INTRODUCTION OF ELASTOLYTIC ACTIVITY INTO ALKALINE PROTEINASE FROM *ASPERGILLUS SOJAE* VIA ELECTROSTATIC CHANGES DUE TO MODIFICATION OF CARBOXYL GROUPS

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Received 16 September 1971

1. Introduction

The absorption of elastase and other basic proteins on elastin was found to be a non-specific, salt-dependent electrostatic process that occurs between the carboxyl groups of elastin and the positively charged groups of basic proteins [1].

It was also suggested [1] that the elastolytic activity of any basic proteinase depends on two qualities: (i) ability to be adsorbed on elastin in the pH range 7–10; (ii) side chain specificity toward the non-aromatic, non-polar amino acids that form the bulk of the amino acids of elastin.

This suggestion was substantiated by the finding that maleylation of the ε-amino groups of porcine elastase and subtilisin caused a complete loss of their ability to be adsorbed on elastin due to a change in their electrostatic properties [2], resulting in a complete loss of their elastolytic activities while their esterolytic activities were almost unchanged.

The present report describes the introduction of elastolytic activity into alkaline proteinase from *Aspergillus sojae* due to electrostatic changes resulting from modification of its carboxyl groups, with water soluble carbodiimide.

2. Experimental

2.1. *Materials*

Alkaline proteinase from *A. sojae* [3] was a gift of Dr. K. Hayashi, porcine elastase was prepared according to Shotton [4] and crystalline subtilisin (Novo type) was purchased from British Drug House (Poole, England). 1-Ethyl-3-(3-dimethyl-aminopropyl) water soluble carbodiimide (WSC) was obtained from Sigma Chemical Co. (St. Louis, Mo., USA) and N-acetyl-L-alanyl–L-alanyl–L-alanine methyl ester ($AcAla_3OMe$) from Miles-Yeda Inc. (Rehovot, Israel).

2.2. *Assay of enzymatic activities*

The esterolytic activity was estimated titrimetrically [5] using the $AcAla_3OMe$. The substrate concentration was 7 mM. The proteolytic activity was estimated on casein [6]. The elastolytic activity was assayed using the Congo Red Elastin (CR-elastin) method [7] as modified by Gertler and Birk [8] or by the direct spectrophotometric method using elastin [9] as described previously [2]. The activity was expressed in CR-elastin units [8] or A_{276} units [2], respectively.

2.3. *Modification of the carboxyl groups*

The modification was carried out at pH 4.75, 25° for a period of 4 hr. The concentration of the alkaline proteinase from *A. sojae* in the reaction mixture was 2 mg/ml and that of WSC was 25 mM. Aliquots were withdrawn from the reaction mixture during the reaction course for immediate estimation of enzymatic activities. Larger samples were removed from the reaction mixture after 60 and 240 min of reaction. They were dialysed in the cold against distilled water and freeze-dried. These samples were used for electrophoretic and adsorption studies.

2.4. *Electrophoresis on cellulose acetate membranes*

The electrophoresis was performed in the Beckman

Micro-Zone Electrophoresis system Model R-100 in 0.08 M pyridine acetate buffer pH 4.0. Samples of 0.25–0.75 nl of 2% of protein solution were applied to the membrane and electrophoresis was performed for 20 min at a potential of 200 V. The layers were stained with Ponceau-S as described originally [10].

2.5. *Adsorption on elastin*

The adsorption and desorption experiments were performed as described previously [1] with the following changes: the amount of elastin was 150 mg per tube and the final volume was 4 ml.

3. Results and discussion

The effect of time on the modification of alkaline proteinase by WSC is presented in fig. 1. As shown, the modification causes partial loss of esterolytic and proteolytic activities, concomitant with almost parallel introduction and increase of the elastolytic activity, as assayed by the direct spectrophotometric method. However, the K_m value for $AcAla_3OMe$ of the native and modified enzyme was almost the same.

It should be mentioned that similar results were obtained when the reaction mixture contained 250 mM NH_2OH, which is a strong nucleophile [11] and

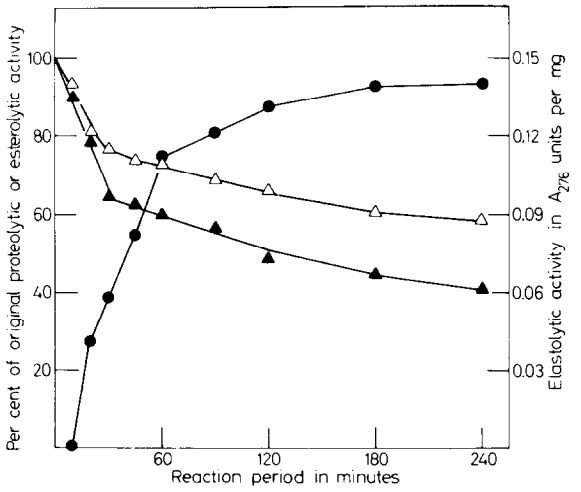

Fig. 1. The time course of the modification of alkaline proteinase from *Aspergillus sojae* by WSC and its effect on the esterolytic (△—△), proteolytic (▲—▲) and elastolytic (●—●) activity.

Table 1
Comparative elastolytic activities of modified alkaline proteinas from *A. sojae*, subtilisin (Novo type) and porcine elastase.

