase activity of the immobilized enzyme preparation.

culated from the binding curves were confirmed by amino acid analysis of the acid hydrolysate of the appropriate saturated S-MDA–protein conjugate. The data are summarized in Table II.

The data of Figure 1 and Table II show that the maximal protein binding capacity of S-MDA resins was two- to threefold higher than that of PAB-cellulose (analogous differences in the diazotization capacities of these resins were pointed out in the preceding section—see Table I). The fraction of the enzyme activity retained by the bound protein was, however, only slightly lower for PAB-cellulose (Table II). The capacities of the S-MDA resins calculated from the binding curves were in good agreement with those calculated from amino acid analysis of saturated S-MDA—protein conjugates (Table II).

No difference in protein binding capacity could be found between two S-MDA samples of relatively high diazotization capacity (designated in the preceding section as limiting diazotization capacity resins—see data in Table I). Thus, S-MDA (3) (of diazotization capacity 0.26 mequiv/g, Table I) and S-MDA (5) (of diazotization capacity 0.33 mequiv/g) gave identical binding curves with mercuripapain and attained the same saturation protein content—10 mg of protein/100 mg of resin (see Figure 1).

In the comparative studies on the preparation and properties of water-insoluble S-MDA and PAB-cellulose conjugates of papain, trypsin, and subtilopeptidase A described below, S-MDA (3) (see Tables I and II) was used routinely unless specified otherwise. In these investigations only saturated samples were employed.

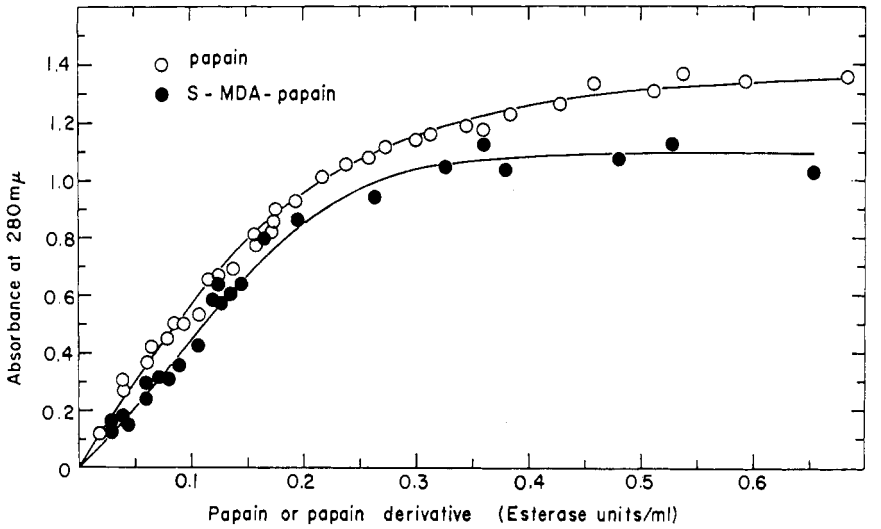


FIGURE 3: Digestion of casein by papain and S-MDA–papain. The test solutions (2 ml) contained 5 mg/ml of heat-denatured casein in 0.05 M phosphate buffer, pH 7.5. The reaction mixtures were incubated at 37° with the specified amount of papain or S-MDA–papain (expressed in esterase units (see above)) for 20 min; 3 ml of a 10% solution of trichloroacetic acid was then added, the precipitate was filtered off, and the absorbance at 280 mμ of the trichloroacetic acid soluble fraction was determined.

Water-Insoluble S-MDA–Papain Conjugates. The coupling of crystalline papain to the polydiazonium salts derived from S-MDA resins or PAB-cellulose led to insoluble derivatives in which the recovery of enzymic activity varied considerably with different batches of enzyme. In order to increase the reproducibility of the binding procedures as well as the recovery of activity in the insoluble papain derivatives, the mercury-blocked enzyme, mercuripapain (Kimmel and Smith, 1958), was employed as starting material.

The binding curves for papain with S-MDA (3) and for mercuripapain with S-MDA (3) and S-MDA (5) and with PAB-cellulose are shown in Figure 1. The data show that the saturation binding capacity of these two S-MDA resins [S-MDA (3) and S-MDA (5)] for mercuripapain was the same and amounted to about 10 mg of protein/100 mg of resin (Table II). A similar value was obtained for the binding capacity of S-MDA (3) for crystalline papain. The saturation binding capacities of S-MDA resins estimated from the binding curves were confirmed by amino acid analysis of acid hydrolysates of saturated S-MDA-mercuripapain preparations (Table II). The enzymic activity retained by the bound protein was 55–60% when mercuripapain was used; it was only 25–30% with crystalline papain (Table II).

The saturation binding capacity of PAB-cellulose was about 4.5 mg of mercuripapain/100 mg of resin. The activity retained by the bound protein was about 45% (Table II).

The pH–activity profile of S-MDA–papain, using Bz-L-ArgOEt as substrate, was similar to that of crystalline papain (Figure 2A). The pH optimum was in the region of pH 6.0 to 6.5. When BzGlyOEt was used as substrate (at $\Gamma/2 = 0.01$) the pH–activity curve of S-MDA–papain was displaced toward more alkaline pH values by about one pH unit with an optimum at pH 7.0–7.5 (Figure 2B). The displaced pH–activity profile was essentially unaffected by increasing the ionic strength. The apparent Michaelis constant, $K_{m,app}$, of S-MDA–papain, with Bz-L-ArgOEt as substrate, [$K_{m,app} \approx 1.9 \times 10^{-2}$ M at pH 6.3] was similar to that of crystalline papain (Whitaker and Bender, 1965). With Bz-GlyOEt as substrate, the value of the Michaelis constant of S-MDA-papain [$K_{m,app} \approx 3.4 \times 10^{-2}$ M at pH 7.0, the optimal pH] was slightly higher than that determined for crystalline papain at its optimal pH [$K_{m,app} \approx 1.8 \times 10^{-2}$ M] (Whitaker and Bender, 1965). The kinetic behavior of a cellulose–benzylazopapain preparation paralleled that of S-MDA–papain.

