AN ASPARTIC ACID RESIDUE AT THE ACTIVE SITE OF *RHODOTORULA GLUTINIS* ACID PROTEASE

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1. Introduction

A new extracellular acid protease from *Rhodotorula glutinis* [1,2] is rapidly inactivated by specific esterification of a carboxyl group at the active site with diazoacetyl-D, L-norleucine methyl ester and related diazoacetyl compounds [3] as porcine pepsin. It is of great interest, the coloured inhibitor, *N*-diazoacetyl-*N'*-2,4,-dinitrophenylethylenediamine [4] has an absorption band at 360 nm, which is used to detect the labeled residue and to determine the active-site amino acid sequence of the enzyme. Stepanov et al. [4,5] showed specifically inhibiting porcine pepsin with *N*-diazoacetyl-*N-'*2,4-dinitrophenylethylenediamine (DDE), and this result facilitates determination of the active-site amino acid sequence of porcine pepsin. Thereafter, Kovaleva et al. [6] also showed that *N*-diazoacetyl-*N-'*2,4-dinitrophenylethylenediamine inactivated an acid protease (awamorin) from *Aspergillus awamori*, and the result led them to determine the active-site amino acid sequence of awamorin.

In the presence of cupric ions, *N*-diazoacetyl-*N-'* 2,4-dinitrophenylethylenediamine also inactivated completely this acid protease, and only 1 eq of DDE is introduced per molecule of the enzyme.

In order to determine the amino acid sequence around the active site of *Rhodotorula glutinis* acid protease, we used DDE as a reagent to elucidate the active site sequence of this acid protease.

In this paper, we describe the identification of the amino acid residue of *Rhodotorula glutinis* acid protease which is related to the reaction of this enzyme with DDE.

2. Materials and methods

Acid protease of *Rhodotorula glutinis* (twice crystallized) used throughout this experiment was kindly given by Professor Murao. DDE was prepared by the method of Stepanov et al. [4]. Other reagents obtained were the highest purity and used without further purification.

The protein concentration of the enzyme solution was determined by measuring the absorbance at 280 nm using $A^{1\%}_{1\,cm} = 14$ [2], and the concentration of inhibitors in DDE-modified enzyme and the concentration of DDE-modified peptide were measured the absorbance at 360 nm using M = 15 000 [4] that was caused by the dinitrophenylamino group of inhibitor.

3. Results and discussion

DDE-modified *Rhodotorula glutinis* acid protease was obtained as follows: To the solution of 60 mg (4×10^{-5} M in a final volume) of *Rhodotorula glutinis* acid protease in 25 ml of 0.1 M acetate buffer, pH 5.5, and then 12.5 ml of 0.016 M cupric acetate (4×10^{-3} M in a final volume) and 12.2 ml of water were added with stirring, after 10 min, at 22°C, 17.64 mg (1.2×10^{-3} M in a final volume) of DDE in 0.3 ml methanol was added to the solution, made to a final volume of 50 ml (enzyme 1: reagent 30 : Cu^{2+} 100). After 1 hr, the reaction mixture devoid of proteolytic activity was filtered through thick filter paper to remove the fine precipitate. The clear solution was filtered through a Sephadex G-25 column

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(3 X 63 cm) equilibrated and eluted with water. The first fraction which revealed two maxima in UV spectrum - at 280 and 360 nm – were collected and lyophilized to give about 45 mg of DDE-modified *Rhodotorula glutinis* acid protease. Above experiment was repeated 7 times in order to prepare 315 mg of DDE-modified enzyme.

300 mg of DDE-modified *Rhodotorula glutinis* acid protease was subjected to peptic hydrolysis after short incubation in slightly alkaline solution to ensure the denaturation of this DDE-modified enzyme, and was dissolved in 45 ml of 0.05 M Tris buffer pH 7.4 after 10 min 11.5 ml of 0.05 N HCl was added to lower pH of the mixture to 3.2, then 10 mg of pepsin in 1 ml of water was added. The mixture was kept at 20° C for 24 hr. After 1 hr of the incubation the initial turbidity of the mixture disappeared, after 6 hr another 10 mg portion of pepsin was added.

