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Research Note

Proteolysis of Collagen by a Thiol Protease from *Paragonimus westermani* Metacercariae

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Paragonimus westermani metacercariae penetrate to the mammalian host tissues at the initial step of infection. It was clearly illustrated that a protease secreted by the metacercariae facilitated its penetration (Hamajima *et al.,* 1985). In a recent study, the protease activity against elastin and type IV collagen has been detected in *Schistosoma mansoni* cercariae (McKerrow *et al.,* 1985). Landsperger *et al.* (1982) and Tzeng *et al.* (1983) have characterized the specificity of the cercarial protease activity from *S. mansoni* for connective tissue molecules. While, acid-soluble and fibrous collagens were reported to be resistant to the protease from the cercariae (Dresden and Asch, 1972), it was shown that an enzyme from *Clostridium histolyticum* degraded native collagens (Bond and Van Wart, 1984). The detailed mechanism of proteolysis of collagen by the protease from parasites at the molecular level has not been clarified. In this study, we attempted to elucidate the ability of the protease from *P. westermani* metacercariae to hydrolyze an acid-soluble collagen and an insoluble collagen, both of which are major structural components of connective tissues.

Paragonimus westermani (3n) metacercariae were isolated from *Eriocheir japonicus.* Purification of the thiol protease from the metacercariae was achieved by Ulutrogel AcA-54, arginine-Sepharose and DEAE-cellulose column chromatography. Acid-soluble atelocollagen was prepared from guinea-pig skin essentially by the method of Bazin and Delaunay (1976), and insoluble collagen was purified from guinea-pig lungs essentially according to the procedure of Deyl and Adam (1976). To prepare the FITC-labeled substrate for the enzymatic assay, fluorescein isothiocyanate (FITC, Sigma Chemical Co.) was covalently coupled to the acid-soluble collagen and insoluble collagen essentially by the method of Goldstain *et al.* (1961). Collagenolytic activity of the enzyme to the FITC-labeled collagen was compared in terms of the time course by the FITC-peptide release assay. The incubation mixture, in triplicate, consisted of 0.1 μg-enzyme, 0.1 mg-FITC-labeled substrate (acid-soluble collagen and insoluble collagen), 0.1 ml of 0.1 M-imidazole-HCl, pH 7.5 and 5 mM-cysteine in a final volumes of 0.2 ml. Incubations were carried out for 1–9 hr at 35°C, and then 1 ml of 0.1 mM-leupeptin was added for the termination of the reaction. After the centrifugation at 3500 rpm for 10 min, the released FITC-peptides were measured fluorometrically at

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495 nm for exitation and at 520 nm for emission. The rate of proteolysis of the substrate in the above assay was expressed as a percent of a total fluorescence. As indicated in Fig. 1 the protease, when it was incubated with the substrates, hydrolyzed FITC-acid-soluble collagen up to 52% and FITC-insoluble collagen up to 42%. The proteolysis gradually increased during the incubation.

To analyze peptides cleaved in the collagen digestion, insoluble collagen was employed as a substrate. Incubations were carried out using 1 μg-enzyme, 1 mg-insoluble collagen in 0.25 ml of 10 mM-acetic acid, 0.25 ml of 0.1 M-imidazole-HCl, pH 7.5 and 2.5 mM-cysteine at 35°C for 1 hr. The activity was terminated by centrifuging the mixture at 3500 rpm for 10 min, and the supernatant was mixed with equal volume of 0.2%-trifluoroacetic acid (TFA). The soluble products from the enzyme reaction were assayed by high-performance liquid chromatography (HPLC); analysis the application of the reverse-phase HPLC on a column of SynCropak RP-8 (4.1 x 250 mm), elution at a flow rate of 1.0 ml/min with 0.1%-TFA during 10 min followed by a liner gradient elution of acetonitrile (0–35%) on 0.1%-TFA. Absorbance of a eluate was monitored at 210 nm. Fig. 2 shows elution profile of reverse-phase HPLC of released peptides from insoluble collagen by the enzyme reaction. The HPLC analysis of the reaction products using gradient system of acetonitrile resulted in separation of major cleaved fragments from the substrate at retention times of 26–42 min and minor fragments at retention times of 5–26 min and 42–65 min. While control experiments, no peak corresponding to the above elution profile was detected.

