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Purification and characterization of chymotrypsin inhibitors from marine turtle egg white

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Abstract. The egg white of marine turtle (*Caretta caretta* Linn.) contains two chymotrypsin inhibitors and one trypsin inhibitor. The two chymotrypsin inhibitors were purified to homogeneity, as judged by ion-exchange chromatography, Polyacrylamide gel and sodium dodecyl sulphate-gel electrophoresis, isoelectric focusing, immunochemical tests and sedimentation in the ultracentrifuge. Their sedimentation coefficient values were independent of protein concentration. Their amino acid composition was similar, and contained seven disulphide bonds, and methionine and carbohydrate moiety were absent. Each inhibitor consisted of a single polypeptide chain of 117 amino acids. The average molecular weight of each inhibitor, calculated from sedimentation and diffusion coefficient values, amino acid composition and sodium dodecyl sulphate-gel electrophoresis was 13000. Both the inhibitors were stable over the pH range of 2–11. They inhibited α-chymotrypsin by forming enzyme-inhibitor complexes at a molar ratio of unity. The dissociation constant of each complex was $1{\cdot}06 \times 10^{-10}$ M. Both the inhibitors were indistinguishable in their physical, chemical and inhibitory properties except for their isoelectric points which were pH 5·23 for inhibitor *A* and pH 6·0 for inhibitor *B.* Chemical modification of all amino groups with trinitrobenzene sulphonate had no effect on their inhibitory activity.

Keywords. Protease inhibitor; chymotrypsin; turtle egg white.

Introduction

In contrast to the extensive studies with protease inhibitors from avian egg whites (Laskowski and Sealock, 1971; Lin and Feeney, 1972), very little information is available on inhibitors from reptilian egg whites. Recently the purification and characterization of an acidic trypsin inhibitor from tortoise egg white were reported by Ray *et al.* (1982), and the primary structure of a basic trypsin inhibitor from Red Sea turtle egg white by Kato and Tominaja (1979).

In a previous communication (Ray *et al.,* 1982) we reported the presence of anti-chymotryptic activity in the egg white of marine turtle (*Caretta caretta* Linn.). In the present paper we describe the purification and properties of two chymotrypsin inhibitors obtained from the same source. Unlike avian ovomucoids and ovoinhibitors, the inhibitors from turtle egg white had lower molecular weight and were devoid of a carbohydrate moiety.

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Abbreviations used: CM, Carboxymethyl; SDS, sodium dodecyl sulphate.

Materials and methods

Eggs of freshly killed turtles *(Caretta caretta* Linn.) were collected from local markets.

Bovine α-chymotrypsin (thrice crystallized) and trypsin (twice crystallized) were purchased from Worthington Biochemical Corporation, New Jersey, USA; *p*-tosyl-L-arginine methyl ester, N-acetyl-L-tyrosine ethyl ester and *p*-nitrophenyl acetate from Sigma Chemical Company, St. Louis, Missouri, USA; Sephadex G-50 (fine) and Carboxymethyl (CM) Sephadex C-50 from Pharmacia Fine Chemicals, Uppsala, Sweden; ampholine (pH 5-8) from LKB Produkter AB, Stockholm, Sweden. Other reagents were commercial preparation of the highest purity available.

Purification of chymotrypsin inhibitors

Step 1. Egg whites from a single turtle (*C. caretta*) were filtered through cheese cloth, and the filtrate was treated with solid $(NH_4)_2SO_4$ to 85 % saturation. The precipitate was collected by centrifugation and dissolved in a minimum volume of water.

Step 2. The resulting solution was applied to a column (3·5 × 75 cm, bed volume 720 ml) of Sephadex G-50 (fine) equilibrated with 0·1 M sodium acetate buffer, pH 4·2. Elution was carried out at room temperature with the same buffer. Active fractions containing both chymotrypsin and trypsin inhibitors were pooled and concentrated by precipitation with $(NH_4)_2\ SO_4$ (85 % saturation). The precipitate was dissolved in and dialysed against 0·1 M sodium acetate buffer, pH 5·0 in the cold.

Step 3. The dialysed solution was applied to a column (2·5×23 cm, bed volume 110 ml) of CM-Sephadex C-50 equilibrated with 0·1 M sodium acetate buffer, pH 5·0 and a linear gradient from 0 to 0·3 M NaCl in the equilibration buffer was used. First two active fractions designated as inhibitors *A* and *B* were separately pooled and rechromatographed under the same condition. They were dialysed exhaustively against water and freeze-dried.

