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IMMOBILIZED ENZYMES. SYNTHESIS OF A NEW TYPE OF POLYANIONIC AND POLYCATIONIC RESINS AND THEIR UTILIZATION FOR THE PREPARATION OF WATER-INSOLUBLE ENZYME DERIVATIVES*

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SUMMARY

1. A new type of hydrophilic, anionic and cationic, polymeric reagents, which contain diazonium or acylazide functional groups were synthesized.

2. Coupling of a copolymer of ethylene and maleic anhydride (EMA) with *p,p'*-diaminodiphenylmethane (MDA) or with hydrazine led to high capacity anionic, arylamine (EMA-MDA) or acylhydrazide (EMA-hydrazide) resins. The anionic resins could be cationized to the appropriate amidopropyldimethylamino derivatives.

3. The EMA-MDA and EMA-hydrazide resins were converted to the polymeric diazonium salts or acylazides, respectively, and coupled to trypsin, chymotrypsin, subtilisin Novo, subtilisin Carlsberg and papain, yielding highly active water-insoluble derivatives of these enzymes.

4. The immobilized enzyme derivatives exhibited enhanced temperature and lyophilization stabilities. Relative to the native enzymes, the anionic derivatives were more stable in the alkaline pH range, while the cationic derivatives were more stable in the acidic pH range.

INTRODUCTION

Immobilized enzyme derivatives serve as specific, easily removable catalysts, that can be used repeatedly in columns and in batch reactors. A large number of immobilized enzyme systems have been described in the literature[1–3].

Most of the natural hydrophylic polymers used for the covalent binding of

Abbreviations: EMA, ethylene-maleic anhydride (1:1) copolymer; MDA, *p,p'*-diaminodiphenylmethane (methylenedianiline); DCC, *N,N'*-dicyclohexylcarbodiimide; EMA-MDA and EMA-hydrazide are used to designate the resins obtained by the coupling of EMA with *p,p'*-diaminodiphenylmethane or hydrazine respectively (see Scheme I). S-MDA designates the insoluble condensation product of dialdehyde starch with *p,p'*-diaminodiphenylmethane[5].

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enzymes are polysaccharides (*e.g.* derivatized celluloses or cross-linked dextrans) and their chemical reactivity and mechanical stability are limited by the properties of the monomer unit. Synthetic polymeric carriers offer a much wider range of properties, which can be adapted to specific needs by appropriate modification of the chemical composition of the material. In many cases, however, derivatives of enzymes bound to synthetic polymers exhibit low temperature and lyophilization stabilities, presumably due to the hydrophobic nature of the support.

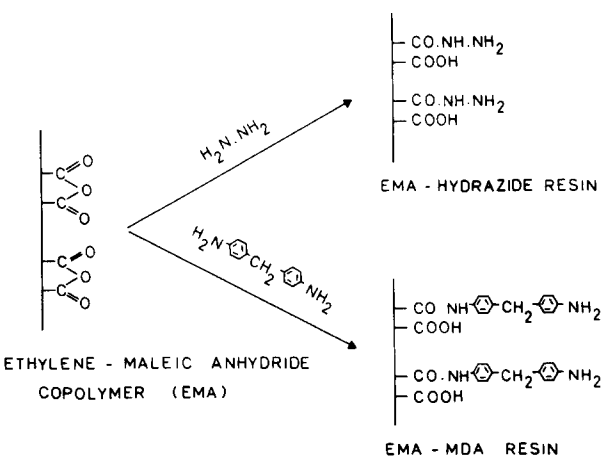
This communication describes the synthesis of a new type of hydrophilic, anionic and cationic, polymeric reagents which contain diazonium or acylazide functional groups. The starting material is a commercially available 1:1 copolymer of ethylene and maleic anhydride (EMA). By coupling EMA with *p,p'*-diaminodiphenylmethane (methylenedianiline, MDA) or with hydrazine, high capacity anionic arylamine or acylhydrazide resins are obtained (the resins are designated EMA-MDA, and EMA-hydrazide, respectively; Scheme I). The anionic resins served as parent compounds for further derivatization into the appropriate cationic amidopropyldimethylamino derivatives. The modification was carried out by coupling the free carboxyls on the resin with *N,N*-dimethyl-1,3-propanediamine in the presence of dicyclohexylcarbodiimide, DCC (Scheme II). The anionic and cationic EMA-MDA and EMA-hydrazide resins were coupled to several enzymes, following activation to the appropriate polymeric diazonium salts or acylazides.

The binding properties of the resins for various proteins and the behavior of the immobilized enzyme derivatives were investigated.

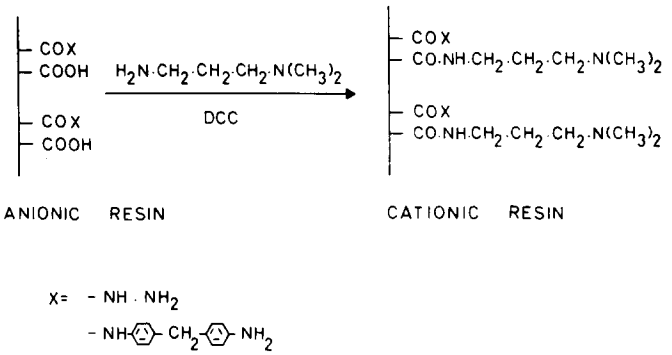
MATERIALS AND METHODS

Chymotrypsin, trypsin and papain were purchased from Worthington Biochemical Corp., Freehold, N.J. Subtilisin Novo and subtilisin Carlsberg were purchased from Novo Industries, Copenhagen.

Ethylene-maleic anhydride (1:1) copolymer, EMA (mol. wt about 20 000) was



Scheme I. Synthesis of EMA-MDA and EMA-hydrazide resins.



