THE SITE OF DIAZOACETYL INHIBITOR ATTACHMENT TO ACID PROTEINASE OF ASPERGILLUS AWAMORI - AN ANALOG OF PENICILLOPEPSIN AND PEPSIN

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SUMMARY It is shown that N-diazoacetyl-N'-2,4-dinitrophenyl-ethylenediamine inactivates specifically the acid proteinase of Aspergillus awamori - awamorin by the attachment to the carboxyl of aspartic acid residue in the sequence Ile-Ala-Asp. This tripeptide coincides with the part of the sequence around the reactive aspartic acid residue in penicillopepsin and resembles the corresponding sequence of pepsin.

The acid proteinases of molds are close to pepsin when the functional properties of the enzymes are compared (1-4).One of them purified from the surface culture of Aspergillus awamori and referred further as awamorin might be inactivated by the treatment with coloured inhibitor - N-diazoacetyl-N'-dinitrophenyl-ethylenediamine (DDE) (5) at pH 5.0 in the presence of Cu^{++}ions.(6). Another enzyme of the same type - an acid protease B from Aspergillus niger kindly supplied by prof. Terui (Japan) might be also inactivated by DDE under the same set of conditions. In both cases only one DDE residue was attached to the enzyme molecule.

The data obtained earlier have clearly shown that DDE (7) as well as the other inhibitors bearing diazoacetyl group inactivate pepsin by the specific interaction with ß-carboxyl group of aspartic acid residue localized in the sequence:

Ile-Val-Asp-Thr-Gly-Thr-Ser-Leu (8,9)

This communication describes the identification of the amino acid residue of awamorin which is responsible for the reaction of the enzyme with DDE. Pure awamorin used throughout this work was prepared from the surface culture by a modification of previously described procedure (5) (Table 1).The purity of the enzyme has been checked by disc-electrophoresis, isoelectrofocusing (authors are indebted to Drs.E.D.Levin and A.Ya.Strongin for these experiments) and chromatography on ECTEOLA-cellulose. The amino acid composition of awamorin corresponds to the formula: Lys_{12},His_3,Arg_3,Asp_{37},Thr_{30},Ser_{40}, Glu_{26},Pro_{18},Gly_{35},Ala_{25},Val_{22},Ile_{15},Leu_{22},Tyr_{13},Phe_{17},Trp_3. These data corresponding to 319 amino acid residues per enzyme molecule might be corrected further. The resemblance of the amino acid composition of awamorin and acid proteinase from Aspergillus saitoi has been discussed earlier (5).

Dinitrophenylation and dansylation as well as the application of Beckman 890 Sequencer failed to reveal N-terminal amino acid of awamorin. Carboxypeptidase A at pH 8 splits off the C-terminal alanine and some other amino acids from C-terminal sequence of awamorin:

$$-(Gly_1,Leu_1,Ala_2,Phe_1)\text{-Ala COOH}$$

DDE-treated awamorin was obtained as follows: To the solution of 200 mg of awamorin in 150 ml of water 1.5 ml of 4 M acetate buffer, pH 5.5 and 2 ml of copper acetate (0.1 M) were added. After 10 min. at 20° 40 mg of DDE in 0.5 ml of wet acetone was added to the solution. After 1 h. the reaction mixture devoid of proteolytic activity was filtered through thick paper filter to remove the fine precipitate. The clear solution was filtered through Sephadex G-25 column (63x4.5cm) washed with water. The first fractions which revealed two

Table 1. The isolation of awamorin from the surface culture of Asp.awamori.For detailed description see ref.5. Chromatography on Amberlite IRC-50 has been introduced to improve the purification.

Step	Total protein content in g	Activity in units (5)	Specific activity units/mg
Water extraction pH 3.0	619	322000	0.52
1st precipitation with ammonium sulfate (0.8 satd.)	97.4	263000	2.7
2nd precipitation with ammonium sulfate (0.8 satd.)	81.1	243000	3.0
Precipitation with 64 per cent C_2H_5OH	30.7	153000	5.0
Chromatography on Amberlite IRC-50, pH 3.4 - 5.5	10.2	143000	14
Chromatography on Sephadex G-75	-	-	22
Chromatography on ECTEOLA-cellulose	1.49	44700	30

maxima in UV spectrum - at 280 and 360 nm were collected and lyophilized to give 150 mg of DDE-modified awamorin.

