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QUARTERLY

Purification and characterization of cystatin from duck egg white

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It is the second peptidase inhibitor, after ovostatin, which showing the same antipapain activity in egg white in different avian species implies differences in amino -acid sequences. Cystatin from duck egg white was purified by carboxymethylpapain affinity chromatography and size-exclusion HPLC. The purified inhibitor which showed partial identity in the immunodiffusion test with chicken egg white cystatin, had an apparent molecular mass of 9.3 kDa as determined by SDS/PAGE. IEF analysis revealed five molecular forms of pI in the range 7.8–8.4. The obtained cystatin was neither glycosylated nor phosphorylated as it is in the case of chicken cystatin. The determined K_i (0.005 ± 0.001 nM) was similar to that reported for human and chicken cystatin C.

Peptidase inhibitors: ovomucoid, ovoinhibitor, cystatin and ovostatin (ovomacroglobulin) consist a major group of proteins in egg white [1]. The cystatins are a superfamily of proteins that inhibit the lysosomal cysteine peptidases, cathepsin B, H and L [2]. Chicken cystatin from egg white was one of the first cysteine peptidase inhibitors to be identified and characterized [3, 4]. It belongs to family 2 of cystatins, which are extracellular proteins of about 120 amino-acid residues with two disulphide bridges [2], and occurs in two major isoelectric forms: non-phosphorylated form 1 with pI of 6.5 and phosphorylated form 2 with pI of 5.6 [5].

Cystatins from other than chicken bird species have not been studied so far. Some reports have dealt with purification and properties of duck ovostatin [6, 7]. The latter exhibited some properties closer to those of α_2-macroglobulin than of chicken ovostatin. In order to find whether such differences among bird species concern other peptidase inhibitors, we have studied the cystatin isolated from duck egg white, and compared its properties with those of chicken cystatin and human cystatin C.

MATERIALS AND METHODS

Materials. Duck fresh, infertile eggs were purchased from a local farm. Sepharose 4B was from Pharmacia Fine Chemicals (Sweden). BANA (*N*α-benzoyl-DL-arginine-2-naphthylamide) and Brij 35 were from Serva Feinbiochemica, Heidelberg/New York. Papain, alkaline phosphatase (from bovine intestinal mucosa), E-64 (*trans*-epoxy-succinyl-L-leucylamido(4-guanidino)butane), Z-Phe-Arg-NMec were from Sigma Chemical Company (U.S.A.).

Abbreviations used: BANA, *N*α-benzoyl-DL-arginine-2-naphthyl-amide; Cn-papain, S-carboxy methyl papain; E-64, *trans*-epoxy-succinyl-L-leucylamido(4-guanidino)butane; HPLC, high pressure liquid chromatography; IEF, isoelectric focusing; SE-HPLC, size exclusion HPLC; Z-Phe-Arg-NMec, Benzyloxy-carbonyl-phenylalanyl-arginyl-7-amino-4-methyl coumarin.

Chicken egg white cystatin was prepared according to Anastasi *et al.* [4].

Enzyme and inhibitor assay. Papain activity was determined according to Barrett [8] using BANA as a substrate. One unit of the enzyme activity hydrolysed 1 μmol of the substrate/ /min under the reaction conditions. The anti-papain activity of cystatin was measured after preincubation with the enzyme for 5 min at 40°C before addition of the substrate. One inhibitor unit corresponds to the amount that decreases the papain activity by one enzyme unit.

Protein assay. This was performed by the method of Lowry *et al.* [9] and, for comparison, by the method of Bradford [10] with bovine serum albumin as a standard.

Carbohydrate determination. The phenol method was used for determination of neutral sugars [11].

