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COUPLING OF THE *PENICILLIUM DUPONTI* ACID PROTEASE TO ETHYLENE-MALEIC ACID (1 : 1) LINEAR COPOLYMER

PREPARATION AND PROPERTIES OF THE WATER-SOLUBLE DERIVATIVE

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Summary

The coupling of the thermostable acid protease (EC 3.4.23.-) of *Penicillium duponti* K 1014 to ethylene-maleic acid (1 : 1) linear copolymer in the presence of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide at pH 3.0, afforded a soluble enzyme derivative with a protein incorporation yield of 67% under optimal conditions. The protein content of the enzyme-polymer complex, the molecular weights of the reactants, and the mean value of 2.2 lysine residues per mol of enzyme found in amide linkage to the matrix, support a structure consisting of two polymer chains per mol of protease, each chain acylating a single lysine residue of the enzyme. The isoelectric point of the coupled enzyme was found to be 3.47, a value lower than that measured on the free protease (3.81). The specific activity of the bound protease against casein, at pH 3.7 and 30°C, was 34% of that of the free enzyme, and at 75°C increased to 70%. The increased size of the coupled enzyme resulted in an improved retention of activity by ultrafiltration membranes over that observed with free protease, alone or in admixture with ethylene-maleic acid copolymer.

A water-soluble, coupled pepsin was prepared in 43% yield on protein basis by using the aminoethylmonoamide of ethylene-maleic acid copolymer and the same water-soluble carbodiimide.

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Abbreviations: EMA, ethylene-maleic anhydride (1 : 1) linear copolymer; EMAC, ethylene-maleic acid linear copolymer; CDI, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate.

Introduction

The immobilization of enzymes on synthetic polymers has been the subject of numerous studies, covering preparative methods and novel bioengineering applications in food processing, synthesis of pharmaceuticals and waste treatment systems [1].

From previous studies on the continuous hydrolysis of soybean proteins by the acid protease (EC 3.4.23.-) of *Penicillium duponti* K 1014 in membrane reactors [2], it became of interest to experiment with membranes of increased porosity, thus requiring the preparation of a water-soluble immobilized protease derivative with substantially higher molecular weight than the free enzyme in order to retain enzymatic activity. The choice of coupling technique was limited by the instability of the *P. duponti* acid protease above pH 6 [3] and by the required stability of the covalent bonds linking the enzyme to the matrix under the operating conditions of proteolysis, e.g. a pH range 2.5—4.0 and temperature of 60—65°C. In this paper we discuss the covalent coupling of *P. duponti* acid protease to ethylene-maleic acid (1 : 1) linear copolymer (EMAC), in the presence of the water-soluble 1-cyclohexyl-3(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate (CDI). EMAC and the aminoethylmonoamide were prepared from the commercially available anhydride EMA (Fig. 1). The use of both polymers for the preparation of water-soluble derivatives of pepsin is also described.

Materials and Methods

Materials. The crystalline acid protease of *P. duponti* K 1014 was prepared as described previously [3]. Twice crystallized swine pepsin (lot No. 2M-2LB) was purchased from Worthington Biochemicals, Freehold, N.J. Hammersten quality casein and hemoglobin (standardized for protease assay) were obtained from Nutritional Biochemicals Corp. EMA (Grade 21, average molecular weight about 30 000; Grade 31, average molecular weight about 60 000) were products obtained from Monsanto Company, St. Louis, Mo. Sephadex G-100 was obtained from Pharmacia Fine Chemicals Inc. CDI was purchqsed from Aldrich Chemical Co. Ethylenediamine and *N,N*-dimethylformamide were products of Fisher Scientific Co. and Aldrich Chemical Co., respectively. All other reagents were either reagent grade or the best grade available. Deionized water used throughout all the experiments was prepared with an ion exchanger Research Model II from Illinois Water Treatment Co. Ultrafiltration equipment was purchased from Amicon Corp., Lexington, Mass.