The modified awamorin was subjected to peptic hydrolysis after short incubation in slightly alkaline solution to ensure the denaturation of this derivative. 300 mg of DDE-treated awamorin 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 8 mg of pepsin in 1 ml of water was added. The mixture was kept for 22 h. at 20°. After 1 h. of the incubation the initial turbidity of the mixture disappeared, after 6 h. another 8 mg portion of pepsin was added.

Chromatography of the hydrolysate on Sephadex G-25 column (3x150 cm) gave four yellow fractions (Fig.1). Fraction 3 contained coloured neutral peptide which was purified by paper electrophoresis at pH 2.2 and 3.5 (25 v/cm) and paper chromatography on Whatman 3MM paper in methylethylketone-t.-butanol-water (2:1:1 by vol.). After acid hydrolysis (5.7 N HCl,105°,24 h.) of the aliquote of this peptide containing 100 nmol of DDE residue the following quantities of amino acids were found (in nmol): Asp 110, Ala 98, Ile 95. Evidently, the inhibitor is attached to the β-carboxyl group of aspartic acid residue in this tripeptide.

It was shown by DNP-method that isoleucine is the N-terminal amino acid 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°,24 h. The separation of the hydrolysate by paper electrophoresis at pH 3.5 revealed the presence of free inhibitor residue, of aspartic acid and of neutral peptide which did not contained the inhibitor residue. After additional purification by paper electrophoresis at pH 5.6 this peptide was hydrolysed (5.7 N HCl,110°,48 h.). In the hydrolysate 45 nmol of alanine and 41 nmol of isoleucine have been 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:

The ester bond connecting DDE residue with aspartic acid is rather unstable which might be ascribed not only to its

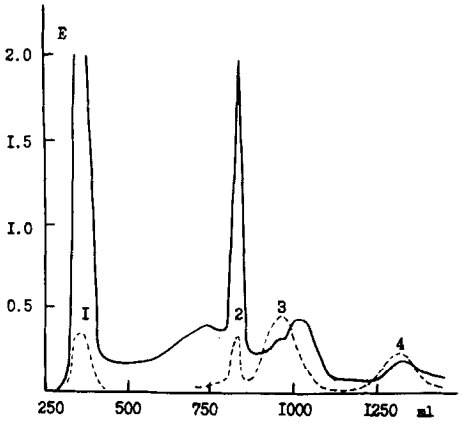

Fig.1. Chromatography of peptic hydrolysate of DDE-treated pepsin on Sephadex G-25 column (3x150 cm). Solid line - absorbancy at 280 nm Dotted line - absorbancy at 360 nm.

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activation by the carbonyl of glycolic acid but also by intramolecular catalysis with the participation of α-carboxyl group of aspartic acid. It is worthwhile to mention that pepsin cleaves the peptide bond at the α-carboxyl of aspartic acid residue modified by the attachment of rather bulky hydrophobic DDE residue.

Hence, DDE - the inhibitor containing diazoacetyl group as a reactive center interacts in swamorin with β-carboxyl group of the unique aspartic acid residue which belongs to the sequence Ile-Ala-Asp. This reaction leads to inactivation of the enzyme. As in the case of pepsins the high specificity of the reaction points on the functional importance of the carboxyl group which becomes labeled. The generality of this reaction common to a number of acid proteinases - various pepsins, acid proteinases of molds (5, 10), catepsin D (11,12) supports the conclusion that the carboxyl group of aspartic acid has to be considered as an

essential part of the catalytic sites of all pepsin-like acid proteinases for which the name "aspartate-type proteinases" might be suggested.

The tripeptide sequence including the "active" aspartic acid residue of awamorin coincides with the corresponding part of penicillopepsin sequence (13):

Awamorin	Ile-Ala-Asp
Penicillopepsin	Ile-Ala-Asp-Thr-Gly-Thr-Thr-Leu
Swine pepsin	Ile-Val-Asp-Thr-Gly-Thr-Ser-Leu

Hence, the strong analogy of functional properties of acid proteinases of molds (at least of awamorin and penicillopepsin) is based on the analogy of their active sites and, very probably, on the more or less pronounced homology of primary structures. The homology shown by Šodek and Hofmann for swine pepsin and penicillopepsin (13) might be extended on the case of awamorin.

Without precise knowledge of eventual differences between acid proteinases of various species of Aspergilli it would be premature to rename awamorin "aspergillopepsin". Nevertheless, presented data definitely show that awamorin has to be classified as pepsin-like enzyme.

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