Scheme II. Cationization of EMA-MDA and EMA-hydrazide resins.

obtained from Monsanto Company, St. Louis, Mo. The EMA samples were dried *in vacuo* over P_2O_5, at 110 °C for 48 h before use. All other reagents, substrates and buffers were of the best grade available.

EMA-hydrazide resins

Preparation of anionic EMA-hydrazide resins. Oven-dried EMA (12.6 g; 0.1 base mole anhydride) was dissolved with stirring in redistilled dimethylsulfoxide (100 ml). A solution of hydrazine hydrate in the same solvent (1.5 g in 50 ml; 0.03 mole) was then added slowly with strong stirring. The reaction mixture solidified immediately into a glassy gel. The solid was broken with a glass rod and left at room temperature for 24 h in a closed vessel, to allow the completion of the cross-linking reaction. The solid glassy material was covered with excess dry acetone, stirred for a few minutes and allowed to settle. The liquid was decanted and the procedure repeated. The solid material was again suspended in acetone, ground in an "omnimixer" homogenizer (Sorval) and allowed to settle. The grinding was repeated several times with fresh portions of acetone until the solid material acquired hard resinous texture as judged by its ease of filtration on a suction filter. After air drying for a few minutes on the filter to remove the acetone, the resin was suspended in 100 ml, dimethylsulfoxide reacted again with hydrazine (10 g hydrazine hydrate in 100 ml; 0.2 mole) and left stirring overnight at room temperature.

The reaction mixture was poured into acetone, the liquid decanted, and the solid ground several times with acetone, washed with acetone on a suction filter and air dried. Traces of solvent were removed by leaving the material over P_2O_5 in a dessicator connected to a high vacuum pump for a few hours. The net weight of dry EMA-hydrazide resin was 10–12 g.

Sometimes on mixing the crosslinked-EMA suspension with the second portion of hydrazine, lumping of the resin was observed. In such cases, the reaction mixture was returned to the homogenizer, ground to break the pieces and then allowed to stir overnight, as described above.

Cationization of EMA-hydrazide resins. Anionic EMA-hydrazide resin (2 g; $1.3 \cdot 10^{-2}$ base mole carboxyl) was suspended in a dimethylsulfoxide solution of dicyclohexylcarbodiimide (6 g in 60 ml; $3.0 \cdot 10^{-2}$ mole). *N,N*-Dimethyl-1,3-propanediamine (10 ml; 0.1 mole) was slowly added to the magnetically stirred suspension,

The reaction mixture was left stirring overnight at room temperature. The resin was separated by centrifugation, resuspended in dimethylsulfoxide, stirred magnetically for a few minutes and centrifuged down again. The procedure was repeated twice to ensure the removal of reagents. The cationized resin was then suspended in acetone, stirred magnetically for a few minutes, and separated on a suction filter. After several washings with acetone the resin was air-dried and left overnight over P_2O_5 in a thoroughly evacuated dessicator, to remove traces of solvent.

Activation of EMA-hydrazide resins. EMA-hydrazide resin (100 mg) was suspended in 50% acetic acid, 0.1 M in HCl (8 ml) and stirred for 30 min at room temperature. The stirred suspension was cooled to 4 °C, aqueous sodium nitrite (40 mg in 1 ml water) was added dropwise, and the mixture stirred for 1 h over ice. The activated resin was separated on a suction filter, exhaustively washed on the filter with cold water, resuspended in water and brought to pH $\approx$ 9 by the dropwise addition of 0.5 M NaOH, crushed ice being added to keep the temperature down. The resin was separated by filtration, washed with 0.2 M borate buffer, pH 8.4, resuspended and used directly in the coupling experiments.

Coupling of anionic EMA-hydrazide resins to proteins. A cold freshly prepared solution of enzyme in 0.2 M borate buffer pH 8.4 (10–40 mg in 2 ml) was slowly added to the magnetically stirred suspension of activated EMA-hydrazide (100 mg) in the same buffer (5 ml). The reaction mixture was left stirring overnight at 4 °C. The water-insoluble EMA-hydrazide enzyme derivative was separated by centrifugation and washed with water, 1 M KCl again with water and then suspended in water.

Coupling of cationic EMA-hydrazide resins to proteins. This was perfo:med in 0.2 M borate, pH 8. The subsequent washings of the insoluble enzyme derivative were carried out as described for the anionic EMA-hydrazide resin.

Determination of the binding capacity of EMA-hydrazide resins. (a) Capacity for amino acids. Activated EMA-hydrazide resin (50 mg) was suspended in 0.2 M borate buffer pH 8.4 (2 ml). A 0.2 M solution of glycine or alanine in the same buffer (8 ml) was added slowly. The reaction mixture was stirred overnight in the cold room. The resin was centrifuged down, dialyzed exhaustively against deionized water and lyophilized. A sample of the lyophilized powder (3–4 mg) was hydrolyzed in an evacuated, sealed tube using 6 M HCl (24 h; 110 °C), the insoluble material was removed by strong centrifugation and the glycine (or alanine) content determined in the amino acid analyzer[4].

(b) Capacity for proteins. The maximal binding capacity of the EMA-hydrazide resins for a specific protein was estimated from the binding curves obtained by coupling the polymeric acylazide derived from the EMA-hydrazide resin (50 mg) with varying amounts of protein (1–25 mg). The amount of bound enzymically-active protein was calculated from the enzymic activity of the insoluble enzyme derivatives. The data was plotted as amount of active bound protein *vs* amount of protein in the coupling mixture. The maximal binding capacity of the EMA-hydrazide resin for a specific protein was estimated from the region where the binding curve levelled off[5] and confirmed by amino acid analysis.

