Biochimica et Biophysica Acta, 611 (1980) 379—383
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BBA Report

BBA 61365

PURIFICATION AND PARTIAL CHARACTERIZATION OF A PROTEASE INHIBITOR FROM *DROSOPHILA MELANOGASTER*

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(Received August 1st, 1979)

Key words: Protease inhibitor; Chymotrypsin; (D. melanogaster)

Summary

A protein from *Drosophila melanogaster* which inhibits bovine α-chymotrypsin activity was purified using an extensive extraction procedure, SP-Sephadex column chromatography and affinity column chromatography. The inhibitor has an estimated molecular weight of approx. 12 000 and is extremely pH and heat stable. It did not exhibit any inhibitory activity against trypsin from numerous sources nor mosquito larval chymotrypsin but did inhibit adult mosquito chymotrypsin. Chymotrypsin-like activity has not been found in *Drosophila* and therefore the function of the inhibitor is unknown. Preliminary work indicates that it effectively inhibits cathepsin D activity from a nematode parasite and rabbit liver.

We have previously identified two protease inhibitors from *Drosophila melanogaster*, one which was specific for bovine trypsin and another, which only inhibited bovine α-chymotrypsin [1]. The latter, called Larval Chymotrypsin Inhibitor, was purified by conventional procedures in what proved to be a long and tedious process [2]. In this report we describe the purification of larval chymotrypsin inhibitor by affinity chromatography which considerably shortens the procedure from seven major steps to three.

The source of larval chymotrypsin inhibitor was third instar larvae of wild-type Oregon R. *Drosophila melanogaster*. Large quantities of these insects were reared and harvested as previously described [2]. The esterolytic activities of chymotrypsin and trypsin were determined using *N*-benzoyl-L-tyrosine ethyl ester hydrochloride (Bz-Tyr-OEt-HCl) and *P*-toluenesulphonyl-L-arginine methyl ester (Tos-Arg-OMe), respectively, as substrates [3,4].

Protease inhibitor activities were determined by incubating the inhibitor

and the respective enzyme for 5 min at room temperature prior to the addition of substrates. One unit of inhibitor activity was defined as the amount of inhibitor which inhibited 1 unit of protease activity. Protein concentrations, for the determination of specific activities were obtained using the method of Lowry et al. [5].

The extraction of the active inhibitor from 50 g larvae was accomplished as follows: an acetone powder was prepared using cold acetone (—10°C). The dried powder was homogenized in 20 volumes (w/v) of 0.043 M acetic acid (pH 3.6) for 3 min at 24°C. The homogenate was brought up to 70°C and maintained at this temperature for 2 min. The hot homogenate was then quickly cooled to 24°C and then centrifuged at 40 000 × *g* for 15 min at 0°C. The precipitate was discarded and the supernatant filtered through glass wool. An equal volume of 5% trichloroacetic acid was slowly added with constant stirring for 1 h. The precipitate was removed by centrifugation and $(NH_4)_2 SO_4$ (60% saturation) was added to the remaining 2.5% trichloroacetic acid supernatant. The resulting precipitate was allowed to settle overnight at 24°C and was then collected by centrifugation. It was then suspended in 15 ml 0.043 M acetic acid containing 0.2 M NaCl and dialyzed against this solution for 48 h at 4°C. Any precipitate that formed during dialysis was removed by centrifugation, and the resulting supernatant, designated Fraction I, was used as the starting material.

Fraction I was further purified via SP-Sephadex chromatography, shown in Fig. 1. It can be seen that activity is recovered after the addition of 0.5 M NaCl to the column. Tubes 98—108 were pooled and designated Fraction II. Fraction II was concentrated by lyophilization before being chromatographed on a chymotrypsin-Sepharose column. In order to prepare this column, pure bovine α-chymotrypsin (Worthington Biochemical Corporation) was insolubilized by coupling it to CNBr-activated Sepharose 6B, by the method of Axén and Ernback [6]. After coupling chymotrypsin to the matrix, the