The caseinolytic activity of S-MDA–papain was similar to that of the crystalline enzyme (Figure 3). The limiting 280-mμ absorbance, attained with large excess of enzyme, was lower by about 20% for the insoluble papain derivative.

The activity retained by an S-MDA–mercuripapain preparation after incubation for 30 min at 37°, at various pH

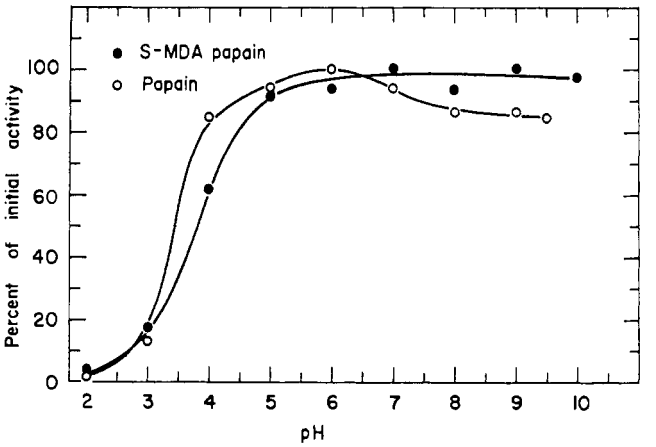


FIGURE 4: Effect of pH on the stability of papain and S-MDA–papain. The test solutions (0.5 ml) in the appropriate buffer and containing papain or S-MDA–papain (about 15 esterase units/ml) were incubated at 37° for 30 min; 0.1-ml aliquots were withdrawn and the esterase activity was determined by the standard procedure (see Experimental Section). The following buffer solutions were used to cover the pH range investigated: pH 3.0–6.3, 0.005 M citrate–phosphate; pH 7.0–9.0, 0.05 M Tris; pH 10.0–10.7, 0.05 M carbonate–bicarbonate.

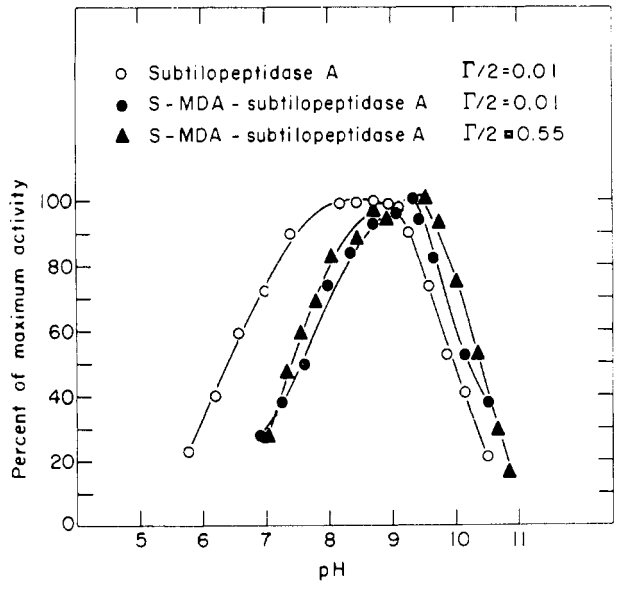


FIGURE 5: pH–activity curves for subtilopeptidase A and S-MDA–subtilopeptidase A acting on acetyl-L-tyrosine ethyl ester at different ionic strengths (Γ/2). The reaction mixtures (5 ml) were 0.018 M in *N*-Ac-LTyrOEt and 0.01 M in KCl (○ and ●) or 0.5 M in KCl and 0.02 M in borate (▲). Amounts of subtilopeptidase A equivalent to 2 esterase units were used for each assay. Substrate hydrolysis was determined potentiometrically using 0.1 N NaOH as titrant.

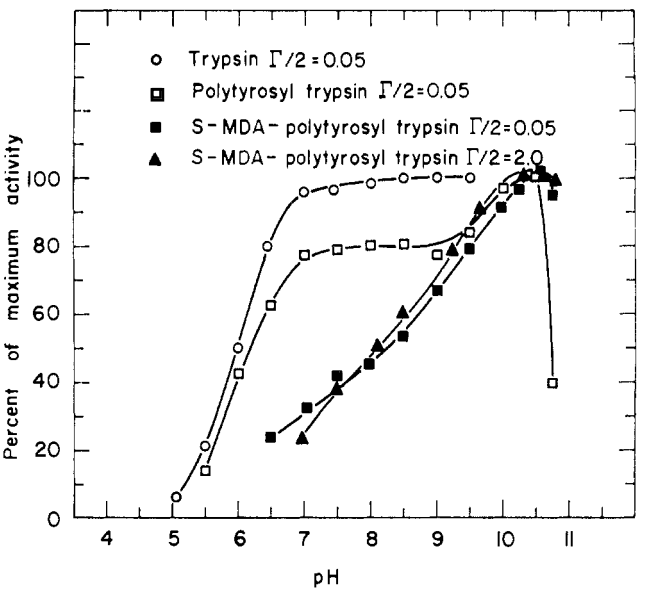


FIGURE 7: pH–activity curves for trypsin, polytyrosyl trypsin, and S-MDA–polytyrosyl trypsin acting on benzoyl-L-arginine ethyl ester at different ionic strengths (Γ/2). The reaction mixture (5 ml) was 1.16×10^{-2} M in Bz-L-ArgOEt and 0.05 M, or 2 M, in KCl. Amounts of trypsin, polytyrosyl trypsin, and S-MDA–polytyrosyl trypsin equivalent to 2 esterase units were used for eash assay. Substrate hydrolysis was determined potentiometrically using 0.1 N NaOH as titrant.

values is shown in Figure 4. The water-insoluble papain derivative was stable in the pH range 4 to 10. Crystalline papain exhibited a similar pH-stability pattern (Figure 4).