EMA-MDA resins

Preparation of anionic EMA-MDA resins. Oven-dried EMA (12.6 g; 0.1 base mole anhydride) was dissolved with stirring in redistilled dimethylsulfoxide (100 ml).

A dimethylsulfoxide solution of *p,p'*-diaminodiphenylmethane (39.6 g in 200 ml; 0.2 mole) was added to the vigorously stirred EMA solution. The reaction mixture solidified after 2–3 min. The solid was broken with a glass rod and left standing at room temperature for 24–48 h in a closed vessel. The hard, glassy gel was covered with acetone, stirred for a few minutes, the solid was allowed to settle and the liquid decanted. This procedure was repeated twice. The hardened, opaque material was then ground with acetone, the liquid was filtered off on a suction filter and the grinding repeated several times until a fine, hard powder of resin-like texture was obtained. The powder was washed with acetone on the filter and air-dried. Traces of organic solvent were removed by leaving the material for a few hours over P_2O_5 in a dessicator connected to a high-vacuum pump. The net weight of dry EMA-MDA resin was about 28 g.

Cationization of EMA-MDA resins was carried out as described for the EMA-hydrazide resins.

Diazotization of EMA-MDA resins. EMA-MDA resin (100 mg) was suspended in 50% acetic acid 0.1 M in HCl (8 ml) and stirred for about 30 min at room temperature. The stirred suspension was chilled to 4 °C and aqueous sodium nitrite (40 mg in 1 ml) was added dropwise. The diazotization mixture was stirred for 1 h over ice, filtered with suction and washed with cold water to remove the acid. The resin was resuspended in water and brought to alkaline pH ($\approx$ 10) by the dropwise addition of 0.5 M NaOH, crushed ice being added to keep the temperature down. The dark red-brown polydiazonium resin was separated by filtration, washed with cold 0.2 M phosphate buffer pH 7.8, resuspended and used directly in the coupling experiments.

Coupling of anionic EMA-MDA resins to proteins. A cold solution of enzyme in 0.2 M phosphate buffer pH 7.8 (10–40 mg in 2 ml) was slowly added to a magnetically stirred suspension of diazotized EMA-MDA (100 mg) in the same buffer (4 ml). The reaction mixture was left stirring overnight at 4 °C. The water-insoluble EMA-MDA enzyme derivative was separated by centrifugation or filtration, washed with water, 1 M KCl and again with water and resuspended in water.

Coupling of cationic EMA-MDA resins to proteins was carried out as described for the polyanionic EMA-MDA resins.

Determination of the binding capacity of the EMA-MDA resins. (a) Diazotization capacity. An aqueous solution of *p*-bromophenol (75 mg dissolved in 2 ml water by the dropwise addition of 2 M NaOH) was added to a diazotized EMA-MDA resin sample (50 mg) suspended in cold 0.2 M phosphate buffer pH 7.8 (9 ml). The reaction mixture was stirred overnight at 4 °C. The resin was separated on a funnel, washed with 0.05 M carbonate buffer pH 10.5, or water brought to the same pH, then with deionized water and finally with methanol and dried *in vacuo* over P_2O_5. The nitrogen and bromine contents of the dry material were determined by the Dumas and Schöniger combustion methods respectively[6]. From these two values and a knowledge of the nitrogen content of the EMA-MDA resin, the diazotization capacity of the various resins could be estimated.

(b) Capacity for proteins. The maximum capacity of the EMA-MDA resins for a given protein was estimated as described for the EMA-hydrazide resins.

Determination of the free carboxyl groups content

Glycine ethyl ester·HCl (325 mg; 2.5 mmoles) was dissolved in 2.5 ml dimethyl-

sulfoxide. An equimolar amount of triethylamine was then added to liberate the ester in the free-base form and the reaction mixture left stirring over ice for 1 h. The solution was filtered to remove the triethylamine·HCl precipitate and added to a test tube containing the appropriate EMA-MDA or EMA-hydrazide resin (50 mg). A solution of dicyclohexylcarbodiimide in dimethylsulfoxide (325 mg in 3 ml) was added to the magnetically stirred suspension and the reaction mixture left at room temperature for 16 h. The highly swollen resin was separated by centrifugation, resuspended in dimethylsulfoxide (5 ml) and centrifuged again. The washing with dimethylsulfoxide was repeated 3 times, and was followed by 3 washings with acetone. The hardened resin was filtered, air-dried and left over P_2O_5 *in vacuo* for 24 h. A weighed amount of the resin (2–4 mg) was hydrolyzed with 6 M HCl (48 h, 110 °C) and the amount of glycine determined in the amino acid analyzer[4].

Determination of the protein content of the EMA-MDA and EMA-hydrazide conjugates of the various enzymes

The EMA-MDA or EMA-hydrazide protein conjugate (10–12 mg) was hydrolyzed in an evacuated, sealed tube using 6 M HCl (24 h; 110 °C). 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 material was removed by strong centrifugation (Sorval; 12 000 rev./min). Amino acid analysis was carried out employing an automatic amino acid analyzer[4]. The amount of protein was calculated from the amounts of alanine, leucine, glycine, valine, glutamic acid and aspartic acid[7].

Determination of enzyme activities

The activities of the various enzymes and water-insoluble derivatives were determined at 25 °C by the pH-stat method[8] using the appropriate ester substrates. A Radiometer pH-stat consisting of the SBR 2c/SBU-1/TTA3 titration assembly and the PHM 26C pH meter was used. The titrant was 0.1 M NaOH. 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 per min under the specified assay conditions. Trypsin and papain were assayed using benzoyl-L-arginine ethyl ester as substrate. The assay mixture (5 ml) was $1.16 \cdot 10^{-2}$ M benzoyl-L-arginine ethyl ester, 0.01 M KCl for trypsin and 0.05 M benzoyl-L-arginine ethyl ester, 0.005 M cysteine, 0.002 M EDTA for papain[5]. Chymotrypsin, subtilisin Novo and subtilisin Carlsberg were assayed using acetyl-L-tyrosine ethyl ester ($1.18 \cdot 10^{-2}$ M in 0.01 M KCl, 5 ml) as substrate[5]. The optimal pH values of activity for trypsin, chymotrypsin, subtilisin Novo, subtilisin Carlsberg and papain and their immobilized derivatives are given in Table VII.