Protein estimation

Protein concentrations in egg whites and in fractions obtained in the early stages of purification were estimated according to Lowry *et al.* (1951) using bovine serum albumin as a standard. The concentrations of both inhibitors *A* and *B* were determined from their absorbance at 280 nm using a value of $E^{1\%}_{1cm}$ =11·5 determined experimentally. α-Chymotrypsin concentration was estimated by active site titration with *p*-nitrophenyl acetate (Kézdy and Kaiser, 1970).

Assay of enzymic and inhibitory activities

α-Chymotrypsin and trypsin activities were determined at pH 7·5 and 27°C in the absence and presence of inhibitor by the pH-stat method (Walsch and Wilcox, 1970) using N-acetyl-L-tyrosine ethyl ester and *p*-tosyl- L-arginine methyl ester, as described earlier (Ray *et al.,*1982). The inhibitory activity was calculated as the difference between enzyme activity in the absence and presence of inhibitor. One unit of inhibitory activity is expressed as a mg enzyme inhibited by the inhibitor. Specific activity is expressed as units of inhibitory activity per mg inhibitor.

Polyacrylamide gel electrophoresis

Disc Polyacrylamide gel electrophoresis was performed at pH 4·5 by the method of Reisfeld *et al.* (1962). Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was carried out according to Weber and Osborn (1969).

Immunodiffusion and Immunoelectrophoresis

These were done according to Hammarstrom and Kabat (1969) using rabbit antiserum to purified inhibitor *B*.

Isoelectric focusing

This was performed according to Vesterberg and Svenson (1966) in an LKB 8100 electrofocusing column using 1% ampholine in the pH range of 5–8. The sample was allowed to focus at 600 V for 72 h at 4°C. After focusing, fractions of 1·9 ml were collected, analysed for pH and absorbance at 280 nm and assayed for inhibitory activity.

Ultracentrifugation

For the determination of sedimentation coefficients and diffusion coefficients of inhibitors, ultracentrifugal analyses were carried out with a Spinco Model E analytical ultracentrifuge at 59780 revolutions/min and 8766 revolutions/min respectively, as described previously (Ray *et al.,* 1982).

Partial specific volumes were calculated from the amino acid composition according to Cohn and Edsall (1943) and the value of 0·705 ml/g was obtained for both inhibitors *A* and *B*.

Amino acid analyses

These were performed according to Spackman *et al.* (1958) on a Beckman Multichrom amino acid analyser. Samples were hydrolysed with 6 M HCl at 110°C for 24, 48 and 72 h under vacuum. The total cysteine content was determined as cysteic acid after performic acid oxidation (Hirs, 1967). Tryptophan content was determined after hydrolysis with *p*-toluenesulphonic acid in the presence of 3-(2-amino ethyl) indole (Liu and Chang, 1971).

Modification of free amino groups

This was performed by reaction with 2, 4, 6-trinitrobenzene sulphonic acid at pH 8·5 according to the method of Habeeb (1966).

Check for carbohydrate content

Attempts were made to measure neutral sugar by phenol- H_2SO_4 method (Dubois *et al.,* 1956) and amino sugar by a modification of Elson-Morgan reaction (Davidson, 1966) using glucose and glucosamine, respectively as standards.

Results and discussion

Purification

Turtle egg white contained two chymotrypsin inhibitors and one trypsin inhibitor. Their separation and purification were achieved by gel filtration through Sephadex G-50 followed by ion-exchange chromatography on CM-Sephadex C-50 (figure 1). Both chymotrypsin inhibitors *A* and *B* on rechromatography gave single peaks with constant inhibitory activity across the peaks, indicating that preparations were probably homogeneous. A summary of the purification procedure is given in table 1. The yield of inhibitor *A* was about one-third of that of inhibitor *B*.