Purification of cystatin. The inhibitor was purified in two main steps: affinity chromatography and SE–HPLC (Size-Exclusion High Pressure Liquid Chromatography). Affinity chromatography on Cm-papain Sepharose 4B column was performed in the manner described for purification of chicken egg white cystatin [4]. The obtained effluent was dialyzed against 25 mM imidazole/HCl buffer, pH 7.4, containing 0.5 M NaCl and concentrated to 2 ml by ultrafiltration using an Amicon cell fitted with a YM-3 membrane. Samples of 200 μl were run on GF-250 column (9 mm × 250 mm, Dupont) equilibrated with 25 mM imidazole/HCl, 0.5 M NaCl, pH 7.4, at a flow rate of 1 ml/min. Four separate peaks of protein detected by A_{280} of the effluent were collected. The fractions from separate runs were combined. The obtained solutions were dialyzed against 25 mM imidazole/HCl, 20% glycerol, pH 7.4, using Spectrapor MWCO 3000 dialysis tubing, and stored at –20°C.

Antibody production. Antiserum directed against chicken cystatin was raised in rabbit by repeated immunization with 50 μg of pure cystatin (mixed form) in complete Freund's adjuvant subcutaneously and intravenously.

Electrophoresis. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (SDS/PAGE) was performed in 15% (w/v) slab gels by the method of Laemmli [12], at pH 8.3. The gels were silver stained [13].

Double immunodiffusion. Immunodiffusion was performed in 1% agarose in phosphate buffered saline, pH 7.4, according to Ouchterlony [14].

K_i determination. The inhibition constant for papain was determined in stopped fluorometric assay with defined active concentration of enzyme and inhibitor according to Thiele *et al.* [15]. Papain used in this experiment was repurified on Hg-Sepharose by the method described for human cathepsin B1 [16] and was 43% active. Fluorescence was measured in a Perkin Elmer spectrofluorometer LS 50 B.

Alkaline phosphatase treatment. To 60 μg of cystatin dissolved in 900 μl of 25 mM imidazole/HCl buffer, pH 7.4, 10 μg of alkaline phosphatase in 100 μl of 0.2 M diethanolamine, 4 mM $MgSO_4$ solution was added. The mixture was incubated for 6 h at 37°C and subsequently applied to isoelectric focusing.

Isoelectric focusing. Isoelectric focusing was performed in 7.5% (w/v) polyacrylamide gel in 5 mm × 75 mm glass tubes at 4°C using Servalyte 2–11 or 7–9 to achieve suitable gradients. Polymerization was catalyzed chemically with ammonium persulphate [17]. The gels were stained with Coomassie Brilliant Blue R-250. For detection of the inhibitory activity zones

Table 1
Purification of cystatin from duck egg white

Purification step	Total protein	Total activity	Specific activity	Purification factor	Recovery
	(mg)	(U)	(U/mg protein)	(-fold)	(%)
Egg white (30 eggs) plus NaCl	97800.0	100.0	0.001	1	100.0
Cm-papain Sepharose 4B eluate	8.8	23.1	2.6	2600	23.1
GF-250 HPLC (low M_r peak)	1.1	11.4	10.3	10300	11.4

the gels were sliced into 2.5 mm pieces and after 24 h extraction with 1 ml of papain-assay buffer/activator, the inhibitory activity of each extract was determined as described above.

RESULTS

Purification of cystatin

Table 1 summarizes quantitative results of a typical procedure with 30 egg whites, as described in Materials and Methods. The yield and enrichment were calculated on the basis of inhibitory activity against papain.

Affinity chromatography on Cm–papain Sepharose 4B yielded a product consisting mainly of four protein peaks with an antipapain activity, differing in retention time in SE–HPLC on Zorbax GF-250 column, corresponding to molecular mass of about: 800 kDa, 80 kDa, 22 kDa and 12 kDa (not shown). The first three peaks (in order of elution from the column) appeared to be glycoproteins as they gave a colour in the phenol-sulphuric acid reaction. We did not characterize them further in this study as cystatins do not contain carbohydrates. The inhibitor of about 12 kDa obtained with the yield of 11% and called by us duck cystatin was the object of this paper.