FITC-labeled collagens, which were used in this studies, have some advantages over the isotope-labeled collagen. As shown in this text, it is simple procedure for preparation of FITC-collagen and assay of the collagenolytic activity. Therefore, in the enzyme analysis using the FITC-labeled substrate is convenient for interpretation of results of the activity. Thiol

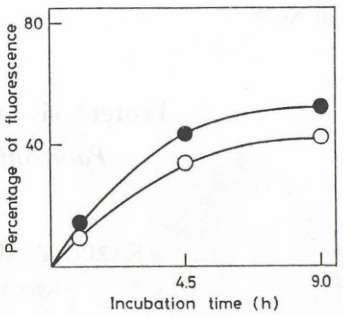

Fig. 1 Time course of proteolysis of a FITC-acid-soluble collagen and a FITC-insoluble collagen by the thiol protease from metacercariae of *P. westermani*. 0.1 mg-FITC-labeled collagen and 0.1 μg-thiol protease were used in each assay system. Proteolysis was performed at 35°C as described in Text. In this experiment, enzyme or substrate alone was used as a control. •: FITC-acid-soluble collagen, ○: FITC-insoluble collagen.

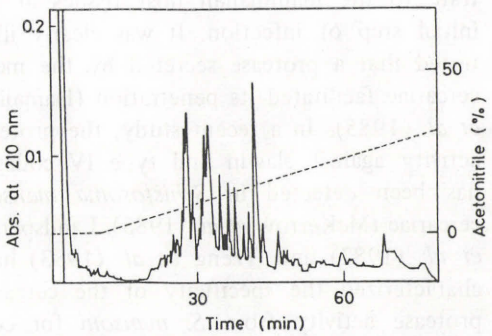

Fig. 2 Reverse-phase HPLC of proteolytic fragments of insoluble collagen on a SynCropak RP-8. Sample was eluted during 10 min with 0.1%-TFA; a gradient elution was then performed as described in Text. ——: absorption at 210 nm, - - - - -: concentration of acetonitrile.

protease from *P. westermani* metacercariae of specificity for collagen was characterized in the above results. Dresden and Asch (1972) as well as McKerrow *et al.* (1985) reported that *S. mansoni* cercarial protease failed to hydrolyze acid-soluble collagen and insoluble collagen. Whereas, on the HPLC analysis, the digestion study of the insoluble collagen by the metacercarial protease was analogous to the results of proteolysis of collagen by *Clostridium* collagenase (Bond and Van Wart, 1984). Therefore, this finding indicate that the metacercarial

protease cleaves the substrate to low molecular weight fragments. On the other hand, the size of the fragments resulted from the enzyme reaction differs far from those produced by mammalian collagenase (Welgus *et al.*, 1985). Although the enzyme from *Paragonimus* metacercariae can hydrolyze insoluble collagen, a protease from *S. mansoni* cercariae can not degrade it, probably because of differences of the specificity for connective tissue proteins (McKerrow *et al.*, 1985). The present results support that the protease of metacercariae recognizes collagen in the several connective tissue proteins.

The protease activity of metacercariae is likely to be of importance in relation to its potential ability to act in the host where it could assist the penetration in solubilizing collagen fibrils. Therefore, the role of the metacercarial protease suggests that its activity may exhibit a physiologically specificity for several connective tissue proteins in the host. Studies on the role of the thiol protease from *P. westermani* metacercariae in the proteolysis of collagen and the other connective tissue proteins from the host are now under way.

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短　報

ウェステルマン肺吸虫メタセルカリアにおけるチオール プロテアーゼのコラーゲンに対する水解活性について

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ウェステルマン肺吸虫メタセルカリアの終宿主における感染機構を明らかにすることを目的として，本幼虫のチオール　プロテアーゼのコラーゲンに対する特異性について検討した．その結果，本酵素はFITC-acid-soluble collagen および FITC-insoluble collagen　に対して顕著な水解活性を示した．また insoluble collagen の本酵素による反応生成物の逆相 HPLC 分析において，その水解はこの基質から短時間内に多数のペプタイドを遊離させていた．