Preparation of EMAC. The polymeric carboxylic acid EMAC was prepared from the anhydride EMA (grades 21 and 31) by boiling 1% solutions in water for 20 min. Unless otherwise mentioned, EMAC refers to the acid prepared from grade 21 EMA.

Preparation of EMAC-aminoethylmonoamide hydrochloride. EMA (1.0 g, corresponding to 0.0079 mol anhydride) in 100 ml *N,N*-dimethylformamide was added dropwise with stirring to a solution of ethylenediamine (0.44 g, 0.0073 mol) in 100 ml *N,N*-dimethylformamide. After being stirred overnight at 25°C, the precipitate was collected by centrifugation and washed twice with

100-ml portions of the same solvent. The product was dissolved in 50 ml water, adjusted to pH 2.0 with 6 M HCl and dialyzed against 0.01 M HCl at 25°C for 3 days, with frequent changes of the dialysis bath. Finally, the retentate was freeze-dried, to yield 1.29 g dry product or 74% of theory. Calculated for $C_8H_{15}O_3N_2Cl$: N, 12.6. Found: 13.2.

Proteolytic activity assays. The activity of free and coupled *P. duponti* protease was assayed against casein by the method described previously [3], except that the substrate was prepared in 0.05 M sodium citrate/HCl buffer (pH 2.0). The activity of pepsin and its coupled derivatives was assayed using hemoglobin [3].

pH vs. activity curves of free and EMAC-bound P. duponti protease. Samples were assayed at 0.5 pH unit intervals, between pH 1.0 and 4.5, following the above procedures, except that hemoglobin concentration was 1.2%, and the reactions were terminated with 7.2% trichloroacetic acid. Buffers used: pH 1, adjusted with HCl; pH 1.5—4.5, 0.05 M sodium citrate/HCl.

pH vs. stability of free and EMAC-coupled P. duponti protease. Enzyme (0.125 mg on a protein basis) in 0.5 ml of buffer was incubated at 30°C for 24 h, and at 60°C for 1 h, using a series of buffers between pH 1.5 and 7.5, at 0.5 pH unit intervals. The enzyme solution was then brought to 0°C, diluted with 0.05 M sodium citrate/HCl buffer (pH 2.0), and the remaining activity assayed against casein substrate by the standard method. Buffers used: pH 1.5—2.0, 0.025 M sodium citrate/HCl; pH 2.5—7.5, 0.04 M Na_2HPO_4/0.02 M citric acid.

Thermal stability of native and EMAC-bound P. duponti protease. Enzyme (0.125 mg on a protein basis) in 0.5 ml of 0.04 M Na_2HPO_4/0.02 M citric acid (pH 4.5) was incubated 1 h at temperatures between 40 and 80°C, at 5°C intervals. The enzyme solution was brought to 0°C, diluted with 0.05 M sodium citrate/HCl buffer (pH 2.0) and the remaining activity assayed by the standard method.

Determination of protein concentration. The concentration of all free and coupled enzymes was determined spectrophotometrically at 280 nm as described before [3]. The background absorbance of EMAC at 280 nm was routinely deducted and, at the most, amounted to 5% of the total.

Relative specific activity of coupled enzymes. This quantity, defined as the ratio of the specific activity of the bound to that of the free form times 100, measured at the same temperature, was calculated from the ratio of the respective proteolytic activities at equal protein concentration.

Preparation of EMAC-coupled P. duponti protease. A solution containing 30 mg of protease and 30 mg EMAC in 6 ml water was adjusted to pH 3.0, and allowed to react with 42 mg CDI for 1 h at 25°C, followed by overnight stirring at 4°C. The precipitate that formed was redissolved by adding 0.3 ml of 1 M acetate buffer (pH 4.5) containing 2 M KCl, and the reaction mixture was subjected to gel filtration at 4°C on a Sephadex G-100 column (2.5 × 100 cm) previously equilibrated with 0.05 M acetate buffer (pH 4.5) containing 0.1 M KCl. The first peak containing the coupled protease was pooled, dialyzed against water for 2 days at 4°C, with frequent changes of dialyzing bath, and lyophilized.