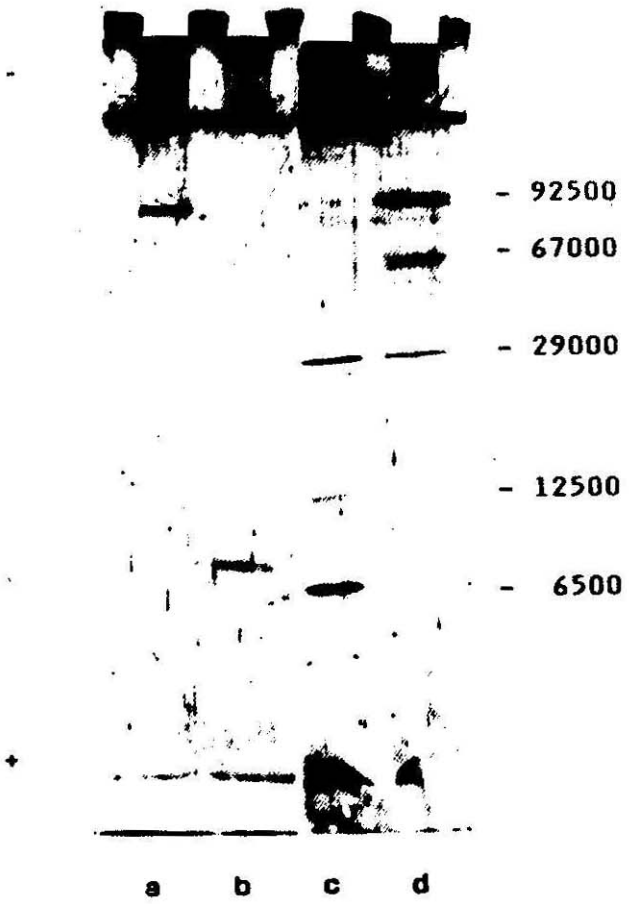

Fig. 1. SDS/PAGE of duck cystatin in 15% gel under non-reducing (lane a), and reducing (lane b) conditions, and standards: phosphorylase B (92.5 kDa), bovine albumin (67 kDa), chicken albumin (29 kDa), cytochrome c (12.5 kDa), aprotinin (6.5 kDa) (lanes c, d). The gel was silver stained.

Properties of duck cystatin

In SDS/PAGE (with reduction) duck cystatin migrated to the position corresponding to molecular mass of 9.3 kDa (Fig. 1, lane b). Under non reducing conditions the inhibitor tended to aggregate (Fig. 1, lane a).

Duck cystatin did not contain neutral sugars as shown by the phenol-sulphuric acid reaction, and was stable to heating at 80°C for 10 min at pH 6.0.

The preparation was heterogeneous in isoelectric focusing at pH 2–11. Several bands stained with Commassie Brilliant Blue were visible (Fig. 2). However, the inhibitory activity was detected only in slices of pI values from 7.5

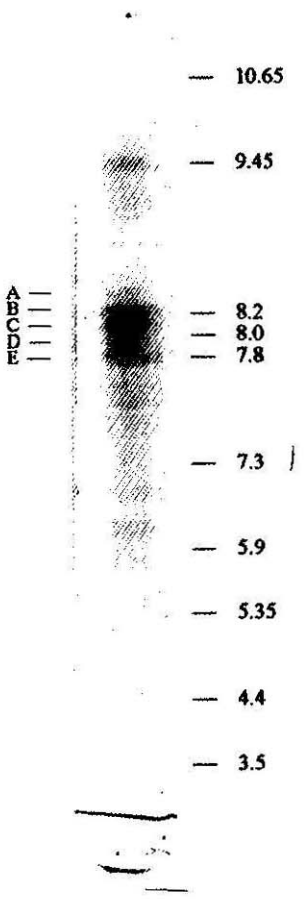

Fig. 2. Isoelectric focusing of duck cystatin (50 µg) using 6.8% polyacrylamide gel and Servalyte 2–11.
Gel was stained with Coomassie Briliant Blue R-250. Five active molecular forms of the inhibitor are designated as A, B, C, D and E.

to 8.5. When a narrower gradient (pH 7–9) was applied we found five separate bands exhibiting the inhibitory activity at pI: 7.8, 7.9, 8.1, 8.3 and 8.4. The pI values corresponded to those of five bands detected with Coomassie Brilliant Blue in the gel shown in Fig. 2 (A, B, C, D, and E, respectively).