The protease activities of the various enzymes and of their immobilized derivatives were determined at pH 7.5, 37 °C, by the casein digestion method[9]. The amounts of enzyme or insoluble enzyme derivative added to the digestion mixture were expressed in units of esterase activity. The reaction mixtures containing immobilized enzyme were stirred magnetically to ensure effective mixing of the reagents.

RESULTS AND DISCUSSION

Preparation and characterization of EMA-MDA and EMA-hydrazide resins

Two types of reactions can take place in a competitive fashion between polymeric, polyfunctional molecules such as EMA and bifunctional reagents, in our case *p,p'*-diaminodiphenylmethane or hydrazine (Scheme I): (a) crosslinking of the polymeric chains by the bifunctional reagent (not shown in Scheme I), (b) one-point attachment of the bifunctional reagent. Crosslinking predominates when excess polymer is present in the reaction mixture. One-point attachment is the main reaction when the bifunctional reagent is in excess.

The conditions under which one could obtain anionic EMA-MDA resins of maximal binding capacity in parallel with satisfactory mechanical properties (dependent on the degree of crosslinking) were determined. A series of EMA-MDA samples were prepared by adding increasing amounts of *p,p'*-diaminodiphenylmethane to EMA in dimethylsulfoxide solution. The base mole ratios of anhydride to *p,p'*-diaminodiphenylmethane in the reaction mixture were varied from 1:1 to 1:5 and the yield, nitrogen content and binding capacities of each sample determined. At a 1:1 molar ratio, very hard, non-swelling resins of low diazotization and protein binding capacities were obtained. Maximal diazotization and protein binding capacities were attained with EMA-MDA samples prepared at a 1:2 molar ratio of anhydride to *p,p'*-diaminodiphenylmethane. The resins swelled in 50% acetic acid 0.1 M in HCl, and after diazotization, at slightly alkaline pH-values. Further increase in the amount of *p,p'*-diaminodiphenylmethane led to no significant increase in the binding capacities of the EMA-MDA resins, and to no detectable changes in their mechanical and swelling properties. The anionic EMA-MDA resins were therefore routinely prepared at a 1:2 molar ratio of anhydride to *p,p'*-diaminodiphenylmethane. Nitrogen analysis and determination of the diazotization capacity of a standard anionic EMA-MDA preparation (Table I), showed that about 20% of the *p,p'*-diaminodiphenylmethane incorporated in the resin was available for diazotization. The diazotization capacity of the anionic EMA-MDA resin (0.80 mequiv/g) was considerably higher than that of a commercial *p*-aminobenzyl cellulose sample (Cellex PAB, Bio Rad Laboratories; 0.09 mequiv/g) or that of a synthetic uncharged diazotizable resin, S-MDA, previously reported (0.30 mequiv/g)[5].

TABLE I

COMPOSITION AND PROPERTIES OF ANIONIC EMA-MDA AND EMA-HYDRAZIDE RESINS

Resin	*Nitrogen content of resin** (%)*	*p,p'-Diamino-diphenylmethane or hydrazine content*** (mole/g)*	*EMA to p,p'-diaminodiphenyl-methane or hydrazine ratio*	*Coupling capacity**** *(equiv/g)*
EMA-MDA	6.54	$2.34 \cdot 10^{-3}$	1.83	$0.80 \cdot 10^{-3}$
EMA-hydrazide	10.5	$3.75 \cdot 10^{-3}$	1.86	$0.122 \cdot 10^{-3}$

* Determined by the Dumas combustion method (see Materials and Methods).

** Calculated from the nitrogen content of the resins.

*** Estimated, in the case of EMA-MDA, from the nitrogen and bromine content of the conjugate of the diazotized resin with *p*-bromophenol; in the case of EMA-hydrazide, from the glycine content of the conjugate of the activated resin with glycine.

The direct coupling of EMA with hydrazine at 1:1 to 1:3 molar ratios of anhydride to hydrazine under the conditions employed for the preparation of the EMA-MDA resins, led to soft, rubbery materials, indicating a low degree of crosslinking. Better control of the degree of crosslinking of the EMA-hydrazide resins could be gained by a two-step procedure, *i.e.* adding first 0.3 mole hydrazine per mole of anhydride and allowing sufficient time (15–20 h) for the crosslinking reaction to go to completion; the crosslinked resin was then reacted with a two-fold molar excess of hydrazine to complete the reaction. EMA-hydrazide resins, comparable in their mechanical properties to the EMA-MDA resins, could be obtained by this procedure. The binding capacity of anionic EMA-hydrazide (0.122 mequiv/g; Table I) was higher than that of a commercial carboxymethylcellulose hydrazide, Enzyte (supplied by Miles-Seravac; 0.028 mequiv/g).

Both types of anionic resins could be cationized by coupling the free carboxyls with *N,N*-dimethyl-1,3-propanediamine *via* activation with dicyclohexylcarbodiimide. No less than 97% of the total carboxyls on the resin were modified by this method (see Materials and Methods). The change in the charge characteristics of the resins was reflected in their mechanical and swelling properties. Excessive swelling of cationic EMA-MDA and cationic EMA-hydrazide was observed in 50% acetic acid, 0.1 M in HCl (the activation solvent); the swollen, activated cationic resins could not, in most cases, be filtered and were separated from the activation reaction mixture by centrifugation. Raising the pH prior to coupling the polydiazonium salt or polymeric acylazide to proteins, led to a contraction of the resin particles; at this stage the resins were easily filterable.