Aqueous suspensions of S-MDA–papain, S-MDA–mercuripapain, and cellulose–benzylazomercuripapain could be stored at 4° for 6 months without significant loss of activity. The cellulose–benzylazomercuripapain suspensions showed a tendency to liberate some yellow color on prolonged storage. On lyophilization, S-MDA–mercuripapain preparations retained about 30% and cellulose–benzylazomercuripapain about 20% of their activity. Dry powders of S-MDA–mercuripapain and cellulose–benzylazomercuripapain could also be obtained by air drying on a Büchner funnel or by washing with 70% methanol followed by air drying. The dry S-MDA–mercuripapain samples thus obtained retained 50–60% of their enzymic activity. Air dried cellulose–benzylazomercuripapain samples retained about 30% of their activity. The dried powders could be stored at 4° for 6 months without loss of activity. Dry samples of S-MDA– and cellulose–benzylazomercuripapain stored at room temperature for 6 months retained about 70% of their activity.

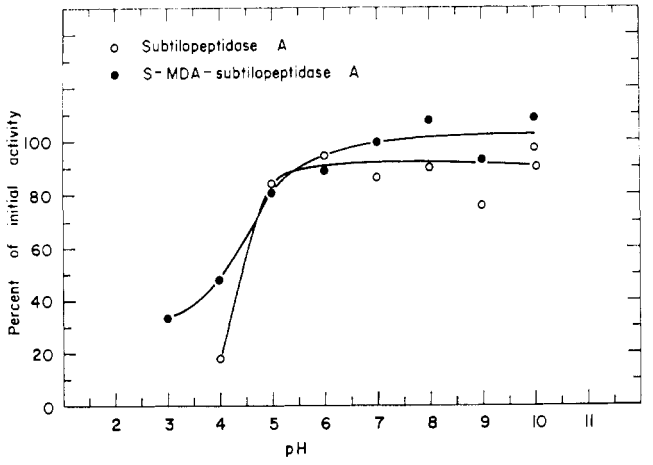


FIGURE 6: Effect of pH on the stability of subtilopeptidase A and S-MDA–subtilopeptidase A. For experimental details, see Figure 4.

Water-Insoluble S-MDA Subtilopeptidase A Conjugates. The saturation binding capacity of S-MDA (3) for subtilopeptidase A, estimated from the appropriate binding curves, was about 8 mg of protein/100 mg of resin. A somewhat lower protein binding capacity (5.7 mg of protein/100 mg of resin) was calculated from the amino acid content of a saturated S-MDA–subtilopeptidase A preparation (Table II). The enzymic activity retained by the bound protein was 30–40% (Table II). When PAB-cellulose was used as starting material for the polydiazonium carrier, the enzymic activity recovered in the insoluble subtilopeptidase A preparations was less than 10%. Cellulose–benzylazosubtilopeptidase A conjugates were therefore not investigated further.

The pH–activity profile of S-MDA–subtilopeptidase A, with *N*-Ac-L-TyrOEt as substrate, was displaced, at low ionic strength (Γ/2 = 0.01), by about one pH unit toward more alkaline pH values, as compared with the crystalline enzyme (Figure 5). The pH optimum of the insoluble subtilopeptidase A derivative was in the range of pH 9.0–9.5. The displaced pH–activity profile was not affected by increasing the ionic strength and the addition of buffer (0.02 M borate, see Figure 5). The apparent Michaelis constant, $K_{m,app}$, of S-MDA–subtilopeptidase A acting on *N*-Ac-L-TyrOEt was 1.7×10^{-2} M (determined at the optimum, pH 9.25). This value was slightly

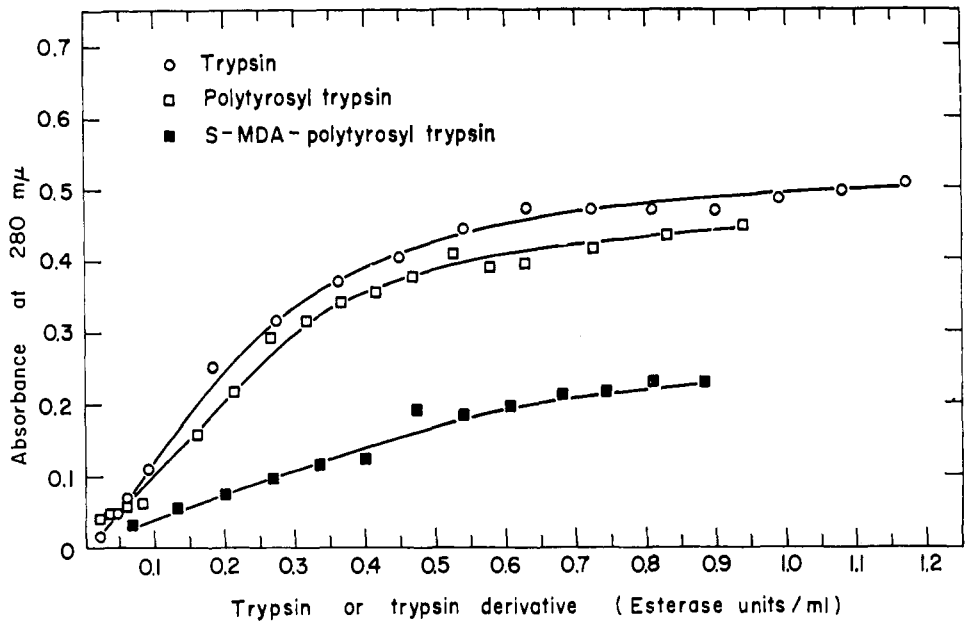


FIGURE 8: Digestion of casein by trypsin, polytyrosyl trypsin, and S-MDA–polytyrosyl trypsin. For experimental details, see Figure 3.

higher than that of $K_{m,app}$ of the crystalline enzyme (0.94×10^{-2} M) (Glazer, 1967).

