SERINE-CONTAINING ACTIVE CENTER OF THE TRYPSIN-LIKE PROTEASE OF CRAYFISH *ASTACUS LEPTODACTYLUS*

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1. Introduction

The protease isolated from the digestive tract of The crayfish *Astacus leptodactylus* displays the same specificity as bovine trypsin and is inhibited both by naturally-occurring and synthetic trypsin inhibitors [1]. It was therefore named the "trypsin-like protease". The protease is also strongly inhibited by phenylmethylsulfonyl fluoride [2] which has been shown by Gold [3] to react with the active serine residue in the molecule of trypsin. Hence, the trypsin-like protease similarly to trypsin falls into the group of proteases of the serine type.

The aim of the present work was to determine the amino acid sequence around the active serine residue. The results obtained are compared with the known data on the environment of active sites in various proteases.

Abbreviations: DFP - diisopropylphosphofluoridate,
DIP - diisopropylphosphoryl-,
DNS - 1-dimethylamino-naphthalene-5-sulfonyl,
TAME - *Nα-p*-toluenesulfonyl-L-arganine methyl ester,
TPAL - trypsin-like protease from *Astacus leptodactylus.*

2. Materials and methods

The trypsin-like protease was prepared from the cardia fluid of *Astacus leptodactylus* by the method described recently [1, 2]. DFP was a product of Boots Pure Drug Co., Ltd., Nottingham, England. ^{32}P-DFP was purchased from the Radiochemical Centre, Amersham, England, and its specific activity was 0.35 mCi/mg. The esterase activity was determined by measurement of the increase in absorbancy at 247 mμ using TAME as substrate and following the method of Hummel [4].

The radioactivity of dry residues of 50-μl aliquots was measured on aluminum planchets using a Geiger-Müller tube.

2.1. *Inhibition of trypsin-like protease by radioactive DFP*

TPAL (145 mg, 6 μmoles) was dissolved in 9.6 ml of 0.005 M tris-HCl at pH 8.0 and inhibited by the addition of 0.6 ml of isopropanolic solution of ^{32}P-DFP (4.38 mg, 23.6 μmoles, 1.52 mCi). After three hours of reaction at 25°C, 20 mg of nonradioactive DFP in 1 ml of isopropanol was added and the reaction was allowed to proceed for another hour. The course of the inhibition was followed by examination of enzymatic activity of aliquots withdrawn at appropriate time intervals. The inhibited protease was separated from excess DFP by gel filtration on a 2 × 39 cm column of Sephadex G-25 equilibrated with

0.001 M tris-HCl buffer at pH 8.0. The yield of the lyophilized inhibited protease was 131.1 mg.

2.2. *Partial acid hydrolysis of inhibited protease*

Lyophilized ^{32}P-DIP-TPAL (130 mg) was suspended in 12 ml of 6 N HCl and heated 35 min at 100°C.

2.3. *Fractionation of partial acid hydrolysate on Dowex 50*

The dry residue of the partial acid hydrolysate was fractionated on a Dowex 50-X2 (H^+-form) column. The course of the fractionation is shown in fig. 1.

2.4. *Isolation of peptides from fractions*

Fractions obtained by ion-exchange chromatography were subjected to high-voltage electrophoresis [5] in pyridine acetate buffer at pH 3.5 (pyridine - acetic acid - water, 1: 10 : 189) [6] on Whatman no. 3 paper. The radioactivity on paper electropherograms was indicated automatically using a Frieseke-Hoepfner methane through-flow counter.

The peptides isolated were characterized by quantitative amino acid analysis according to Spackman and coworkers [7] and by *N*-terminal end-group analysis using the DNS-method of Gray and Hartley [8] and identification of the DNS-amino acids by thin-layer chromatography [9].

3. Results and discussion

As expected, DFP brings about a rapid inactivation of TPAL. The rate of the inhibition was established by the measurement of esterolytic activity of aliquots of the reaction mixture incubated under conditions described in the experimental part of the paper (concn. of TPAL = 0.59×10^{-3}, concn. of DFP = 2.33×10^{-3} M, pH 8.0, 25°C). In 15 min the degree of inhibition is 86%.