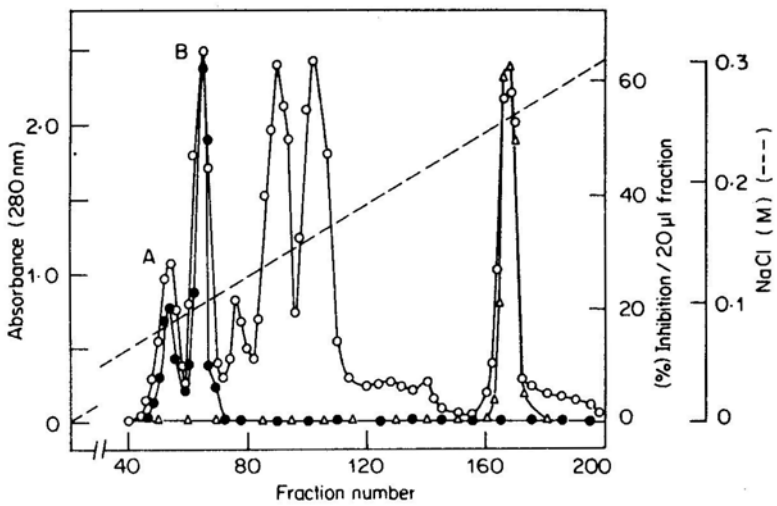

Figure 1. CM-Sephadex C-50 chromatography of active fraction from the gel filtration of turtle egg white. The column (2·5 × 23 cm, bed volume 110 ml) was equilibrated with 0·1 M sodium acetate buffer, pH 5·0. About 900 mg protein was applied to the column. Elution was performed using a linear gradient of 0–0. 3M NaCl in the same buffer. Fractions of 5·0ml were collected at a flow rate of 30 ml per h. (o), absorbance at 280 nm; (●), chymotrypsin inhibition; (Δ), trypsin inhibition.

Homogeneity

Both inhibitors *A* and *B* were homogeneous on Polyacrylamide gel electrophoresis at pH 4·5 and on SDS-gel electrophoresis at pH 7·0 in the presence of 2-mercaptoethanol, as shown in figure 2. Isoelectric focusing of both inhibitors yielded single peaks with constant inhibitory activity across the peaks with isoelectric points at pH 5·23 for inhibitor *A,* and at pH 6·0 for inhibitor *B*.

Ultracentrifugal analysis of both inhibitors also showed single symmetrical peaks at different protein concentrations (0·5–1·4%) with sedimentation coefficient ($S_{20,\ w}$) values of 1·63S for inhibitor *A* and 1·66S for inhibitor *B*. Their sedimentation coefficients were essentially independent of protein concentration.

Table 1. Summary of the purification of chymotrypsin inhibitors from turtle egg white.

Step	Chymotrypsin inhibitory activity (units)	Specific activity (units/mg)	Recovery of activity (%)	Purification
Egg white	686	0·07	100	1
$(NH_4)_2SO_4$ precipitation	624	0·08	91	1
Gel filtration through Sephadex G-50	368	0·41	54	6
Chromatography on CM-Sephadex G-50				
Inhibitor *A*	80	1·96	12	30
Inhibitor *B*	200	1·74	29	24
Rechromatography on CM-Sephadex C-50				
Inhibitor *A*	60	2·00	9	30
Inhibitor *B*	170	1·96	26	29

Inhibitor activity and protein content were determined as described under Materials and methods.

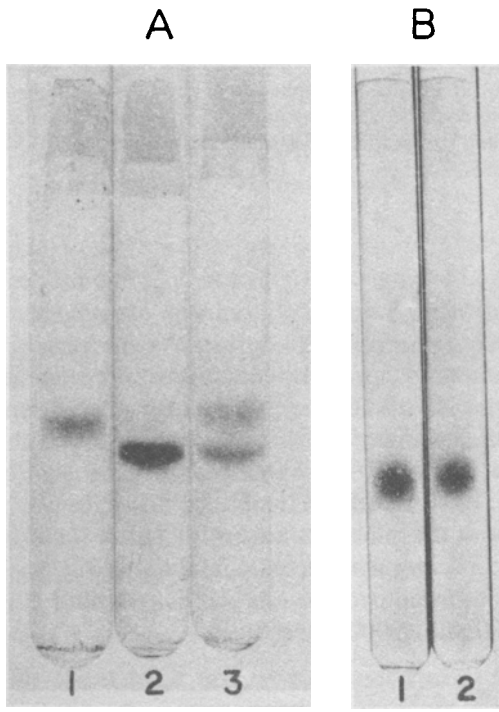

Figure 2. Polyacrylamide gel electrophoresis of chymotrypsin inhibitors of turtle egg white on (A) 10% gel at pH 4·5 and (B) 7·5 % gel containing 0·1 % SDS at pH 7·0. 1, inhibitor *A*; 2, inhibitor *B*; 3, a mixture of inhibitors *A* and *B*.