The initial caseinolytic activity of S-MDA–subtilopeptidase A was about 50% of that of the native enzyme. The limiting absorbance at 280 mμ attained with high concentrations of the insoluble enzyme derivative asymptoted, however, to the value recorded for crystalline subtilopeptidase A.

The activity retained by an S-MDA–subtilopeptidase A preparation after incubation for 30 min at 37°, at various pH values is shown in Figure 6. The water-insoluble subtilopeptidase A derivative was stable in the pH range 5 to 10. Moreover, it was considerably more stable than the crystalline enzyme at low pH values (3–5) (Figure 6).

S-MDA–subtilopeptidase A suspensions in 0.1 M phosphate buffer, pH 7.5, could be stored at 4° for 3 months without significant loss of enzymic activity.

On lyophilization or air drying, S-MDA–subtilopeptidase A preparations were almost completely inactivated.

Water-Insoluble S-MDA–Polytyrosyl Trypsin Conjugates. The direct coupling of trypsin to the polydiazonium salts derived from S-MDA resin or PAB-cellulose led to excessive inactivation of the bound protein. To circumvent this limitation, polytyrosyl side chains were grown onto the protein by initiating the polymerization of *N*-carboxy-L-tyrosine anhydride with the enzyme in an aqueous medium (Glazer *et al.*, 1962; Bar Eli and Katchalski, 1963). Polytyrosyl trypsin, subsequently coupled to the polydiazonium salts derived from S-MDA or PAB-cellulose, gave highly active water-insoluble derivatives.

The saturation binding capacity of the S-MDA (3) resin for polytyrosyl trypsin, estimated from the appropriate binding curves was about 30 mg of protein/100 mg of resin. The protein binding capacity of the S-MDA resin, calculated from the amino acid content of a saturated S-MDA–polytyrosyl trypsin preparation, was 24 mg of polytyrosyl trypsin/100 mg of resin (Table II). The enzymic activity retained by the bound protein was 50% (Table II).

The saturation binding capacity of PAB-cellulose for polytyrosyl trypsin was about 10 mg of protein/100 mg of resin (Table II). The activity retained by the bound protein was about 30%.

The pH–activity profile of S-MDA–polytyrosyl trypsin, with Bz-L-ArgOEt as substrate, was displaced, at low ionic strength ($\Gamma/2 = 0.01$), by about two pH units toward more alkaline pH values, as compared with trypsin or polytyrosyl trypsin (Figure 7). The pH optimum of the insoluble derivative was in the region of pH 10–10.5. The displaced pH–activity profile of S-MDA–polytyrosyl trypsin was essentially unaffected by increasing the ionic strength. Cellulose-benzylazopolytyrosyl trypsin exhibited similar pH–activity characteristics.

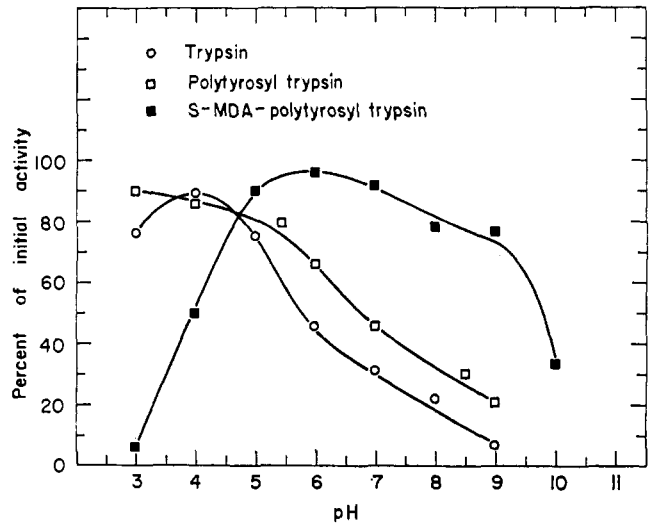


FIGURE 9: Effect of pH on the stability of trypsin, polytyrosyl trypsin, and S-MDA–polytyrosyl trypsin. For experimental details, see Figure 4.

TABLE III: Amino Acid Composition of Papain and S-MDA–Papain.[a]

Amino Acid	Papain		S-MDA–Papain[c]
	Literature[b]	This Study[c]	
Lys	10	9.85	7.6
His	2	1.74	
Arg	12	11.1	4.1
Asp	19	17.8	18.2
Thr	8	7.3	7.95
Ser	13	10.9	14.2
Glu	20	20.2	17.9
Pro	10	10.2	9.8
Gly	28	27.5	27.4
Ala	14	13.9	14.8
Val	18	16.4	14.1
Ile	12	10.9	8.4
Leu	11	11.1	10.7
Tyr	19	19.3	12.1
Phe	4	4.06	3.5

[a] Results are expressed as moles of amino acid per mole of enzyme and are calculated on the basis of the mean values obtained for 14 Ala/mole, 20 Glu/mole, 28 Gly/mole, and 11 Leu/mole. [b] From revised sequence data (Koekoek, 1969). To the original composition given by Light *et al.* (1964), 13 residues were added as a result of X-ray work (Drenth *et al.*, 1968). The composition of the 13 extra residues was established by G. Lowe (private communication) to be: Lys, Arg, Thr, Ser, Glu, Gly, Ala, Val_2, Ile_4. [c] Mercuripapain used (see Experimental Section).