The particle size of the resins was estimated by examination of aqueous suspensions of the powders under the microscope. The mean diameter of the dry resin particles was in the range of 20–40 μm. Following preswelling in 50% acetic acid (see above) or the binding of protein, the mean diameter was found to increase by a factor of 3 to 4.

Binding of proteins

Representative data on the maximal protein binding capacities of anionic EMA-MDA and anionic EMA-hydrazide resins are summarized in Table II. The capacity of EMA-hydrazide resins is considerably higher than that of EMA-MDA resins. This could be due to the higher hydrophilicity of the EMA-hydrazide network, leading to a more open structure, and thus to a higher capacity for proteins.

The EMA-MDA enzyme derivatives had particulate form and were easily filtered. The immobilized enzyme samples derived from EMA-hydrazide were more swollen, of softer texture, and filtered slowly.

The recovery of enzymic activity in insoluble derivatives prepared under standard conditions (10 mg enzyme per 100 mg resins, in the coupling mixture) was higher when the support was EMA-hydrazide (Table III). The total activity recovered in immobilized form was found to depend on the charge characteristics of the support; the anionic resins yielded in general higher total immobilized activity (Table III). Comparison of the data of Tables II and III with values in the literature shows that the recovery of immobilized enzymic activity with EMA-MDA and EMA-hydrazide resins was, in most cases, higher or comparable with the values recorded for other support materials[10–22].

TABLE II

PROTEIN BINDING CAPACITIES OF ANIONIC EMA-MDA AND EMA-HYDRAZIDE RESINS FOR VARIOUS ENZYMES

Enzymes	*EMA-MDA resins*				*EMA-hydrazide resins*			
	Protein content		*Active bound protein***		*Protein content*		*Active bound protein***	
	*Calculated from binding curves** *(mg/100 mg)*	*Calculated from amino acid analyses** *(mg/100 mg)*	*(mg)*	*(%)*	*Calculated from binding curves** *(mg/100 mg)*	*Calculated from amino acid analyses** *(mg/100 mg)*	*(mg)*	*(%)*
Trypsin	10	9.7	3.4	34	30	29	12.0	40
Chymotrypsin	10	9.8	2.0	20	25	24.1	3.2	13
Subtilisin Novo	20	18	5.4	27	10	9.7	1.0	10
Subtilisin Carlsberg	20	24	2.0	10	10	9.8	1.0	10
Papain	30	31	9.4	31.4	45	44.2	27	60

* Details described in Materials and Methods section.
** Estimated from the esterase activity of the immobilized enzyme preparation.

TABLE III

RECOVERY OF IMMOBILIZED ENZYMIC ACTIVITY*

The specific activities of the native enzymes employed were as follows: trypsin, 35 units/mg; chymotrypsin, 350 units/mg; subtilisin Novo, 280 units/mg; subtilisin Carlsberg, 800 units/mg; papain, 16 units/mg.

Enzyme	*EMA-MDA resin*		*EMA-hydrazide resin*	
	Anionic (%)	*Cationic (%)*	*Anionic (%)*	*Cationic (%)*
Trypsin	30	9.5	80	75
Chymotrypsin	28	10	50	30
Subtilisin Novo	27	16	18	12
Subtilisin Carlsberg	14	3.5	34	19
Papain	34	30	50	55

* Immobilized derivatives prepared under standard conditions. 10 mg enzyme per 100 mg resin in the coupling mixture.

The high recovery of enzymic activity observed for the EMA-MDA derivatives of trypsin and chymotrypsin was rather atypical. Several authors describing the coupling of trypsin and chymotrypsin to uncharged, diazotizable resins such as leucine-*p*-aminophenylalanine copolymers[13], *p*-aminobenzyl-cellulose[5,11,12] and a synthetic resin S-MDA[5,22], have reported total loss of enzymic activity in the covalently bound protein. It thus seemed likely that the damage accompanying the binding of trypsin and chymotrypsin to polymeric diazonium reagents was at least partially avoided in the case of the charged diazotizable resins, anionic and cationic EMA-MDA. This assumption could be tested by determining the number and nature of the amino acid residues, forming azo linkages with the polydiazonium reagent. Such

TABLE IV

AMINO ACID COMPOSITION OF TRYPSIN AND SOME OF ITS WATER-INSOLUBLE DERIVATIVES

The results are expressed as moles of amino acid per mole of enzyme, calculated on the basis of the mean values obtained for Ala, Gly, Val, Asp, Glu and Leu.

Amino acid	*Native enzyme*	*S-MDA trypsin*	*Anionic EMA-MDA trypsin*	*Cationic EMA-MDA trypsin*
Lys	14	7.2	11.2	12.6
His	3	1.5	2.2	3.1
Arg	2	1.5	4	2.03
Asp	22	22.1	21.6	22.6
Thr	10	9.0	10	10
Ser	34	33	32.7	31.7
Glu	14	14.5	14.6	13.2
Pro	8	8.3	8.7	8
Gly	25	24.3	25.1	27.2
Ala	14	13.7	14.3	13.5
Val	17	15.7	16	18.1
Ile	15	14	14.7	15.2
Leu	14	13	14.2	14.3
Tyr	10	2.1	2.8	7.4
Phe	3	2.2	2.6	3.25

TABLE V

AMINO ACID COMPOSITION OF CHYMOTRYPSIN AND SOME OF ITS WATER-INSOLUBLE DERIVATIVES

The results are expressed as moles of amino acid per mole of enzyme, calculated on the basis of the mean values obtained for Ala, Gly, Val, Asp, Glu and Leu.