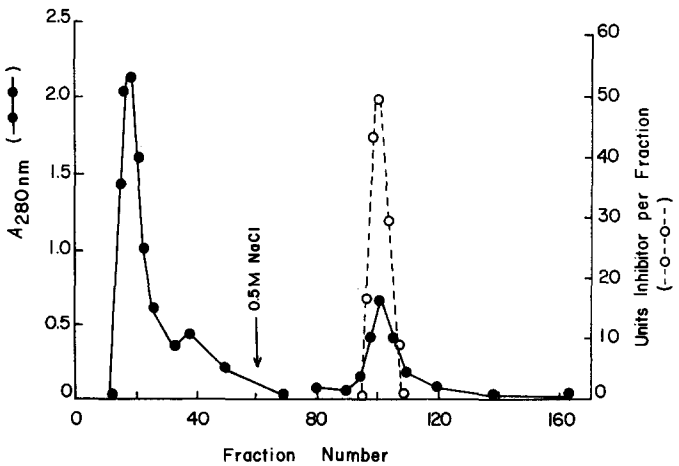

Fig. 1. Chromatography of 7.5 ml of Fraction I on sulfopropyl-Sephadex C-25 (40 × 2.5 cm). The column was equilibrated and eluted with 0.043 M acetic acid containing 0.2 M NaCl (pH 3.6). At fraction 60 (arrow) elution of the inhibitor was initiated with the same eluent containing 0.5 M NaCl. 5-ml fractions were collected at a flow rate of 20 ml/h at 15°C.

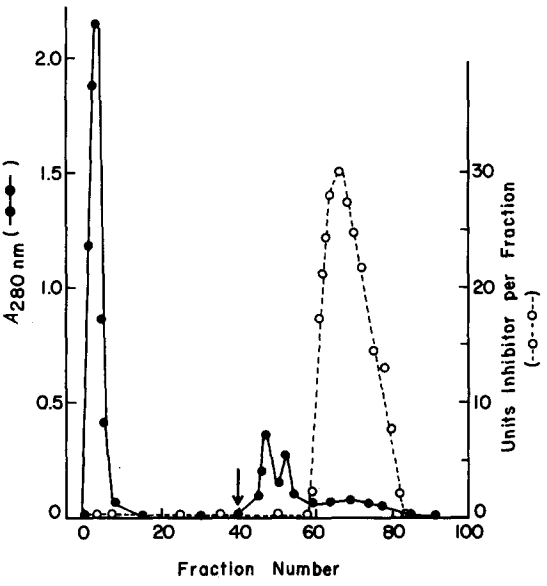

Fig. 2. Affinity chromatography of sulfopropyl-Sephadex fractions on chymotrypsin-Sepharose 6B (12 × 0.9 cm). The column was equilibrated with 0.01 M Tris-HCl buffer (pH 8.0) and then loaded with 2.5 ml of Fraction II, and first eluted with the starting buffer (10-ml fractions). At tube 40 the eluent was changed to 0.5 M KCl adjusted to pH 2.0 with HCl. 2-ml fractions were then collected at a flow rate of 10 ml/h at 15°C.

excess enzyme was removed in 500-ml washes of 0.08 M Tris-HCl buffer (pH 7.8) at 4°C until the absorbance of the wash at 280 nm reached zero. The cleaned chymotrypsin-Sepharose complex was packed into a column which was equilibrated overnight with 0.01 M Tris-HCl buffer (pH 8.0) using a flow rate of 10 ml per h at 15°C before use.

Lyophilized Fraction II was suspended in 50 ml 0.08 M Tris-HCl buffer (pH 7.8) and an aliquot was chromatographed on this column. Fig. 2 shows that larval chymotrypsin inhibitor was observed in tubes 59—83. These were pooled, designated Fraction III, dialyzed against distilled water overnight at 4°C, and lyophilized to dryness until needed. Table I summarizes the results. It should be noted that the final purification factor of 33.9 is

TABLE I

PURIFICATION OF *DROSOPHILA* LARVAL CHYMOTRYPSIN INHIBITOR

50 g of *D. melanogaster* were used. One unit of inhibitor activity is defined as the amount of inhibitor which inhibits one unit of bovine α-chymotrypsin esterolytic activity.