TABLE IV: Amino Acid Composition of Subtilopeptidase A and S-MDA–Subtilopeptidase A.[a]

Amino Acid	Subtilopeptidase A		S-MDA–Subtilopeptidase A
	Literature[b]	This Study	
Lys	9	8.8	7.2
His	5	4.9	4.1
Arg	4	3.6	2.3
Asp	28	24.2	27.9
Thr	19	18.1	17.9
Ser	32	30.2	29.6
Glu	12	12	12.2
Pro	9	10.8	12.5
Gly	36	35.7	37.9
Ala	42	41	39.8
Val	31	29.5	26.6
Met	5	4.4	4.1
Ile	10	8	9.2
Leu	16	16	16.2
Tyr	13	12.4	9.2
Phe	4	3.8	4.1

[a] Results are expressed as moles of amino acid per mole of enzyme and are calculated on the basis of the mean value obtained for 42 Ala/mole, 12 Glu/mole, and 16 Leu/mole. [b] Data of DeLange and Smith (1968).

The caseinolytic activity of S-MDA–polytyrosyl trypsin was about 35% that of crystalline trypsin or polytyrosyl trypsin (Figure 8). The limiting absorbance at 280 mμ attained by the insoluble polytyrosyl trypsin derivative was about 50% of that of trypsin or polytyrosyl trypsin.

The activity retained by S-MDA–polytyrosyl trypsin preparations after incubation for 30 min at 37°, at various pH values is shown in Figure 9. The range of maximum stability of the insoluble polytyrosyl trypsin derivatives was between pH 5 and 9. The stability decreased rather steeply beyond either of these pH values. The region of maximum stability of trypsin and polytyrosyl trypsin, on the other hand, was in the acid pH range. Beyond pH 5, a continuous decrease in their stability was observed. The pH-stability pattern of cellulose-benzylazopolytyrosyl trypsin was similar to that recorded in Figure 9 for the S-MDA derivative.

S-MDA– and cellulose–benzylazopolytyrosyl trypsin suspensions in water or in 0.1 M phosphate buffer, pH 7, could be stored at 4° for 6 months without loss of enzymic activity. The cellulose–benzylazopolytyrosyl trypsin suspensions showed a tendency to liberate some yellow color on prolonged storage.

S-MDA– and cellulose–benzylazopolytyrosyl trypsin preparations were almost completely inactivated on lyophilization or air drying.

Amino Acid Composition of the S-MDA Derivatives of Papain, Polytyrosyl Trypsin, and Subtilopeptidase A. The amino acid composition of acid hydrolysates of S-MDA derivatives of papain, subtilopeptidase A, and polytyrosyl trypsin are given in Tables III, IV, and V. The amino acid compositions determined for the native enzymes and literature values are also given. The data show that the tyrosine, arginine, and lysine contents of the insoluble derivatives were considerably lower than the respective values obtained for the native enzymes; a small decrease in histidine content was also observed in the case of S-MDA–polytyrosyl trypsin.

Experiments on the effects of low molecular weight diazonium salts on proteins have indicated that diazo reagents may couple, in addition to tyrosine and histidine, also to lysine, arginine, serine, and threonine (Howard and Wild, 1957; Sokolovsky and Vallee, 1966, 1967). It has been also shown that acid hydrolysis did not regenerate the free amino acids from monoazo and bisazo derivatives of *N*-acetyltyrosine, *N*-acetylhistidine, and hippuryllysine; moreover, arginine was regenerated in less than stoichiometric amounts from acid hydrolysates of azohippurylarginine derivatives (Sokolovsky and Vallee, 1967). It could therefore be assumed that the amino acid residues of an immobilized protein which participated in the formation of covalent links with the diazotized S-MDA resin could be detected by virtue of their disappearance from the acid hydrolysate.

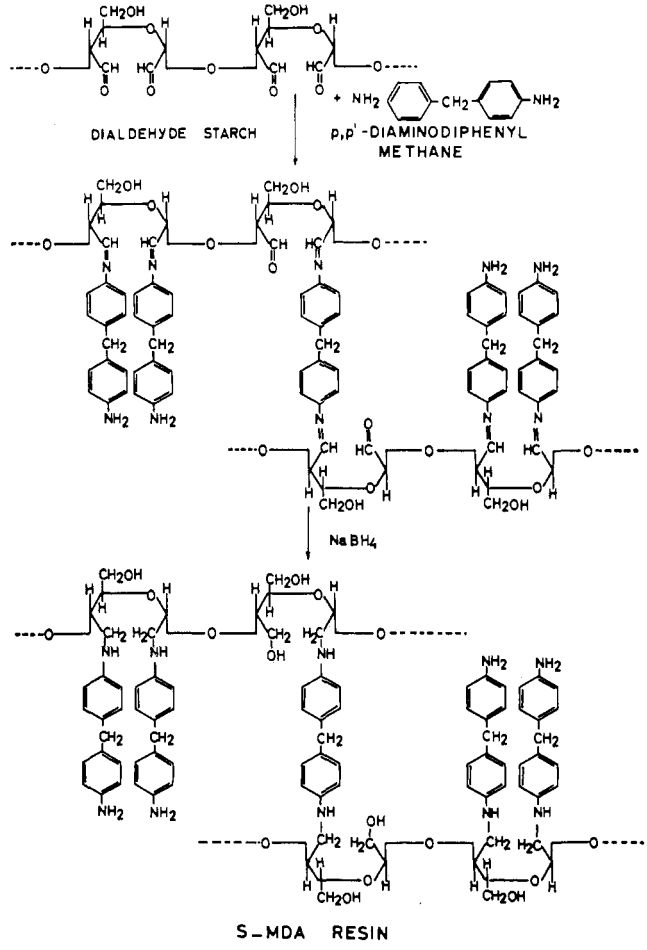
The nature and number of missing amino acids were found to depend on the nature of the immobilized protein. Thus, in the case of S-MDA–papain, 8 arginines, 2 lysines, and 7 tyrosines were found to be missing (Table III). In the case of