Kinetics of casein hydrolysis by EMAC-coupled and free P. duponti protease. To 4 ml of 1.25% casein solution in 0.05 M acetate buffer (pH 3.2) was

added 1 ml of protease solution containing either 1 mg of native enzyme or an equivalent amount of coupled enzyme on activity basis, and the mixture was incubated at 60°C. Appropriate aliquots (0.5 ml) of the reaction mixture were periodically removed, and 0.5 ml of 2% sodium dodecyl sulfate was added to terminate the reaction as well as to solubilize the precipitate that formed during the hydrolysis. The liberation of α-amino groups was measured by the ninhydrin method [4]. The degree of peptide bond cleavage was calculated by comparing the ninhydrin value of the test sample with a reference value given by the equivalent amount of protein hydrolyzed with 6 M HCl, at 110° for 24 h.

Enzyme permeation studies through ultrafiltration membranes. P. duponti protease (10 mg) or an equivalent amount on protein basis of EMAC-coupled derivative was dissolved in 50 ml of 0.05 M acetate buffer (pH 4.5) containing 0.1 M KCl and placed in an Amicon ultrafiltration cell Model 401, fitted with Diaflo membranes of different porosity. The solution was concentrated to 10 ml at 25°C under 20 lb/inch^2 of N_2. Enzyme activity of the filtrate was assayed, and the permeation loss was expressed with respect to the total units initially present in the cell. Mixtures of native protease with EMAC-21 or -31, at concentrations equivalent to those of the derivatives, served as controls.

Isoelectric focusing analysis. The isoelectric point of the EMAC-protease was determined as already described for the native protease [3], on a LKB 8101 column with a pH gradient 3.0—6.6.

Determination of acylated lysine residues in EMAC-coupled P. duponti Protease. The number of lysine residues reacted with the matrix was determined by amino acid analysis of the hydrolysate (6 M HCl, at 110° for 24 h, in vacuum) of a EMAC-protease sample, previously deaminated in duplicate with nitrous acid by the procedure of van Slyke [5]. To 1 ml of enzyme solution containing 0.5% protein, 0.2 ml glacial acetic acid was added, followed by a drop of 1-octanol (anti-foaming agent) and 0.2 ml 60% sodium nitrite. The reaction mixture was left at 25°C for 30 min with occasional stirring; it was then dialyzed against water at 4°C for 2 days, with frequent bath changes. The contents of the bag were taken to dryness in a rotary evaporator in vacuum, at bath temperature of 40°C. The residue was hydrolyzed as mentioned, and the hydrolysate analyzed on a Beckman Amino Acid Analyzer Model 120-C, equipped with a single column of Durrum DC-1A resin and a Beckman Model 125 Integrator. The amino acid compositions of the free, EMAC-coupled and free deaminated protease were also determined. These data were necessary to estimate the lysine content of the deaminated EMAC-protease, expressed as residues per mol of protease, corresponding to those lysyl groups acylated by the matrix and therefore resistant to deamination.

Results

Optimization of the coupling reaction with EMAC. Preliminary experiments had shown that the formation of EMAC-coupled *P. duponti* protease could be followed by gel permeation on Sephadex G-100, by measuring the extent of the new peak that eluted prior to the unreacted free enzyme. Using this technique, the coupling reaction was studied in detail as a function of pH and concentration of reactants. As can be seen in Fig. 2, pH 3.0 was found op-