The homogeneity of both inhibitors was also shown by immunodiffusion and Immunoelectrophoresis where each inhibitor gave a single precipitin line of identity (figure 3). The cross-reactivity of inhibitor *A* against rabbit anti-inhibitor *B* serum indicates the immunological identity of the two inhibitors.

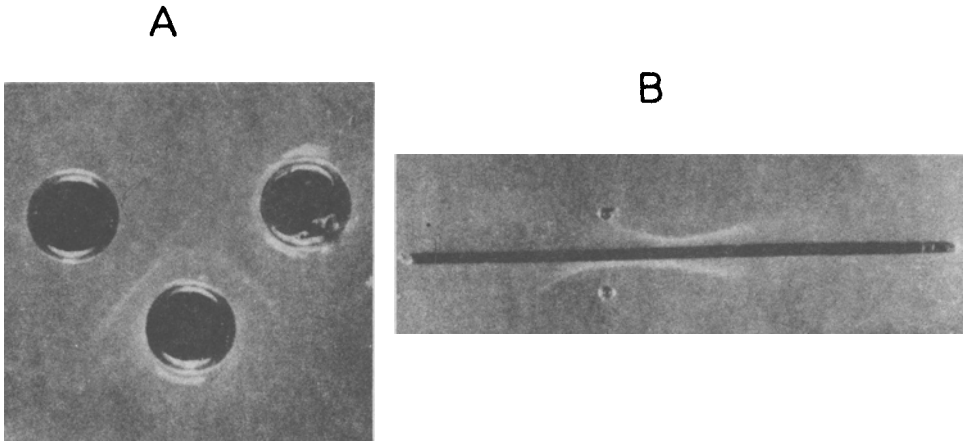

Figure 3. A. Immunodiffusion of chymotrypsin inhibitors of turtle egg white. The central well contained the antiserum to inhibitor *B* and left and right wells contained inhibitors *A* and *B* respectively. **B**. Immunoelectrophoresis of chymotrypsin inhibitors from turtle egg white. The central slot contained the antiserum to inhibitor *B* and the upper and lower slots contained inhibitors *A* and *B* respectively. Electrophoresis was done in 0·05 M veronal buffer, pH 9·0 at 5v/cm and 25° C.

Molecular weight

The molecular weight of both the inhibitors was estimated to be 12900 by SDS-polyacrylamide gel electrophoresis. The diffusion coefficients of inhibitors *A* and *B* calculated from the boundary spreading in the ultracentrifuge (Baldwin, 1957) were $10{\cdot}3 \times 10^{-7}\text{cm}^2\ s^{-1}$, and $10{\cdot}6 \times 10^{-7}\text{cm}^2{\cdot}s^{-1}$ respectively. From the sedimentation-diffusion data using the Svedberg equation the molecular weights were found to be 13000 for inhibitor *A* and 12900 for inhibitor *B*. Amino acid compositions gave a molecular weight of 13100 for both the inhibitors. From the above results the average molecular weight of both the inhibitors was calculated as 13000, which was similar to those of tortoise and turtle egg white trypsin inhibitors (Ray *et al.,* 1982). In contrast, the molecular weights of avian ovomucoids and ovoinhibitor (Lin and Feeney, 1972) were reported as 28000 and 49000 respectively.

Amino acid composition

Table 2 shows the amino acid compositions of inhibitors *A* and *B*. It may be noted that both the inhibitors have identical number of indivisual amino acid residues. Their amino acid composition is markedly different from the compositions of tortoise and

Table 2. Amino acid compositions of chymotrypsin inhibitors *A* and *B* of turtle egg white.