TABLE V: Amino Acid Composition of Trypsin, Polytyrosyl Trypsin, and S-MDA–Polytyrosyl Trypsin.[a]

Amino Acid	Trypsin		Poly-tyrosyl Trypsin	S-MDA–Poly-tyrosyl Trypsin
	Litera-ture[b]	This Study		
Lys	14	12	11.9	10.9
His	3	2.4	2.1	1.3
Arg	2	1.5	0.9	
Asp	22	22.1	21.8	20.4
Thr	9	8.65	9.85	9.3
Ser	33	31	33	32
Glu	14	13.7	14.6	13.7
Pro	7	9.15	9.7	8.2
Gly	25	24.9	26.8	27.6
Ala	14	14	14	14
Val	18	11.3	12.4	12
Met	2	1.8	1.8	1.5
Ile	15	11.7	11.8	11.1
Leu	14	13	12.9	12.4
Tyr	10	8.5	28.4	20.7
Phe	2	2.67	2.66	2.5

[a] Results are expressed as moles of amino acid per mole of enzyme and are calculated on the basis of 14 Ala/mole. [b] Data of Walsh and Neurath (1964).

SCHEME I



S-MDA–subtilopeptidase A, 2 lysines, 4 tyrosines, and possibly 2 arginines were missing (Table IV). In the case of S-MDA–polytyrosyl trypsin, 2 arginines, 1 histidine, 8 tyrosines, and 2–3 lysines were missing (Table V).

Discussion

A new type of insoluble, polyfunctional, diazotizable resins, S-MDA, was prepared by the condensation of dialdehyde starch, DAS, with a bifunctional reagent, *p,p'*-diaminodiphenylmethane (methylenedianiline, MDA), and the subsequent reduction of the Schiff's base-type, highly cross-linked, polymeric product (Scheme I).

In reactions between polymeric, polyfunctional molecules, such as DAS, and bifunctional reagents, two types of reactions are known to take place in a competitive fashion: (a) cross-linking of the polymeric chains by the bifunctional reagent, and (b) one-point attachment of the bifunctional reagent. The former reaction predominates when excess polymer is present in the reaction mixture; the latter is enhanced by increasing the concentration of the bifunctional reagent.

The conditions under which S-MDA resins of minimal cross-linking, and thus of maximal diazotization capacity, could be obtained were determined by varying the ratio of dialdehyde starch to MDA in the reaction mixture (Table I). Limiting-capacity resins (diazotization capacities 0.26 to 0.33 mequiv/g) were obtained at DAS:MDA ratios of 1:3 to 1:5 [S-MDA (3) to S-MDA (5) in Table I].

The *p,p'*-diaminodiphenylmethane content of the high capacity S-MDA resins, estimated from nitrogen analysis [see samples S-MDA (3) to S-MDA (5) in Table I] was about 48% by weight. This value indicated that about one MDA molecule was incorporated in the resin per hexose residue.

The S-MDA resins, following diazotization, were coupled to papain and mercuripapain, subtilopeptidase A, and polytyrosyl trypsin. The properties of the S-MDA resins were compared with those of commercial *p*-aminobenzyl-celluloses. Both the diazotization and protein binding capacities of the S-MDA resins were found to be about threefold higher than those of PAB-cellulose (Tables I and II). Moreover, the insoluble enzyme preparations utilizing PAB-cellulose as carrier had a tendency to liberate color on prolonged storage in suspension in aqueous buffers. This deficiency of PAB-cellulose could be overcome to a considerable extent by refluxing the resin with methanol prior to diazotization; it could not, however, be completely eliminated. The S-MDA–protein conjugates did not liberate color. The recoveries of enzymic activity in the insoluble derivatives of the enzymes investigated were considerably higher when S-MDA resins were used as carrier. The S-MDA derivatives also exhibited somewhat better stability upon storage or drying. The *p*-amino-DL-phenylalanine–L-leucine copolymer described by Bar Eli and Katchalski (1963) was found to be comparable with or even superior to the S-MDA resins as a diazotizable carrier. This material, however, was difficult and expensive to prepare.

The saturation capacities of the S-MDA resins for the specific proteins investigated were estimated from the appropriate binding curves (see Figure 1) and confirmed by amino acid analysis of saturated S-MDA–protein samples. The maximal binding capacity of the S-MDA resins for papain, mercuripapain, and subtilopeptidase A was about 10 mg of protein/100 mg of resin (Table II). With polytyrosyl trypsin, however, a considerably higher value was obtained (24 mg of protein/100 mg of resin; Table II). It appears that size and shape factors, as well as the availability of amino acid residues capable of forming azo bonds with the diazotized resin, play a role in determining the value of the maximal binding capacity of the S-MDA resin for a specific protein.

The amino acid analyses of acid hydrolysates of S-MDA–papain, S-MDA–subtilopeptidase A, and S-MDA–polytyrosyl trypsin revealed, among the missing amino acids, not only tyrosine but also lysine and arginine (Tables III–V). Experiments on the chemical modification of amino acids and proteins by means of low molecular weight diazo reagents, such as diazotized aniline (Howard and Wild, 1957), diazotized arsanilic acid (Tabachnick and Sobotka, 1959), and, more recently, diazo-1*H*-tetrazole (Sokolovsky and Vallee, 1966, 1967), have also shown that lysine and arginine are among the amino acids affected. Moreover, the studies of Sokolovsky and Vallee (1967) have demonstrated that modification of these amino acids by diazo reagents leads to their disappearance from the acid hydrolysate of a protein and is accompanied, in some cases, by the appearance of new, ninhydrin-positive peaks in the amino acid analyzer. It is therefore reasonable to assume that the amino acids missing from the acid hydrolysates of the S-MDA derivatives represent the amino acid residues through which the covalent bonds between the protein and the polymeric, diazonium reagent were formed. An attempt to interpret the modified behavior of the S-MDA derivatives of the proteolytic enzymes investigated has, therefore, to take into consideration the effects of modification of specific amino acids on the properties of the immobilized protein.