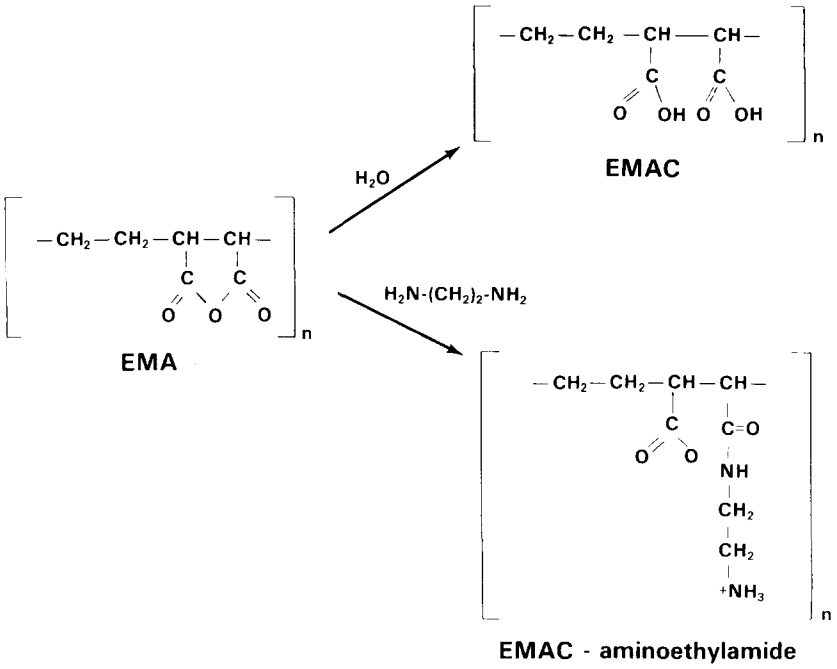

Fig. 1. Synthesis of water-soluble EMA derivatives.

timum for the reaction when the reactants were present at 0.5% concentration; above pH 3.5, the yield of bound enzyme decreased markedly. At all pH values the activity recovered in the bound enzyme fraction was proportional to the amount of protein coupled, that is, the EMAC-enzyme had a constant specific activity. The reaction at pH 3.0 was optimized next in terms of EMAC and CDI concentrations for an enzyme concentration held constant at 0.5%. At

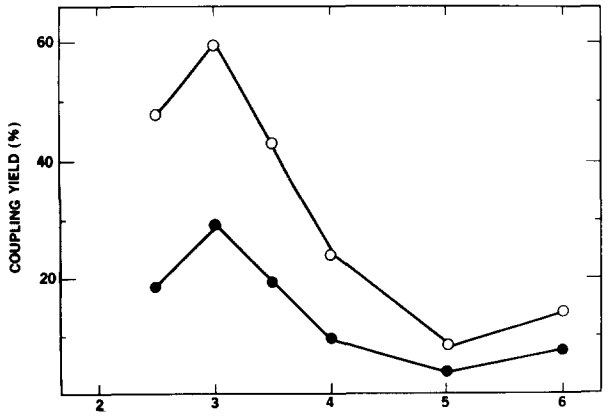

Fig. 2. Effect of pH on the coupling reaction of *P. duponti* acid protease with EMAC. The reaction was carried out at various pH values in 2 ml of the reaction mixture containing 0.5% enzyme, 0.5% EMAC, and 0.5% CDI as described in Materials and Methods. ○, coupling yield of protein; ●, coupling yield of activity.

0.5% EMAC concentration, 0.7% CDI was needed for optimum yield, corresponding to a molar ratio [COOH]/[CDI] of 4.2. On the other hand, for 0.75% EMAC the optimum changed to 1.0% CDI, with a similar molar ratio [COOH]/[CDI] of 4.6. At both optimal conditions the reactions were similar, giving a coupling yield of 67% of the protein and 27% of the activity present, measured at 30°C.