Incubation of the inhibitor with alkaline phosphatase produced no changes in IEF, indicating that none of the molecular forms was a phosphoprotein (not shown). As a control experiment, dephosphorylation of the chicken cystatin form 2 was used.

Inhibition of papain by duck cystatin is presented in Fig. 3. The data set was collected by measuring the enzymatic activity of papain, with fluorogenic substrate Z-Phe-Arg-NMec, at equilibrium with different concentrations of the inhibitor. The inhibition constant was then calculated by non-linear regression analysis using the general equation for tight-binding inhibitors [18]. The determined K_i (0.005 ± 0.001 nM) was identical to that reported for chicken cystatin K_i (0.005 nM) [19].

The immunological properties of duck and chicken cystatins were compared in an Ouchterlony double immunodiffusion test with antiserum against chicken cystatin. As shown in Fig. 4, the proteins revealed partial identity.

DISCUSSION

In this study we have demonstrated the existence of cysteine peptidase inhibitors in duck egg white. Our preliminary experiments have shown 2.5-fold higher antipapain activity in the homogenates of egg white from infertile duck eggs than from fertile ones, therefore we have used the former for the preparation of low molecular mass cystatin.

The procedure used for purification of duck cystatin represents a modified protocol employed earlier by Anastasi *et al.* [4] for chicken egg white cystatin. Following affinity chromatography we did not use chromatofocusing but size-exclusion HPLC separation on a Zorbax GF-250 column. As a result a low molecular mass inhibitor was isolated.

On the basis of the molecular mass, pI, termostability, lack of carbohydrate and precipitation with antiserum directed against chicken egg white cystatin, the inhibitor could be classified as a member of family 2 of the cystatin superfamily. Duck cystatin showed only partial identity with chicken cystatin, pointing to differences in their structure. The molecular mass of duck cystatin, as determined by SDS/PAGE in reducing conditions, is 9.3 kDa. It is lower than that reported for chicken (13.1 kDa) and human cystatin C (13.2 kDa) [19]. In the absence of 2-mercaptoethanol a protein band of

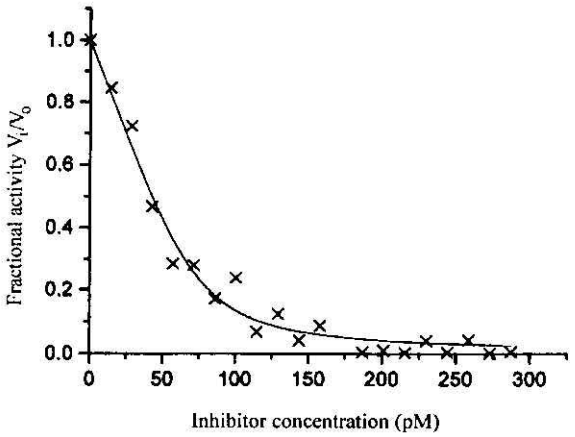

Fig. 3. Inhibition of papain (80 pM) by different concentrations of duck cystatin.
The fractional activity is the reaction rate in the presence of inhibitor divided by the rate in its absence. The curve was evaluated by non-linear regression analysis using the general equation for tight-binding inhibitors [18].