Modification of lysyl and arginyl residues by attachment to the polymeric diazo reagent will result in the disappearance of positive charges and thus in an increase in the net negative charge on the protein.

Attachment of azo groups to phenolic rings has been shown to lower the pK of the phenolic hydroxyl (Tabachnick and Sobotka, 1959; Sokolovsky and Vallee, 1967); Sokolovsky *et al.* (1967) have reported for 3-azotetrazoletyrosine a value of 8.8 for p$K_{a,app}$ of the phenolic hydroxyl as compared with 10.1 for the hydroxyl group of tyrosine (Martin *et al.*, 1958). Coupled tyrosine residues will therefore be more acidic and will be partly ionized at neutral and slightly alkaline pH values, effecting an increase in the net positive charge of the protein in this pH range. The ionization region of the two types of tyrosines (modified and unmodified) would overlap, however, due to the closeness of their pK's.

It could therefore be assumed that the covalent attachment of proteins to the S-MDA resins (*via* azo bonds) would, in all cases, be accompanied by a considerable increase in the net negative charge or the bound protein. The magnitude of this increase would, of course, depend on the nature and number of modified amino acid residues.

The high-storage stability in aqueous suspension of the S-MDA derivatives of the proteolytic enzymes investigated can be attributed mainly to the prevention of autodigestion resulting from the fixation of the enzymes onto the insoluble carrier.

The pH-stability pattern of the S-MDA–protein conjugates depended on the nature of the protein moiety. Thus, the pH-stability curve of S-MDA–papain was essentially identical with that of the crystalline enzyme (Figure 4). S-MDA–subtilopeptidase A showed increased stability in the acid pH region (pH 3–5) (Figure 6). The S-MDA–polytyrosyl trypsin derivatives, on the other hand, showed a pH-stability pattern totally dissimilar to that of both trypsin and polytyrosyl-trypsin (Figure 9), with maximal stability at neutral and alkaline pH values. It should be pointed out that the pH dependence of stability of the S-MDA–polytyrosyl trypsin derivatives resembled that of acetylated and succinylated trypsin (Sri Ram *et al.* 1954, 1962). It seems, therefore, that immobilization *per se* plays a secondary role regarding the pH dependence of stability of an immobilized enzyme derivative. The chemical nature of the individual amino acid residues participating in the link between enzymic protein and polymeric carrier, and their role in the determination and stabilization of the tertiary structure of the native enzyme, appear to be among the main factors which determine the pattern of pH-stability behavior. No simple rules regarding these factors emerge, however, from the known data.

The pH–activity profiles of the S-MDA derivatives of papain, subtilopeptidase A, and polytyrosyl trypsin, acting on Bz-GlyOEt, *N*-Ac-L-TyrOEt, and Bz-L-ArgOEt, respectively, were displaced toward more alkaline pH values as compared with the native enzymes. This effect was essentially independent to the ionic strength of the medium.

The dependence of enzymic activity on pH is commonly ascribed to the dissociation of ionizing groups participating in the enzymic catalysis (Gutfreund, 1955; Bender and Kézdy, 1965). The chemical identity of such an active site ionizing group has, in many cases, been deduced from the value of the dissociation constant, p$K_{a,app}$, calculated from the pH–activity profile of the enzyme. Thus, the dissociation constants calculated from the acid limbs of the pH–rate curves of trypsin, subtilopeptidase A, and chymotrypsin [p$K_{a,app} \approx 7$] have been assigned to active-site histidines (Bender and Kézdy, 1965). In the case of chymotrypsin, the hypothetical histidine residue has been unequivocally identified as histidine-57 on the basis of both chemical and crystallographic evidence (Bender and Kézdy, 1965; Siegler *et al.*, 1968).

The alkaline displacement of the pH–activity profiles of the S-MDA derivatives can thus be interpreted as reflecting an increase in the value of p$K_{a,app}$ of an active-site-ionizing group on the immobilized enzyme, presumably histidine in the case of trypsin and subtilopeptidase A and either a carboxyl or an extremely acidic histidine in the case of papain (Husain and Lowe, 1968a,b; Lowe, 1970).

Several cases in which chemical modifications of an enzymic protein have led to perturbation of its pH–rate characteristics have been recorded in the literature.

The pH–activity profiles of water-insoluble derivatives of trypsin, chymotrypsin, papain, and subtilopeptidase A, in which a copolymer of ethylene and maleic acid was used as carrier, were found to be displaced toward more alkaline pH values (Levin *et al.*, 1964; Goldstein *et al.*, 1964; Goldstein, 1970; L. Goldstein, unpublished data). Polycationic derivatives of the same enzymes showed the opposite behavior, *i.e.*,

displacement of the pH–activity profiles toward more acidic pH values (Goldstein and Katchalski, 1968; L. Goldstein, unpublished data). These effects were abolished at high ionic strength. The phenomena were related to the unequal distribution of hydrogen ions between the charged "solid phase," the polyelectrolyte enzyme particle, and the surrounding solution, resulting from the long-range effects of the electrostatic potential prevailing in the domain of the immobilized enzyme particle (Goldstein *et al.*, 1964; Goldstein and Katchalski, 1968).

The pH–activity curves (or the pK_a's calculated therefrom) of acetylated and succinylated derivatives of trypsin and chymotrypsin were also found to be displaced toward higher pH values (Goldstein *et al.*, 1964; Goldstein and Katchalski, 1968).