Preparation of EMAC-coupled P. duponti protease. Fig. 3 shows the results of a preparative run conducted on 30 mg protease at the optimal conditions, as described in Materials and Methods. The pure EMAC-protease recovered (48.7 mg; coupling yield 58%) had a protein content of 34.1% by Kjeldahl nitrogen and 36.3% by absorbance at 280 nm. The relative specific activity was 39.4% at 30°C.

Enzymatic properties of the EMAC-coupled P. duponti protease. The pH vs. activity profiles of the EMAC-coupled and free protease, determined for the hydrolysis of casein and hemoglobin, showed that for both substrates the pH optima of the EMAC-enzyme were shifted 0.5—1.0 unit to more acid pH values with respect to those of the native enzyme. These pH shifts resulted from to covalent attachment of the EMAC matrix, because a mixture of free protease and EMAC at equivalent concentrations reproduced the activity profiles of the free enzyme alone.

The EMAC-protease was found less stable than the free protease at pH values below 3.5. However, in the range 3.5—7.5, the stability of both enzyme preparations was similar.

The thermal stability of both enzymes was examined by incubation at various temperatures at pH 4.5, pH at which the free protease seems to be most

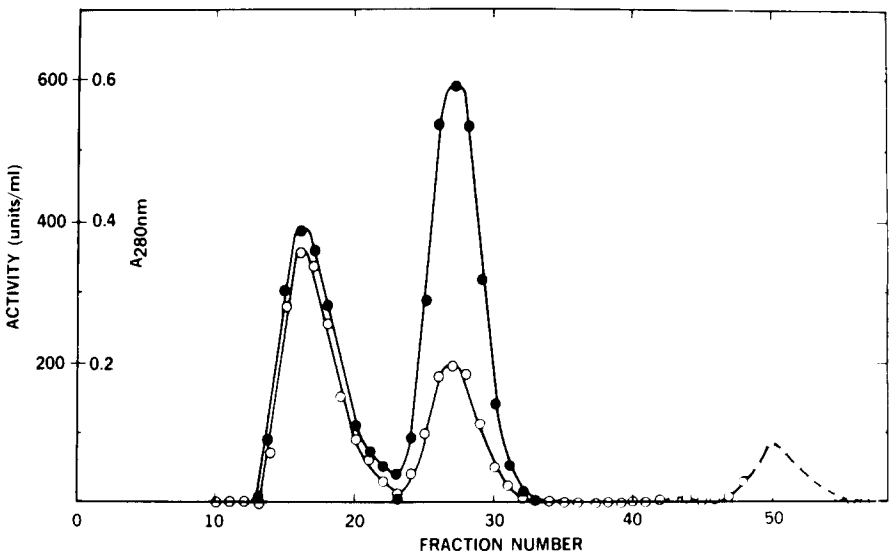

Fig. 3. Separation of EMAC-bound protease of *P. duponti* from unreacted enzyme. Permeation through Sephadex G-100 as described in Materials and Methods. The flow rate was 20 ml/h and 10-ml fractions were collected; ○, $A_{280\ nm}$; ●, protease activity. Fractions (Nos. 14—20) were combined and the product isolated (see text).

stable. Throughout the temperature range 40—80°C both forms exhibited remarkably similar thermostability.

Under the conditions described in Fig. 4, the specific activity of the EMAC-protease measured against casein at pH 3.7 and 30°C was only 34% of that of the free enzyme. However, it showed remarkable enhancement at higher temperatures, rising to 70% at 75°C.

The rate of casein hydrolysis, at pH 3.2 and 60°C, was followed for both proteases by measuring the liberation of α-amino groups as a function of time. At equivalent activities, both enzymes hydrolyzed casein to the same extent, with 18% of the bonds split after 8 h.

Retention of EMAC-coupled P. duponti protease by ultrafiltration membranes. As indicated in Table I, the two types of coupled protease tested, prepared with EMAC-21 and EMAC-31, showed an improved retention over membranes of various porosities, whereas the controls gave permeation values similar to those of the free enzyme. The better retention seen for the EMAC-31 derivative was the result of the increased molecular size imparted by the matrix. In addition, the results demonstrate that covalent binding of the enzyme to the polymeric matrix is essential for the retention of enzyme activity.