Chromatography of the peptic hydrolysate on a Sephadex G-25 column (3 X 150 cm) gave four peaks as shown in fig. 1. Peak 4 contained coloured neutral peptide which was pooled, evaporated to dryness in a rotating evaporator and further purified by high voltage paper electrophoresis at pH 2.2 and 3.5 is shown in fig. 2. The single yellow spot was found to migrate towards the cathode and

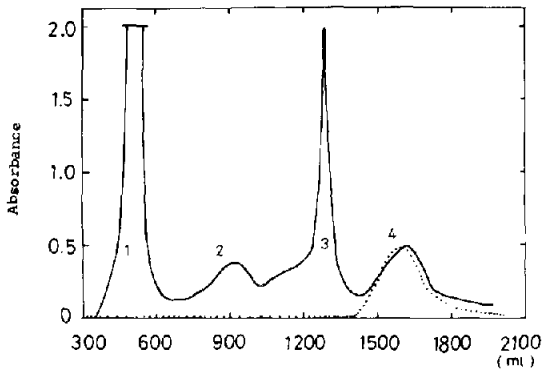


Fig. 1. Chromatography of peptic hydrolysate of DDE-modified *Rhodotorula glutinis* acid protease on a column (3 X 150 cm) of Sephadex G-25. Solid line = absorbance at 280 nm. Dotted line = absorbance at 360 nm. The fraction comprising peak 4 was collected and freeze-dried, and the resulting yellow peptide was dissolved in water and subjected to electrophoresis at pH 2.2 and 3.5.

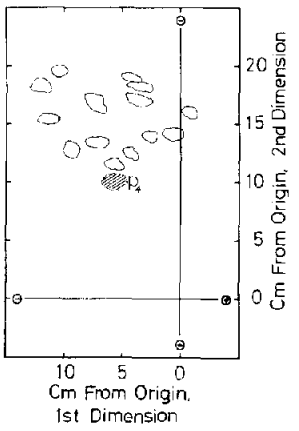
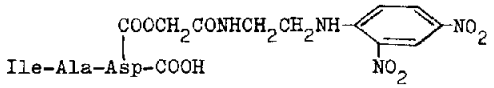


Fig. 2. Two dimensional high voltage electrophoretic pattern of peptide recovered under Peak P4. The first dimension was performed at pH 3.5 in pyridine–acetic acid–water buffer (1:10:89 by vol.), 30 V/cm, 2 hr and the second dimension at pH 2:2 in pyridine–acetic acid–80% formic acid–water buffer (1:10:13.5:89 by vol.), 30 V/cm, 90 min. The position of peptide P4 was observed as yellow spot.

corresponded to a spot that slowly developed a red colour with ninhydrin–cadmiun reagent. The paper corresponding to the single yellow spot was located, cut out, and sewn onto another sheet of Toyo No. 50 paper, and subjected to paper chromatography with methylethylketone–*t*-butanol–water (2:1:1 by vol.) as eluent. The material in this yellow spot was eluted from the paper with water, freeze dried and subjected to amino acid analysis. After acid hydrolysis (6 N HCl, 110° C, 22 hr) of the aliquot of this purified peptide containing 100 nmoles of DDE residue the following quantities of amino acids were found (in nmoles): Asp 101, Ala 99, Ile 96. Apparently, the inhibitor is attached to the β-carboxyl group of aspartic acid residue in this peptide.

It is identified by DNP-method [10] that isoleucine is the N-terminal amino acid residue of this peptide. The tripeptide which turned to be stable to carboxypeptidase A action was subjected to partial hydrolysis with 0.01 N HCl at 110° C for 24 hr. The separation of the hydrolysate by paper electrophoresis at pH 3.5 in pyridine–acetic acid–water (1:10:89 by vol.), 50 V/cm, 80 min, revealed the presence of free inhibitor residue, that of aspartic acid and of neutral peptide which did not contain the inhibitor residue. After additional purification

by paper electrophoresis at pH 5.6 in 0.25 M triethylamine solution adjusted pH with acetic acid, 50 V/cm, 70 min, this peptide was hydrolyzed (6 N HCl, 110° C, 48 hr). In the hydrolysate 45 nmoles of alanine and 43 nmoles of isoleucine were found. As far as the N-terminal position of isoleucine has been proved, the sequence Ile -Ala should be assigned to the dipeptide and the following structure to the tripeptide derivative:



It is worthwhile to mention that porcine pepsin cleaves preferentially the peptide bond at the α-carboxyl group of aspartic acid residue of which the β-carboxyl group is modified by attachment of rather bulky hydrophobic DDE residue. Therefore, DDE, the inhibitor containing diazoacetyl group as a reactive center interacts in *Rhodotorula glutinis* acid protease with β-carboxyl group of the unique aspartic acid residue which belongs to the sequence Ile–Ala–Asp, and this reaction leads to the inactivation of the enzyme.

As in the cases of porcine pepsin and various kinds of acid proteases [5–9] the high specificity of the reaction points on the functional essential carboxyl group which becomes labeled. Thus, there is reason to conclude that the carboxyl group of aspartic acid residue may be considered as an essential part of the catalytic sites of *Rhodotorula glutinis* acid protease and all pepsin-like acid proteases.

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