A plausible explanation of the observed shifts of the pH–activity profiles described in this paper may be that the modification of amino acid residues as a result of the coupling reaction (Tables III–V) affects the pK_a's of active site groups due to the disappearance of positive charges or the lowering of the pK_a's of coupled tyrosines. The almost negligible effect of ionic strength on the observed, altered pH dependencies, suggests more localized electrostatic interactions than those observed in the case of polyelectrolyte–enzyme conjugates.

A qualitative explanation of some of the individual kinetic characteristics of the various S-MDA derivatives could be attempted at this point, taking into account the ideas discussed above.

The diffuse pH–activity profile of S-MDA–polytyrosyl trypsin (Figure 7) may possibly be related to nonuniform electrostatic effects arising from the continuous ionization of tyrosyl residues (modified and unmodified) in the whole pH range of activity of the enzyme.

The symmetrically displaced pH–activity profile of S-MDA–subtilopeptidase A (Figure 5) might be due to an essentially constant change in the state of ionization of the protein resulting mainly from the elimination of cationic groups (compare amino acid composition data for S-MDA–subtilopeptidase A and S-MDA–polytyrosyl trypsin, Tables IV and V).

The apparent discrepancy in the kinetic behavior of S-MDA–papain toward the positively charged substrate Bz-L-ArgOEt, as compared with the uncharged substrate Bz-GlyOEt (Figure 2), may be due to even more specific charge interactions which, however, cannot at this point be more precisely interpreted on the basis of the available data.

It should also be mentioned that some of the diazonium groups on the S-MDA resin might have undergone partial degradation to phenol in the course of the coupling reaction (see, for example, Zollinger, 1961). Such carrier phenol groups could be a factor in the observed kinetic anomalies of the S-MDA-bound enzymes.

Finally, the lower proteolytic activities of the S-MDA derivatives, calculated on the basis of the amount of active bound enzyme, are most probably due to steric diffusional restrictions imposed by the polymeric carrier.

Acknowledgment

The authors are indebted to Miss Sylvia Nemet for excellent technical assistance.

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Synthetic Homologs of Phosphoenolpyruvate and Specificity of Pyruvate Kinase*

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ABSTRACT: Seven homologs of phosphoenolpyruvate were synthesized and characterized. These homologs were synthesized from the corresponding β-bromo-α-keto acids using the Perkow reaction. All of the homologs were evaluated for their abilities to serve as substrates or inhibitors for pyruvate kinase (EC 2.7.1.40). Results of the kinetic studies indicated that none of the seven homologs of P-enolpyruvate will serve as a substrate for rabbit muscle pyruvate kinase. However, P-enol-α-ketobutyrate and P-enol-α-ketovalerate act as competitive inhibitors for the pyruvate kinase–P-enolpyruvate reaction. The other five homologs did not inhibit the reaction of the enzyme. The inhibition constants, K_i, for P-enol-α-ketobutyrate and P-enol-α-ketovalerate were determined by the method of M. Dixon (*Biochem. J. 55*, 170 (1953)). P-enol-α-ketobutyrate had a K_i of 6.5×10^{-5} M, while the K_i of P-enol-α-ketovalerate was 10.7×10^{-4} M. The homologs with six or more carbons or the homolog with two methyl groups on the β-carbon (P-enol-α-ketoisovalerate) do not act as substrates or inhibitors, thus indicating that pyruvate kinase is quite selective for its substrate.

Phosphoenolpyruvate is the only reported compound that is capable of phosphorylating ADP or other nucleoside diphosphates by the action of pyruvate kinase (EC 2.7.1.40). Furthermore, no work has been reported in which enolic phosphates have been examined for their possible activity as substrates or inhibitors of pyruvate kinase. However, Mildvan *et al.* (1967) showed that fluorophosphate inhibits the pyruvate kinase reaction by specifically competing with the P-enolpyruvate and not with ADP. Previous work of Mildvan and Cohn (1965, 1966) indicated that a ternary complex of P-enolpyruvate, Mn^{2+}, ADP, and enzyme was operative. This complex was proposed based on data from kinetic, conformational, and nuclear magnetic resonance studies. Examination of the proposed complex would lead one to suspect the possibility of steric specificity with respect to the site at which the enolic phosphate binds.

The present work reported herein was undertaken to elucidate the specifity of pyruvate kinase using synthetic homologs of P-enolpyruvate.

Clark and Kirby (1964) have developed a convenient method for the synthesis of P-enolpyruvate in which bromopyruvic acid is converted into the P-enolic dimethyl ester by the Perkow reaction using trimethyl phosphite. The methyl groups are removed in aqueous cyclohexylamine to yield the monocyclohexylammonium salt of P-enolpyruvate. It appeared feasible that if β-brominated α-keto acids other than pyruvate (*e.g.*, α-ketobutyric, etc.) were used, a similar reaction would yield corresponding homologs of P-enolpyruvate. The synthesis of β-bromo-α-ketobutyric acid by Sprinson and Chargaff (1946) indicated that the bromination of α-keto acids with a catalytic amount of H_2SO_4 would yield a brominated α-keto acid with a single bromine on C-3 (β-carbon). In this study we have also developed methods of synthesis for the β bromination of several α-keto acids along with the subsequent conversion of these acids into homologs of P-enolpyruvate.

Materials and Methods

Materials. Bromopyruvic, α-ketobutyric, α-ketovaleric, α-ketocaproic, α-ketoisovaleric, and α-ketoisocaproic acids were purchased from Sigma Chemical Co. The α-ketocaprylic acid was obtained from Pfaltz and Bauer Inc. α-Ketodecanoic acid was supplied by Aldrich Chemical Co. The α-ketoisovaleric acid was also synthesized according to the method of Ramage and Simonsen (1935). Trimethyl phosphite was purchased from J. T. Baker Chemical Co. and Eastman

* From the Department of Chemistry, Middle Tennessee State University, Murfreesboro, Tennessee. *Received January 19, 1970.* This work was supported in part by a grant from the Faculty Research Committee, Middle Tennessee State University.