Determination of the isoelectric point. Fig. 5 compares the isoelectric points of the EMAC-bound protease and the free enzyme. In both cases, a symmetrical peak with coinciding 280 nm absorbance and proteolytic activity was formed, containing over 96% of both the activity and 280 nm absorbance applied to the column. The isoelectric point of the EMAC-protease was found to be 3.47, shifted downwards with respect to that of the free protease (3.81). When the EMAC-enzyme recovered from the electrofocusing column was permeated through Sephadex G-100, it was eluted at the same position as before, indicating no change of molecular size occurred as consequence of electrofocusing. This fact is further evidence that the enzyme in the EMAC-protease is covalently bound to the matrix because independent experiments have shown that the permeation properties of the free enzyme through Sephadex G-100 are not changed in the presence of EMAC.

Number of lysine residues acylated in EMAC-coupled P. duponti protease.

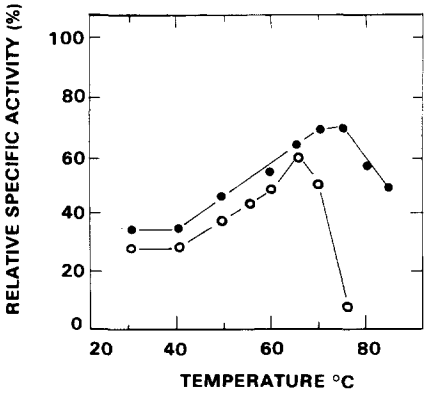

Fig. 4. Effect of temperature upon the relative specific activity of EMAC-immobilized *P. duponti* protease. ○———○, pH 2.5; ●———●, pH 3.7.

TABLE I

ULTRAFILTRATION BEHAVIOR OF NATIVE AND COUPLED *P. DUPONTI* ACID PROTEASE

Experimental details are described in Materials and Methods. Protease activity found in the filtrate is expressed as percentage with respect to the total activity used for the experiment.

Diaflo membrane type	Molecular weight cut-off	Permeation loss of protease activity (%)				
		Free enzyme	Enzyme immobilized with			
			EMAC-21		EMAC-31 *	
			Control	Test	Control	Test
PM-10	10 000	0.2	0.2	0	0.02	0
PM-30	30 000	12.7	14.2	1.0	13.2	0.03
XM-50	50 000	43.3	44.7	10.8	43.8	1.44
XM-100A	100 000	42.1	44.1	13.6	43.0	1.20

* This derivative was prepared by the same method as used for EMAC-21 derivative (see text). Protein content of the coupled enzyme was calculated to be 35.2% from nitrogen content obtained by micro-Kjeldahl method. The relative specific activity was 36.8% at 30°C.