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (-fold)
Fraction I: extract	15	232.2	845.2	3.6	1
Fraction II: SP-Sephadex	50	41.7	548.4	13.2	3.7
Fraction III: affinity column	50	3.3	402.6	122.0	33.9

an underestimate because it is based on Fraction I, a preparation which itself has undergone extensive purification. The purity of Fraction III was determined by polyacrylamide disc gel electrophoresis [7,8]. Electrophoresis was performed in the following gels: (1) 10% acrylamide at pH 4.3; (2) 10% acrylamide at pH 8.9; (3) 15% acrylamide at pH 4.3; 15% acrylamide at pH 8.9. In all cases only one protein band appeared. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in 10% acrylamide at pH 7.0 also served to demonstrate the purity of Fraction III, because again, only one protein band was present.

From gel filtration column chromatography on Sephadex G-75 the molecular weight of the inhibitor was calculated to be 11 500 [9,10]. The molecular weight of larval chymotrypsin inhibitor calculated by SDS-polyacrylamide gel electrophoresis [11] gave values of 11 900, 12 200 and 12 000 in separate experiments.

The pH stability of the inhibitor was tested using a 0.08 M citrate/phosphate/borate buffer at one pH unit intervals between 2.0 and 12.0. Pure larval chymotrypsin inhibitor was incubated for 1 h at 37°C at these different pH values and then tested for activity against bovine α-chymotrypsin. No decrease in activity was observed between pH 2 and 11 under these conditions. However, at pH 12.13 and 24°C, the half-life of the inhibitor was found to be approx. 25 min.

The inhibitor is also extremely heat stable. For example, at pH 3.6 its half-life at 70°C is approx. 5.7 h and even at 100°C, $t_{1/2}$ is about 68 min. Larval chymotrypsin inhibitor was stable in 8 M urea but was irreversibly denatured in urea plus mercaptoethanol. This suggests that larval chymotrypsin inhibitor has specific disulfide linkages which are required in order for it to function as an inhibitor.

Drosophila larval chymotrypsin inhibitor inhibits both the esterolytic and endopeptidase activities of bovine α-chymotrypsin. It has no effect against bovine, *Aedes aegyptii*, *Drosophila* or yeast trypsin and in addition shows no inhibitory activity toward yeast chymotrypsin. However, it is active against adult *Aedes aegyptii* chymotrypsin but, surprisingly, is inactive against *Aedes* larval chymotrypsin. Since no chymotrypsin has been detected in *Drosophila* its function is unknown. Recently however, we have found that *Drosophila* larval chymotrypsin inhibitor is inhibitory toward cathepsin D from both the parasite *Nippostrongylus brasiliensis* and rabbit liver (Kang, Bolla, Weinstein and Fuchs, unpublished). Since catheptic activity has been reported in *Drosophila melanogaster* [12] as well as in other insects [13] it may be that the function of larval chymotrypsin inhibitor is associated with lysosomal mediated histolysis.

This work was supported by NIH grant 2RO1 AI 10707.

References

1 Kang, S.H. and Fuchs, M.S. (1973) Comp. Biochem. Physiol. 46B, 367—374
2 Kang, S.H. and Fuchs, M.S. (1974) Insect Biochem. 4, 1—8
3 Hummel, B.C.W. (1959) Can. J. Biochem. Physiol. 37, 1393—1399
4 Kang, S.H. and Fuchs, M.S. (1973) Anal. Biochem. 54, 262—265
5 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275

6 Axén, R. and Ernback, S. (1971) Eur. J. Biochem. 18, 351—360
7 Reisfeld, R.A., Lewis, J.J. and Williams, D.E. (1962) Nature 195, 281—283
8 Williams, D.E. and Reisfeld, R.A. (1964) Ann. N.Y. Acad. Sci. 121, 371—381
9 Andrews, P. (1964) Biochem. J. 91, 222—233
10 Ensign, J.C. and Wolf, R.S. (1966) J. Bacteriol. 91, 524—534
11 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
12 Mulherbar, L., Kothari, R.M. and Vaidya, V.G. (1972) Wilhelm Roux' Arch. 171, 195—199
13 Houseman, J. (1978) Can. J. Zool. 56, 1140—1143.