Amino acid	Residues/mol		
	Inhibitor *A*	Inhibitor *B*	Integer
Lys	9·1	8·6	9
His	1·8	1·9	2
Arg	2·0	2·0	2
Asp	10·8	10·7	11
Thr[a]	6·7	6·8	7
Ser[a]	7·8	7·9	8
Glu	15·7	16·0	16
Pro	6·3	5·7	6
Gly	12·5	12·8	13
Ala	3·0	3·0	3
½Cys[b]	14·2	13·9	14
Val[c]	2·7	2·9	3
Met	0	0	0
Ile	1·7	1·8	2
Leu	5·6	5·8	6
Tyr	5·1	4·8	5
Phe	8·6	8·6	9
Trp[d]	0·9	0·9	1

[a]Values obtained by extrapolation to zero hydrolysis time.
[b]Determined as cysteic acid after performic acid oxidation.
[c]Values are yields from 72 h hydrolysates.
[d]Determined after hydrolysis with *p*-toluenesulphonic acid.

turtle egg white trypsin inhibitors reported earlier (Ray *et al*., 1982). Both chymotrypsin inhibitors *A* and *B* contained 14 half-cystine residues, and one tryptophan residue, and lacked methionine residue. Free sulphydryl groups could not be detected by Ellman's reaction (Ellman, 1959) even in the presence of 8 M urea. Since SDS-gel electrophoresis in the presence and absence of 2-mercaptoethanol showed the presence of a single polypeptide chain of the same mobility, it was concluded that all of the half-cystine residues were present in both the inhibitors as disulphide bonds. No N-terminal amino acid residue was found in the performic acid oxidized derivatives of both the inhibitors, as judged by dansyl chloride method (Gray, 1967). Like tortoise and turtle egg white trypsin inhibitors, they lacked carbohydrate moiety. In this respect they differ from avian ovomucoids and ovoinhibitors which are glycoproteins.

Inhibitor properties

Both inhibitors *A* and *B* inhibited bovine α-chymotrypsin, but not trypsin. The inhibition of chymotrypsin at pH 7·5 by increasing amounts of inhibitors *A* and *B* is shown in figure 4. It was noted that the interaction of chymotrypsin with inhibitor *A* was identical to that with inhibitor *B*. The titration curve was linear up to 98% inhibition and extrapolation of the data indicated that one mol of the inhibitor reacted

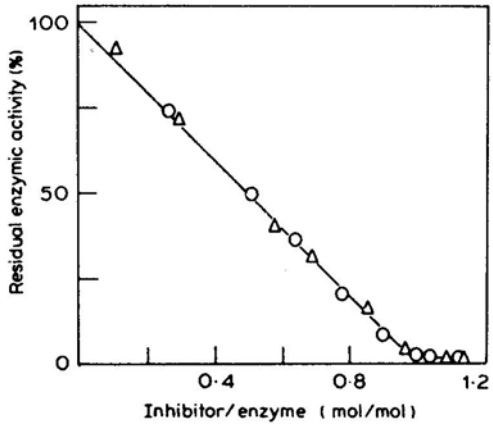

Figure 4. Inhibition of α-chymotrypsin by turtle egg white inhibitor *A* (o) and inhibitor *B* (Δ). Assays were performed on N-acetyl-L-tyrosine ethyl ester at pH 7·5 and 27°C after 2 min preincubation of enzyme and inhibitor.

with one mol of α-chymotrypsin to form a 1:1 inhibitor-enzyme complex. The dissociation constant of the complex calculated according to Green and Work (1953) was found to be $1{\cdot}06 \times 10^{-10}$ M for each inhibitor.

Both the inhibitors *A* and *B* were found to be stable over the pH range of 2–11. No inhibitory activity was lost when each inhibitor was heated to 100°C at neutral pH for 5 h. The high stability was probably due to the presence of seven disulphide bonds, small size and compact nature of these molecules.

Reaction with trinitrobenzene sulphonate led to the complete modification of free amino groups present in the inhibitors, but their inhibitory activities were not affected. This suggested that the amino groups were probably not necessary for the inhibition of chymotrypsin.

Results presented in this study indicated clearly that chymotrypsin inhibitors *A* and *B* of turtle egg white were very similar in their physical, chemical and inhibitory properties, except that inhibitor *A* was more acidic in nature than inhibitor *B*. This difference was reflected in their elution profiles on ion-exchange chromatography, Polyacrylamide gel electrophoresis and isoelectric focusing. Since both the inhibitors contained identical number of individual acidic amino acids and basic amino acids, it was apparent that inhibitor *A* had lesser amide content than inhibitor *B*. These two inhibitors might be considered as iso-inhibitors.

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