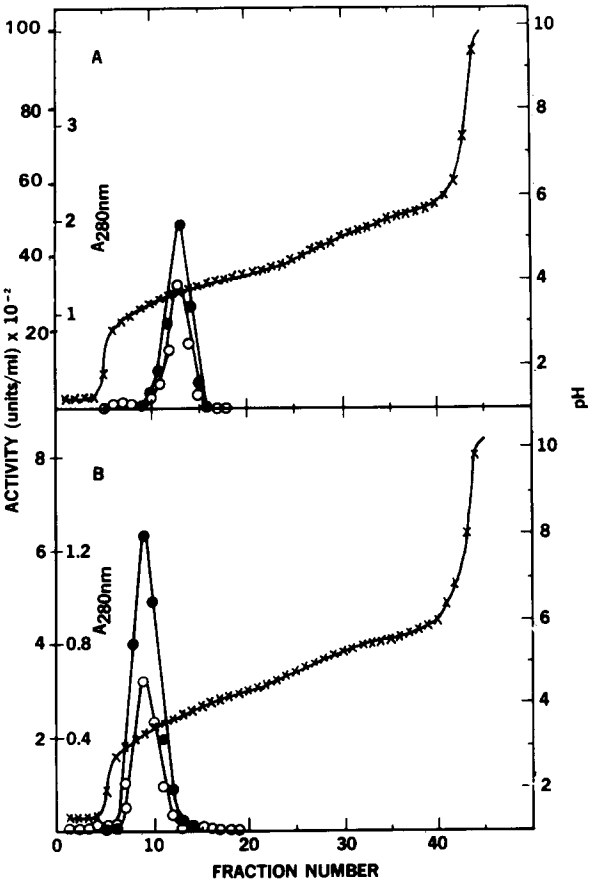

Fig. 5. Isoelectric focusing profiles of free and EMAC-coupled *P. duponti* protease. Experimental details are described in Materials and Methods, using 10 mg of material in each case: ○, $A_{280\ nm}$, ●, protease activity. (A) Free enzyme. (B) Immobilized enzyme.

TABLE II

CONTENT OF SELECTED RESIDUES IN THE VARIOUS *P. DUPONTI* PROTEASE DERIVATIVES

Values expressed as residues per mol of protease ± S.E. of the mean. In case of the derivatives, the residue content refers to the protease moiety of the complex.

Residue	Free protease	EMAC-protease	Deaminated free protease	Deaminated EMAC-protease
Lys	12.5 ± 0.2	13.2 ± 0.1	0 ± 0.0	2.2 ± 0.1
Glu	42.7 ± 0.2	42.5 ± 0.1	44.4 ± 1.1	42.9 ± 0.4
Val	22.1 ± 0.0	22.0 ± 0.2	23.3 ± 0.6	22.5 ± 0.6
Ile	13.0 ± 0.1	13.3 ± 0.1	12.7 ± 0.3	13.8 ± 0.4
Leu	26.9 ± 0.3	26.7 ± 0.2	25.5 ± 0.6	27.1 ± 0.8

The binding was expected to involve the acylation of some of the 12 lysine residues of the enzyme by the CDI-activated carboxylate groups of the matrix. Table II shows that the content of glutamic acid, valine, isoleucine and leucine in the various enzyme derivatives is, within experimental error, unchanged with respect to the free protease. Also, the deamination conditions totally destroyed lysine in the free protease, but in the case of the EMAC-protease, however, it left intact those lysyl residues acylated by the matrix. The value of 2.2 lysine residues per mol of protease found is the mean of four estimates, each one based on the content of the above four amino acids taken as reference.

Immobilization reactions with the aminoethylamide of EMAC. Table III shows that the coupling of pepsin to EMAC in the presence of CDI at pH 3.0 was poor, as expected from the low content of lysine [6]. However, the coupling to the aminoethylamide of EMAC at pH 3.5 proceeded readily, resulting in a 3-fold increase in yield. The coupling of *P. duponti* protease to the aminoethylamide of EMAC at pH 4.0 was found inferior to that observed with EMAC, because of the poorer yield and the 8-fold reduction in relative specific activity.

TABLE III

COUPLING OF *P. DUPONTI* PROTEASE AND PEPSIN TO EMAC AND ITS MONOAMINOETHYLAMIDE

Coupling reactions with EMAC were carried out at pH 3.0; coupling with EMAC-aminoethylamide was conducted at pH 3.5 for pepsin, and at pH 4.0 for *P. duponti* protease, following the general procedure described in Materials and Methods, using 0.5% enzyme, 0.5% matrix and 0.7% CDI.

	Coupling * yield (%)	Relative specific activity (%)
EMAC/pepsin	16.3	25.0 (pH 1.8, 30°C)
EMAC-aminoethylamide/pepsin	43.2	29.6 (pH 1.8, 30°C)
EMAC/*P. duponti* protease	64.7	36.4 (pH 2.0, 30°C)
EMAC-aminoethylamide/*P. duponti* protease	37.3	4.5 (pH 2.0, 30°C)

* Based on protein content.