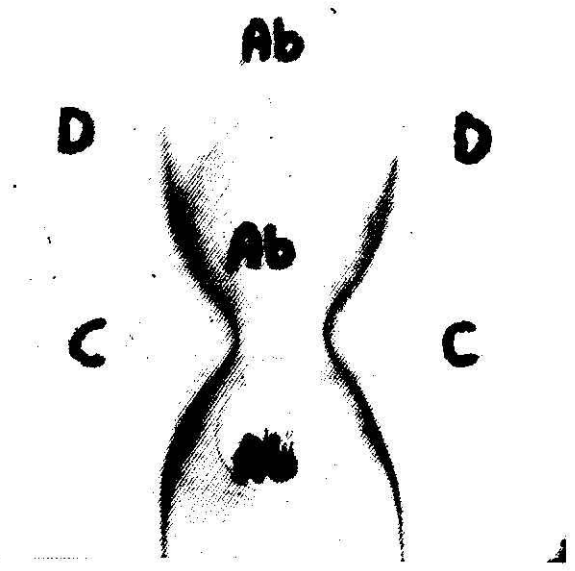

Fig. 4. Immunodiffusion of chicken and duck cystatins towards antiserum against chicken cystatin.
C, chicken cystatin; D, duck cystatin; Ab, antiserum against chicken cystatin.

93 kDa was observed near the top of the gel, probably due to aggregation of the inhibitor molecules. Similar observations have been reported in the case of stefin B [19] and a deletion variant of chicken cystatin obtained from *E. coli* [20].

Like human cystatin C [21], the duck cystatin occurs in several forms with pI in the alkaline pH range. The nature of structural differences underlying the differences in pI between the isoforms has not been elucidated, but degradation of the polypeptide chain could be taken into consideration. N-Terminally truncated, active forms of chicken egg white cystatin [22] as well as human cystatin C [21] have been described. Chicken egg white cystatin has been reported to occur in two major isoelectric forms, which could be separated by ion exchange chromatography [4], but the additional forms, present in smaller quantities, were observed [23].

Laber *et al.* [5] reported that two isoelectric forms of chicken cystatin differ only at the serine residue at position 80, which is phosphorylated in the pI 5.6 form and non-phosphorylated in the pI 6.5 form. On the other hand, human cystatin C is not a phosphoprotein [21]. Our experiment with alkaline phosphatase showed that, under the conditions, when the pI 5.6 form of chicken cystatin was completely transformed to the pI 6.5 form, all forms of duck cystatin remained unchanged. Therefore it may be concluded that duck cystatin is not phosphorylated.

Biological function(s) of phosphorylation of cystatin 2 thus far remain unclear. Regulation of the inhibitor activity seems quite unlikely as the two forms have identical inhibition constants for papain [22]. Laber *et al.* [5] postulated that the P~Ser residue might possibly be involved in the import of cystatin into the developing egg. If this is true, why the duck cystatin is not phosphorylated?

The physiological role of proteinase inhibitors in the eggs has not been elucidated. It is believed that the inhibitors might play a protective role against bacterial proteinases [2]; moreover, they are supposedly related to the regulation of absorption of albumen and yolk proteins into the developing embryos [24].

It can be concluded that the differences between chicken and duck egg white inhibitors concern not only ovostatin but also cystatin. For proper understanding of the physiological basis of such differences among closely related bird species, further studies are required.