Amino acid	*Native enzyme*	*S-MDA chymotrypsin*	*Anionic EMA-MDA chymotrypsin*	*Cationic EMA-MDA chymotrypsin*
Lys	14	11.7	12.6	12.8
His	2	1.8	2.1	2.1
Arg	3	2.76	2.2	3.2
Asp	22	21.3	23	21.7
Thr	22	23	22.2	19.1
Ser	27	27	25	23
Glu	15	15.3	15.8	14.8
Pro	9	9	9	9.4
Gly	23	23.1	21.3	23.8
Ala	22	22.3	23	21.4
Val	23	22.8	23.3	22.6
Ile	10	9.6	9.7	10.8
Leu	19	20	18	18.1
Tyr	4	—	1.01	3.1
Phe	6	5	7.1	4.5

residues can be detected by virtue of their disappearance from acid hydrolyzates of the appropriate immobilized enzyme derivatives[5,22–24].

The amino acid compositions of acid hydrolyzates of anionic and cationic EMA-MDA derivatives of trypsin and chymotrypsin as well as those determined for the native enzymes and for the insoluble, enzymically inactive conjugates of trypsin and chymotrypsin with the uncharged diazotizable resin, S-MDA are given in

Tables IV and V. With the uncharged S-MDA derivatives[5] a considerable decrease in tyrosine and lysine content is observed (4 Tyr and 2 Lys are missing in S-MDA chymotrypsin; 8 Tyr and 7 Lys are missing in S-MDA trypsin); in the case of S-MDA-trypsin, a small decrease in histidine is found. The anionic and cationic EMA-MDA derivatives exhibit lower losses of tyrosine and lysine (3 Tyr and 1 Lys, and 1 Tyr and 1 Lys are missing in anionic and cationic EMA-MDA chymotrypsin respectively; 7 Tyr and 2 Lys and 3 Tyr and 1 Lys are missing in anionic and cationic EMA-MDA trypsin, respectively); a small decrease in the content of hydroxyamino acids can also be detected. In general, the lowest number of missing amino acids is found in the cationic derivatives of both trypsin and chymotrypsin. The data can serve as a reasonable indication for the modified chemical selectivity of the polyelectrolyte EMA-MDA diazonium reagents.

These restrictions on selectivity are probably due to the redirection of the charged polymeric reagent towards the more solvated (hydrophilic) parts of the enzyme molecule, as reflected in the considerable decrease of azo bonds formed with tyrosine, located mainly in the hydrophobic interior of the protein molecule[25].

Properties of the immobilized EMA-MDA and EMA-hydrazide enzyme derivatives

Stability. Aqueous suspensions of anionic EMA-MDA derivatives of chymotrypsin, subtilisin Carlsberg and subtilisin Novo could be stored at 4 °C for 3–4 months without significant loss of activity. Suspensions of EMA-MDA papain and EMA-MDA-trypsin could be stored under the same conditions for periods of up to 8 months with no decrease of activity. The corresponding cationic EMA-MDA derivatives lost 20–30% of their activity under the same conditions.

The EMA-hydrazide derivatives (both anionic and cationic) of all five enzymes exhibited extremely high storage stabilities. No loss of activity was recorded after storing suspensions at 4 °C, for about 1 year, in the presence of a bactericide.

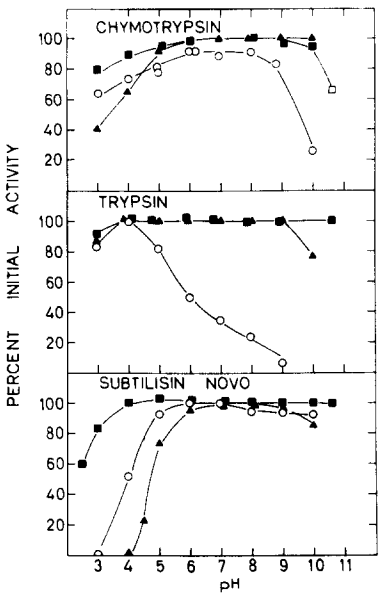
TABLE VI

RECOVERY OF ACTIVITY ON LYOPHILIZATION

	EMA-MDA resin		*EMA-hydrazide resin*	
	Anionic (%)	*Cationic (%)*	*Anionic (%)*	*Cationic (%)*
Trypsin	76	46	100	93
Chymotrypsin	30	8	50	91
Subtilisin Novo	60	5	70	98
Subtilisin Carlsberg	27	22	70	86
Papain	42	—	90	—

The enzymic activity retained by the various EMA-MDA and EMA-hydrazide derivatives following lyophilization is given in Table VI. Storage of the lyophilized powders in a dessicator at 25 °C for about 1 year was accompanied by no loss of activity.

The activity retained by several representative anionic and cationic EMA-hydrazide derivatives after incubation for 30 min at 37 °C, at various pH values, is shown in Fig. 1. Relative to the corresponding native enzymes all anionic EMA-



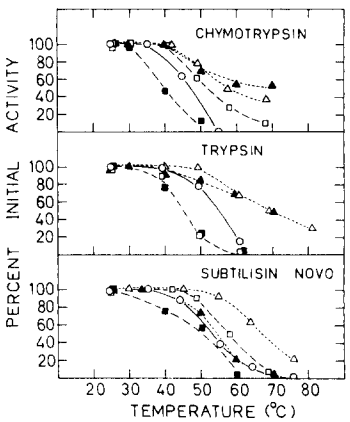


Fig. 1. Effect of pH on the stability of anionic and cationic EMA-hydrazide derivatives of chymotrypsin, trypsin and subtilisin Novo. ○, native enzyme; ▲, anionic EMA-hydrazide derivative; ■, cationic EMA-hydrazide derivative. The test solutions (0.5 ml) in the appropriate buffer, containing enzyme or immobilized enzyme derivative (about 15 esterase units per ml) were incubated at 37 °C for 30 min; 0.1 ml aliquots were withdrawn and the residual esterase activity determined by the standard procedures (see Materials and Methods). The following buffer solutions were used to cover the pH range investigated: pH 3.0, 0.05 M citrate; pH 4–5, 0.1 M acetate; pH 6–9, 0.05 M phosphate; pH 10–10.7, 0.05 M carbonate.