Enzyme assayed	Substrate used	
	Elastin (units*/mg enzyme)	Congo Red Elastin
Modified alkaline proteinase (60 min modification)	0.110	68
Modified alkaline proteinase (240 min modification)	0.140	70
Subtilisin	0.328	115
Porcine elastase	1.410	304

* A_{276} units for elastin [3] and CR elastin units [8] for Congo Red elastin.

replaces the carbodiimide from the carboxyl groups. However, if the reaction is performed in the presence of 250 mM ethylenediamine (H_2N-CH = CH-NH_2) it results in a complete loss of all the enzymatic activities. It seems, therefore, that when the carbodiimide is replaced by a rather small and non-charged residue, the enzyme is affected very little, but when the introduced group changes the charge from negative to positive, the conformational change is probably so drastic that the enzyme becomes denaturated.

The elastolytic activities of the modified alkaline

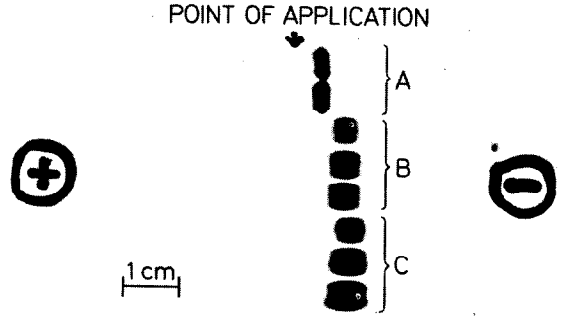

Fig. 2. Electrophoresis of the native and modified alkaline proteinase from *A. sojae* on cellulose acetate membranes in 0.08 M pyridine–acetate buffer pH 4.0 for 20 min at 200 V. A = native proteinase; B = modified proteinase (60 min modification); C = modified proteinase (240 min modification). The amount of protein applied in each band varied from 5–15 ng.

Table 2
The effect of ionic strength and enzyme concentration on the adsorption of modified alkaline proteinase (240 min modification) on elastin at pH 8.8.

Adsorption medium		Enzyme concentration (mg/tube)	Per cent activity not adsorbed on elastin as measured on:		
Na-borate buffer pH 8.8	+NaCl		$AcAla_3OMe$	Casein	Elastin
5 mM	none	0.20	26	27	25
10 mM	none	0.20	30	28	23
20 mM	none	0.20	32	36	27
20 mM	50 mM	0.20	73	71	65
20 mM	100 mM	0.20	101	100	97
10 mM	none	0.40	35	36	37
10 mM	none	0.32	32	30	28
10 mM	none	0.24	26	28	29
10 mM	none	0.16	22	20	22
10 mM	none	0.08	16	17	not tested

proteinase as compared to those of porcine elastase and subtilisin are shown in table 1. The modified enzymes exhibit 10% of elastolytic activity as compared to porcine elastase when the assay was done on elastin and 23% when the assay was performed on Congo Red elastin. Similar relative elastolytic activities, as measured by the latter method were observed in 2 elastolytic proteinases from *Streptomyces griseus* [12].

Fig. 2 presents the electrophoretic properties of the native and modified enzymes. As expected, the modification makes the enzyme more basic due to blocking of its carboxyl groups. After modification periods of 60 or 240 min all the enzyme is modified and no traces of the native enzyme could be observed. Small differences can be also seen between the enzyme modified for 60 and 240 min, the latter one being more basic. It should be noted that the bands of the modified enzymes are more diffused than those of the native enzyme. It is difficult therefore to conclude whether the enzymatic activities of the modified enzyme are unique properties of each molecule, or they rather represent an average of the whole population.

The effect of the ionic strength and enzyme concentration on the adsorption of the modified alkaline (240 min modification) proteinase on elastin is summarized in table 2. The results show clearly that the adsorption process is salt-dependent and has probably a non-specific electrostatic nature similarly to other basic proteins [1], and occurs likewise between the carboxyl groups of elastin and positively charged groups of the modified enzyme. It should be remembered that the native alkaline proteinase does not adsorb at all on elastin because of its slightly acidic pI and the adsorption is achieved due to the at least partial blocking of its carboxyl groups that makes this protein more basic. As shown in table 2 the extent of adsorption is never 100% and is influenced by the enzyme concentration. As reported previously [1] trypsin, elastase and bovine pancreatic trypsin inhibitor were fully adsorbed on elastin under similar experimental conditions. It may be therefore concluded that the interaction between the elastin and the modified alkaline proteinase is relatively weaker, since the modified enzyme is relatively less basic, thus resembling the behaviour of α-chymotrypsin [1].

As can be also seen the percent of enzymatic activities non-adsorbed on elastin were quite similar when assayed with 3 different substrates. This indicates that even if the population of the modified molecules is heterogeneous, it is still basic enough to be adsorbed on elastin. Moreover, all the adsorbed activities could be quantitatively recovered by extracting the enzyme adsorbed on elastin with Na-borate buffer, pH 8.8, containing 200 mM NaCl.

All the presented results emphasize the fact that the adsorption of elastolytic enzymes on elastin is essential for the elastolysis. Furthermore, the results substantiate our former hypothesis [1, 2] that any

alkaline proteinase, which possesses side chain specificity toward the non-aromatic non-polar amino acids and is basic enough to be adsorbed on elastin at slightly alkaline pH, will solubilize this insoluble protein.

Acknowledgements

The author is indebted to Prof. Y. Birk for her interest and valuable comments, to Miss Geta Tinman for her excellent technical assistance, and to Dr. K. Hayashi for the sample of alkaline proteinase.

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