REFERENCES

1. Lewis, S. (1991) Egg white proteins. *Comp. Biochem. Physiol.* **100** B, 1–9.
2. Bobek, L.A. & Levine, M.J. (1992) Cystatins–-inhibitors of cysteine proteinases. *Crit. Rev. Oral Biol. Med.* **3**, 307–332.
3. Fossum, K. & Whitaker, J.R. (1968) Ficin and papain inhibitor from chicken egg white. *Arch. Biochem. Biophys.* **125**, 367–375.
4. Anastasi, A., Brown, M.A., Kembhavi, A.A., Nicklin, M.J.H., Sayers, C.A., Sunter, D.C. & Barrett, A.J. (1983) Cystatin, a protein inhibitor of cysteine proteinases. Improved purification from egg white, characterisation and detection in chicken serum. *Biochem. J.* **211**, 129–138.
5. Laber, B., Krieglstein, K., Henschen, A., Kos, J., Turk, V., Huber, R. & Bode, W. (1989) The cysteine proteinase inhibitor chicken cystatin is a phosphoprotein. *FEBS Lett.* **248**, 162–168.
6. Nagase, H., Harris, E.D., Jr. & Brew, K. (1986) Evidence for a thiol ester in duck ovostatin (ovomacroglobulin). *J. Biol. Chem.* **261**, 1421–1426.
7. Ruben, G.C., Harris, E.D., Jr. & Nagase, H. (1988) Electron microscopic studies of free and proteinase–bound duck ovostatins (Ovomacro-globulins). *J. Biol. Chem.* **263**, 2861–2869.
8. Barrett, A.J. (1981) Cystatin, the egg white inhibitor of cysteine proteinases. *Methods Enzymol.* **80**, 771–778.
9. Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.
10. Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72**, 248–254.
11. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. & Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28**, 350–355.
12. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685.
13. Morrissey, J.K. (1981) Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* **117**, 307–310.

14. Ouchterlony, O. (1949) Antigen–antibody reaction in gels. *Acta Pathol. Microbiol. Scand.* **26**, 507–509.

15. Thiele, U., Assfalg-Machleidt, I., Machleidt, W. & Auerswald, E.A. (1990) N-Terminal variants of recombinant stefin B: effect on affinity for papain and cathepsin B. *Biol. Chem. Hoppe-Seyler* **371**, 125–136.

16. Barrett, A.J. (1973) Human cathepsin B1. Purification and some properties of the enzyme. *Biochem. J.* **131**, 809–822.

17. Haglund, H. (1971) Isoelectric focusing in pH gradient; in *Methods of Biochemical Analysis* (Glick D., ed.) vol. 19, pp. 82–85, Wiley, Interscience.

18. Bieth, J. (1974) Some kinetic consequences of the tight binding of protein-proteinase-inhibitors to proteolytic enzymes and their application to the determination of dissociation constants; in *Bayer-Symposium V "Proteinase Inhibitors"* (Fritz, H., Tschesche, H., Greene, L.J. & Truscheit, E., eds.) pp. 463–469, Springer-Verlag Berlin.

19. Barrett, A.J. & Salvesen, G. (1986) Proteinase inhibitors; in *Research Monographs in Cell and Tissue Physiology* (Dingle, J.T., Gordon, J.L., eds.) vol. 12, p. 533, Elsevier, New York.

20. Auerswald, E.A., Nägler, D.K., Schulze, A.J., Engh, R.A., Genenger, G., Machleidt, W. & Fritz, H. (1994) Production, inhibitory activity, folding and conformational analysis of an N-terminal and an internal deletion variant of chicken cystatin. *Eur. J. Biochem.* **224**, 407–415.

21. Popović, T., Brzin, J., Ritonja, A. & Turk, V. (1990) Different forms of human cystatin C. *Biol. Chem. Hoppe–Seyler* **371**, 575–580.

22. Machleidt, W., Thiele, U., Laber, B., Assfalg--Machleidt, I., Esterl, A., Wiegand, G., Kos, J., Turk, V. & Bode, W. (1989) Mechanism of inhibition of papain by chicken egg white cystatin: Inhibition constants of N-terminally truncated forms and cyanogen bromide fragments of the inhibitor. *FEBS Lett.* **243**, 234–238.

23. Turk, V., Brzin, J., Longer, M., Ritonja, A. & Eropkin, M. (1983) Protein inhibitors of cysteine proteinases. III. Amino-acid sequence of cystatin from chicken egg white. *Hoppe-Seylers Z. Physiol. Chem.* **364**, 1487–1496.

24. Sugimoto, Y., Hanada, S., Koga, K. & Sakaguchi, B. (1984) Egg-yolk trypsin inhibitor identical to albumen ovomucoid. *Biochim. Biophys. Acta* **788**, 117–123.