Fig. 2. Temperature stability of chymotrypsin, trypsin and subtilisin Novo and of their anionic and cationic EMA-MDA and EMA-hydrazide derivatives, ○, native enzyme; △, anionic EMA-hydrazide derivative; ▲, cationic EMA-hydrazide derivative; □, anionic EMA-MDA derivative; ■, cationic EMA-MDA derivative. The test samples (0.5 ml) containing enzyme or immobilized enzyme derivative (about 15 esterase units per ml) in a buffer of pH of optimal stability (see Fig. 1) were incubated at the specified temperature for 15 min; 0.1 ml aliquots were withdrawn and the residual activity determined by the standard procedures at 25 °C (see Materials and Methods).

hydrazides, as well as the anionic EMA-MDA derivatives (not shown in the figure), exhibited higher stabilities in the alkaline pH-range. Enhanced stabilities in the acid pH-range were found for the cationic EMA-hydrazide and EMA-MDA derivatives. The modified patterns of stability as a function of pH, have been attributed to local-pH effects stemming from the redistribution of hydrogen and hydroxyl ions in the domain of immobilized polyelectrolyte enzyme derivatives[26] (see also section on pH-dependence of activity, below).

The temperature stability of the anionic and cationic EMA-MDA and EMA-hydrazide derivatives is illustrated in Fig. 2. All anionic EMA-hydrazide derivatives were more stable than the respective native enzymes. The anionic EMA-MDA derivatives were, in all cases, less stable than the corresponding EMA-hydrazide derivatives. With the cationic resins, decrease in thermal stability relative to the anionic analog was found in all cases, the cationic EMA-MDA derivatives being less stable than the native enzymes. It is of interest to point out the similarity be-

tween the temperature stabilities of the various derivatives (Fig. 2) and their stability to lyophilization (Table VI). These findings can be accomodated in a general framework by considering the chemical composition of the various support materials; the EMA-MDA resins contain bulky aromatic groups (see Scheme I) and are thus considerably more hydrophobic than the corresponding EMA-hydrazide resins. Experience has established that the temperature and lyophilization stabilities of many immobilized enzymes can be qualitatively related to the chemical nature of the support material. Thus the polyanionic, highly hydratable, ethylene-maleic acid-copolymer derivatives of several enzymes have been found to have high temperature and lyophilization stabilities[14,22,26]. By introducing hydrophobic residues, *e.g.* by using styrene-maleic acid or isobutylvinyl ether-maleic acid copolymers as supports, both the temperature and lyophilization stabilities of the enzyme derivatives are drastically lowered[27]. Similar lowering of stability has been found for enzymes immobilized on *p*-aminobenzyl-cellulose and other cellulose derivatives, leucine-*p*-aminophenylalanine copolymers and S-MDA resins[5,22]. Hydrophilic carriers such as Sephadex, Sepharose or polyacrylamide on the other hand, yield enzyme derivatives of relatively high stability[20,28,29]. Although the effects of cationization *per se* on the stability of immobilized enzymes have not been established, it is reasonable to assume, in view of the foregoing, that the decreased temperature stability of the cationic derivatives might be due to the presence of additional hydrophobic residues—the $-(CH_2)_3-$ side chain and the dimethylamino end group.

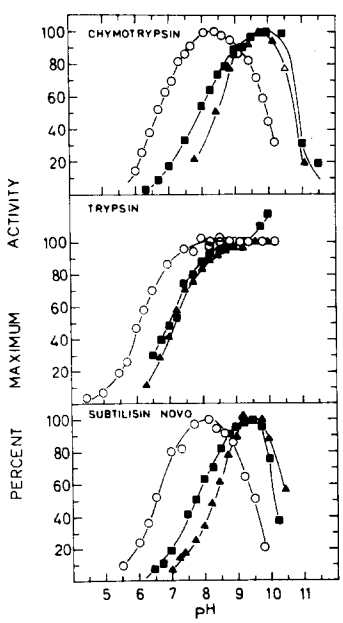


Fig. 3. pH-Activity curves for the anionic and cationic EMA-hydrazide derivatives of chymotrypsin, trypsin and subtilisin Novo. ○, native enzyme; ▲, anionic derivative; ■, cationic derivative. The initial rates of substrate hydrolysis were determined by the pH-stat method using the appropriate substrate (see Methods). All test solutions were 0.01 M in KCl.

Kinetic properties

The pH-activity profiles of both the anionic and the cationic EMA-MDA and EMA-hydrazide derivatives of all five enzymes investigated were displaced towards more alkaline pH-values, relative to the corresponding native enzymes (Fig. 3). The perturbed pH-activity profiles were essentially independent of the ionic strength of the medium. The pH-optima of activity of the various derivatives are summarized in Table VII.

The perturbation of the pH rate profiles of immobilized polyelectrolyte enzyme derivatives has been extensively investigated[2,3,26,30]. Such phenomena, as well as the modified pH-dependence of the stability of polyelectrolyte enzyme derivatives, have been attributed to electrostatic carrier-induced effects. These result in an unequal distribution of hydrogen and hydroxyl ions between the polyelectrolyte "immobilized enzyme phase" and the bulk solution, *i.e.* lower or higher "local pH" in the domain of an anionic or a cationic enzyme derivative, respectively. The pH-dependence of the stability of the anionic and cationic derivatives shown in Fig. 1, obeys this simple model. The data of Fig. 3 and Table VII show, however, that the pH-activity profiles of the cationic derivatives of all five enzymes investigated, as well as those of the anionic derivatives, are displaced towards more alkaline pH values. The electrostatic model is therefore not sufficient for a unified interpretation of the data, additional effects taking place in the presence of substrate.