Discussion

The coupling of the acid protease of *P. duponti* to EMAC in the presence of the water-soluble carbodiimide CDI has been optimized in terms of pH and concentration of reactants. The optimal pH for the reaction was found to be 3.0, with a marked drop in yield occurring on either side of this point. This behavior could be interpreted by assuming that the ionic attraction of the reactants precedes and, hence, facilitates acylation. At pH 3.0 the ionic interaction between enzyme and matrix should be maximal, decreasing above the isoelectric point (pH 3.8) where the enzyme acquires negative charge, and below pH 3.0 when the negative charge of EMAC (pK_{a1} 4.35) [7] is lost by protonation.

The acylation reaction conducted under optimal conditions at pH 3.0 required 112 activated carboxylates in the matrix per mol of enzyme, a molar excess calculated from the ratio of [carboxylate] to [CDI] in the reaction mixture. Coupling proceeded, however, in a highly restricted manner, involving the acylation of only 2.2 lysine residues out of the 12 present in 1 mol of enzyme.

The protein content of the EMAC-protease, the molecular weights of the reactants, the gel permeation behaviour of the coupled enzyme on Sephadex G-100, and the 2.2 lysine residues per mol found in amide linkage to the matrix, support a structure consisting of two EMAC chains per mol of protease, each chain acylating one lysine residue.

The pH shift of optimal activity against casein and hemoglobin is a direct result of the covalent attachment of the enzyme to the polycarboxylic acid matrix. The pH shift could be due to the negatively charged micro environment [8] surrounding the immobilized enzyme; this effect disappears at low values of pH, since the overall negative charge is diminished through protonation.

The increase of the relative specific activity of the EMAC-protease with temperature (Fig. 4) observed at pH 2.5 and 3.7 is a remarkable phenomenon. This apparent thermal activation of the coupled enzyme can be rationalized in terms of an "unwrapping effect" of the two EMAC chains triggered by hydrogen-bond breaking as the temperature is increased, thus leading to a more open conformation with a higher accessibility of the active site of the enzyme to the substrate.

The degree of hindrance (electrostatic, steric) of the substrate-enzyme interaction introduced by the polyanionic matrix should be further explored with the aid of specific, low molecular weight substrates, having better access to the occluded active site. Although so far no suitable synthetic dipeptide has been found to function as a substrate for the *P. duponti* acid protease, the use of diphenyl sulfite for this purpose may be fruitful, because this compound is a synthetic substrate for pepsin [9].

On an isoactivity basis, the EMAC-coupled *P. duponti* protease hydrolyzed casein at pH 3.2 and 60°C to the same extent as the free protease. This fact, along with the 3-fold increase in molecular weight of the immobilized enzyme, could allow peptide size regulation during continuous proteolysis in membrane reactors, as suggested earlier [10].

The coupling experiments with pepsin have shown the advantages of the aminoethylamide of EMAC over EMAC itself for the synthesis of water-soluble derivatives of proteins of low lysine content in good yield, in excellent agree-

ment with previous observations [11]. The similar relative specific activity found for both pepsin derivatives (Table III) suggests that the coupling of the enzyme to EMAC-aminoethylamide does not involve the aspartyl residue present in the active site, which is essential for catalysis. In the case of the *P. duponti* protease, however, the 8-fold reduction in relative specific activity observed for the EMAC-aminoethylamide protease with respect to the EMAC derivative, strongly suggests that in the first instance the essential aspartyl group [3] may have reacted with the matrix.

The characterization of these bound enzymes with the aid of specific inactivators, inhibitors and synthetic substrates will indicate the changes created by binding and possibly will reveal further differences between pepsin and the fungal acid proteases.

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