The degradation of ^{32}P-DIP-TPAL was effected by partial acid hydrolysis. It can be assumed, of course, that an enzymatic degradation might have yielded a longer peptide derived from the environment of the active serine. Since, however, the time for the experiments was limited in view of the short half-life of ^{32}P, we decided to carry out the degradation by partial acid hydrolysis because the fractionation of the hydrolysate is usually easier.

The isolated radioactive peptides, their structure and designation are shown in table 1. The yield of each isolated peptide was of the same order.

Table 1
Radioactive peptides isolated from partial acid hydrolysate of ^{32}P-DIP-TPAL.

Peptide	Structure	Color with ninhydrin
II 3	Ser*	
III 1	(Ser*, Gly)	
III 3	Asp (Ser*, Gly)	Orange
IV 5	Asp (Ser*, Gly)	Blue-gray
IV 6	Asp. Ser*	Orange
IV 7	Asp. Ser*	Yellow to violet

Asp. Ser*. Gly.

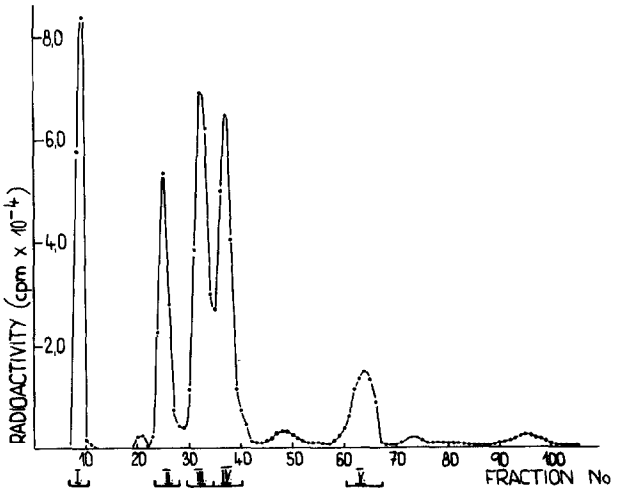

Fig. 1. Ion-exchange chromatography of partial acid hydrolysate of ^{32}P-DIP-TPAL on 1.2 X 45 cm column of Dowex 50-X2, 200–400 mesh. Eluted with 0.01N HCl. Fractions were collected at 15 min intervals. The radioactivity was determined with 50-µl aliquots of each fraction. The fractions were pooled as shown in the figure.

Fraction I when subjected to separation on paper yielded one intensive ninhydrin-negative, radioactive spot. Since no amino acid was detected in its total acid hydrolysate, the spot obviously contains radioactive phosphoric acid arising from the liberation of the DIP-group during partial acid hydrolysis.

As can be seen in table 1, two pairs of peptides were isolated which show identical structures and differ in electrophoretic mobilities and in the color with

ninhydrin. As described in detail by Naughton and coworkers [6], the diisopropyl group can by hydrolyzed to different degrees during partial acid hydrolysis and aspartic acid can undergo α-β shift.

The tripeptide sequence around the active serine residue of TPAL is identical with amino acid sequences around active serine in trypsins, chymotrypsins, elastase, and thrombin of mammalian origin (bovine, sheep, pig), though TPAL is a representative of a protease isolated from invertebrates at a rather low level of phylogenetic development. Mammals and the decapode crayfish have persued a separate line of evolution for at least 500 millions of years. Nevertheless, the amino acid sequence around the active serine indicates that TPAL can be put side by side with proteases which have been referred to as serine proteases of the mammalian type. On the other hand, in microbial proteases a Thr. Ser. Met sequence has been shown, though in two cases, i.e., the α-lytic protease from *Sorangium*, and the protease from *Streptomyces griseurs* the Asp. Ser. Gly sequence has also been reported (cf. review in [10]).

Recently Gibson and Dixon [11] isolated a chymotrypsin-like protease from sea anemone, *Metridium senile*. The mechanism of activation of this protease and its serine and histidine active centers are identical with mammalian chymotrypsins. The sea anemone (Coelenterate) as well as the crayfish (Arthropoda) belong to the Protostomia branch of phylogenetic development, which has separated very early from the Deuterostomia branch, which leads to the mammals. When compared with mammalian proteases, the findings of Gibson and Dixon as well as the results obtained by us show the identity in amino acid sequences of the active centers of proteases from animals which represent very different stages of phylogenetic development.

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