When the medium contains substrate, concentration gradients of substrate and product are generated in the enzyme particle, as the reaction proceeds. The steady state distribution of concentrations of substrate and product in the immobilized enzyme particle could perturb its pH-activity profile even in the absence of carrier-induced polyelectrolyte effects, since the enzymic hydrolysis of esters such as acetyl-L-tyrosine ethyl ester or benzoyl-L-arginine ethyl ester leads to the liberation of hydrogen ions. The protons produced within the enzyme particle would lower the local-pH and lead to a displaced pH-activity curve.

The plausibility of such a model could be demonstrated by a qualitative estimation of the effective pH within the enzyme particle with an indicator. When the indicator Neutral Red was added to aqueous suspensions of anionic or cationic EMA-hydrazide derivatives of trypsin or chymotrypsin, both the enzyme particles and the external solution were yellow at pH values above 7.0. On addition of the appropriate ester substrate, the enzyme particles separated by centrifugation were red, although the supernatant solution remained yellow. This was true for both anionic and cationic derivatives.

The high values of K_m(app.), obtained for all EMA-MDA and EMA-hydrazide enzyme derivatives tested (Table VII) could be attributed in a similar fashion to substrate concentration gradients, established in the domain of the enzyme particles as a result of local consumption of substrate. Saturation of the immobilized enzyme would hence occur at overall substrate concentrations higher than those required for the saturation of the native enzyme. The swollen enzyme particles thus seem to resemble enzyme membranes in their kinetic behavior[31–33].

The caseinolytic activities calculated on the basis of the amount of active bound enzyme were lower than those of the corresponding native enzymes for all derivatives. The limiting absorbance at 280 nm, attained with high concentration of insoluble enzyme derivative, was also lower than the value recorded for the native

TABLE VII

KINETIC PARAMETERS OF THE EMA-MDA AND EMA-HYDRAZIDE ENZYME DERIVATIVES

	Trypsin		*Chymotrypsin*		*Subtilisin Novo*		*Subtilisin Carlsberg*		*Papain*	
	Optimal pH	K_m*(app.) (mM)*	*Optimal pH*	K_m*(app.) (mM)*	*Optimal pH*	K_m*(app.) (mM)*	*Optimal pH*	K_m*(app.) (mM)*	*Optimal pH*	K_m*(app. (mM)*
Native Enzyme	8.0	$< 10^{-5}$	8.3	0.7 ± 0.1	8.2	54.5 ± 10.0	8.6	26 ± 8	6.3	10.2 ± 1.0)
EMA-MDA										
Anionic	9.0	0.36 ± 0.10	9.5	7.9 ± 0.8	9.2	66 ± 12	9.4	34 ± 12	7.5	41 ± 5
Cationic	9.0	0.22 ± 0.15	9.2	1.45 ± 0.8	9.0	126 ± 25	9.2	130 ± 30	7.5	60 ± 8
EMA-hydrazide										
Anionic	9.2	0.28 ± 0.10	9.7	4.7 ± 0.9	9.5	> 100	9.25	76 ± 18	7.5	30 ± 4
Cationic	9.2	2.8 ± 0.8	9.7	15 ± 3	9.5	> 100	9.2	84 ± 21	—	—

enzyme. Representative data on the caseinolytic activity of subtilisin Novo and its EMA-MDA and EMA-hydrazide derivatives are shown in Fig. 4. The low proteolytic activities are most probably due to steric restrictions imposed by the polymeric carrier.

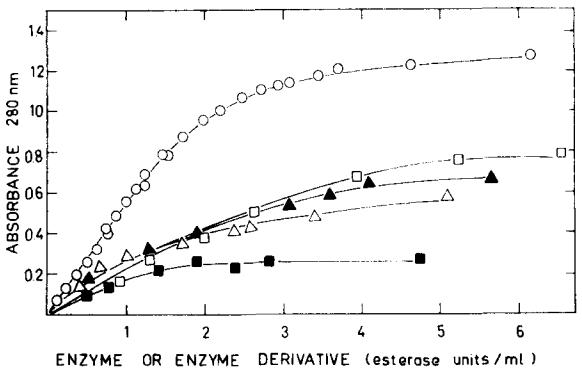


Fig. 4. Digestion of casein by subtilisin Novo and by several immobilized derivatives of the enzyme. ○, native enzyme; △, anionic EMA-hydrazide derivative; ▲, cationic EMA-hydrazide derivative; □, anionic EMA-MDA derivative; ■, cationic EMA-MDA derivative. The test solutions (2 ml) contained 5 mg/ml of heat-denatured casein in 0.1 M Tris buffer pH 7.5. The reaction mixtures were incubated at 37 °C with the specified amount of enzyme or immobilized enzyme derivative (expressed in esterase units) for 20 min. 3 ml of 10% solution of trichloroacetic acid was then added, the precipitate filtered off, and the absorbance of the trichloroacetic acid-soluble fraction determined at 280 nm.

Concluding remarks

The synthetic method described in this article is not limited to the cases discussed. By proper choice of bifunctional reagent (*e.g.* aliphatic diamines, aminoalcohols *etc.*) EMA-derived resins of a wide range of chemical and charge characteristics can be devised. Such resins can be considered as potential supports both for the insolubilization of enzymes, where a given property required for the immobilized enzyme can be built-in in the carrier polymer, and in affinity chromatography where a property of the carrier, *e.g.* charge, can be superimposed on the specificity of an immobilized ligand to fit specific needs.

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