A Novel Cysteine Protease Inhibitor of the Egg of Chum Salmon, Containing a Cysteine-rich Thyroglobulin-like Motif

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ECI, one of three isoforms of cysteine protease inhibitor found in chum salmon eggs was purified to homogeneity, and its complete amino acid sequence was determined. The primary structure of ECI did not resemble those of other cysteine protease inhibitors of the cystatin superfamily but did resemble that of a cysteine-rich motif found as a repetitive structural element in thyroglobulin and several other proteins. The function of the cysteine-rich motif is not yet hypothesis. Two cysteine motif proteins, thyroglobulin and entactin, were tested for papain inhibitory activity and found to have none.

Proteinaceous inhibitors of cysteine proteases are widely distributed in various animal and plant tissues (1). Cystatins are known to form a superfamily of structurally homologous proteins and are grouped into three families: intracellular cystatin, extracellular cystatin, and kunitz. In addition to the cystatin superfamily, calpastatins are found to specifically inhibit calpains in animal cells (1).

Recently, a novel type of cysteine protease inhibitor was isolated from the eggs of chum salmon (2). Two isoforms of the proteins had molecular weights of 16,000 and 11,000, respectively, and showed inhibitory activities against papain, cathepsin B, and cathepsin L. Since partial N-terminal amino acid sequences of these salmon proteins were distinct from cystatins and calpastatins, they were considered to differ from so far known cysteine protease inhibitors.

The purpose of this paper is to present the primary structure of the cysteine protease inhibitor in salmon eggs. The third isoform of the salmon protease inhibitor was purified, and its amino acid sequence was analyzed. The salmon protein was found to be a novel type of cysteine protease inhibitor having a cysteine-rich motif (3-5) of thyroglobulin, IGF-1-binding protein and entactin/nidogen.

EXPERIMENTAL PROCEDURES

Materials—The eggs were taken from a mature chum salmon, Oncorhynchus keta, immediately after it had been caught in the Kujiriver, Ibaraki, Japan and stored at ~80°C until used. Papain, thyroglobulin, rabbit m-calpain, bovine trypsin, and bovine chymotrypsin were purchased from Sigma. Mouse entactin was purchased from Upstate Biotechnology (Sweden).

Purification of Cysteine Protease Inhibitor—Chum salmon eggs (800 g) were homogenized in 4 volumes of 20 mM sodium acetate buffer (pH 6.0) containing 0.1 M NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol (buffer A). The homogenate was centrifuged at 10,000 × g for 30 min. To the supernatant was added 50% (v/v) acetic acid. The mixture was centrifuged at 10,000 × g, and the supernatant was then concentrated in vacuo. The concentrated extract was applied to a C-4 μBondesphere 300-Å column (7.8 × 300 mm, Nihon Waters, Japan) equilibrated with 0.1% trifluoroacetic acid. The desired fraction was eluted by a linear 0–50% acetonitrile gradient and applied to a DEAE-Hitachi gel column (7.8 × 100 mm, Hitachi, Japan) and followed by separation using a C-18 μBondesphere column (3.6 × 200 mm, Nihon Waters, Japan). The targeted component was reduced and used in further experiments.

Polyacrylamide Gel Electrophoresis—SDS-PAGE was performed as described by Laemmli (6). The isoelectric point was determined by isoelectric focusing in a polyacrylamide gel (model 111, Bio-Rad) as described by the manufacturer.

Amino Acid Sequence—After the salmon inhibitor was reduced and S-pyridylethylated, 0.5 nmol of the reduced protein was incubated with 5 pmol of lysyl endopeptidase in 100 μl of 50 mM Tris-HCl buffer, pH 9.0, containing 8 M urea for 12 h at 37°C or with 5 pmol of Staphylococcus V8 protease in 100 μl of 0.1 M ammonium bicarbonate buffer, pH 8.0, containing 4 M urea for 12 h at 37°C. The digested sample was applied to a reverse-phase C18 column (3.6 × 200 mm, 300 Å). Peptides were eluted using an acetonitrile gradient. The amino acid sequences of the S-pyridylethylated protein and peptides digested by lysyl endopeptidase (K1-K3) and Staphylococcus V8 protease (V1-V3), shown by ELSD bars (see Fig. 2), were determined by automated Edman degradation (ABI models 470 and 476).

RESULTS AND DISCUSSION

The cysteine protease inhibitor in chum salmon eggs was purified to homogeneity by chromatographic steps of C-4 reverse phase HPLC, DEAE-Hitachi gel chromatography, and C-18 reverse phase HPLC (Fig. 1 and Table I). The molecular weight of this inhibitor was estimated to be 9000 by SDS-PAGE (Fig. 1), and pI was around 5.8 by isoelectric focusing. The inhibitor showed cysteine protease inhibitory activities against papain at Kᵢ = 0.35 nM and chum salmon cathepsin B (7) at Kᵢ = 15.8 nM while exhibiting no inhibitory activity against rabbit m-calpain, bovine trypsin, and bovine chymotrypsin. The papain (1 mol) was inhibited by about 1 mol of the purified inhibitor.

The analysis of the amino acid sequence of this inhibitor (Fig. 2) showed high homology with the partial N-terminal sequences of other cysteine protease inhibitors (Form I, AIRP-KTPCEDARDQV; Form II, AIRPMTCPERGADAA) in the egg of chum salmon as previously reported by us (2). Therefore, the salmon inhibitor isolated in the present study must be one of the cysteine protease inhibitor isoforms in the egg of chum salmon.

The very first residue at the N-terminal region of the inhibitor was identified to be mainly His and rarely Val. This might bring to proteolytic degradation as known to other proteins. Furthermore, although glycosylation was not analyzed, a putative asparagine-type glycosylation site was found at the Asn-55 residue. This finding suggests the secretory processing during its biosynthesis.

A comparison of the amino acid sequences was made by the
FASTA search (8) of the DDBJ/Swissprot/PIR sequence data bases (Fig. 3). The computer analysis for sequence homology of the salmon inhibitor indicated that the cysteine-rich motif in ECI was very similar to the six motifs of thyroglobulin precursor (3). The thyroglobulin-like motif has been found in a major component of the basement membrane, entactin/nidogen (4), IGF binding proteins, specific binding protein to IGF (5), human testicular proteoglycan, testican (9), and scorpion venom insectotoxin 12 (10). An amino acid sequence comparison of the salmon inhibitor with these homologous proteins containing

| Step                  | Total protein | Total activity | Specific activity | Yield | Purity |
|-----------------------|---------------|----------------|-------------------|-------|--------|
| Acetone extract       | 598           | 228            | 0.38              | 100   | 1      |
| µBondasphere C4       | 20.5          | 29.6           | 1.4               | 13.0  | 3.7    |
| DEAE-Hitachi gel      | 6.3           | 14.7           | 2.3               | 6.4   | 6.1    |
| µBondasphere C18      | 2.0           | 7.8            | 3.9               | 3.4   | 10.3   |

**Fig. 1.** Purification of the cysteine protease inhibitor from chum salmon egg. A fraction obtained by acetone fractionation of salmon egg extract was applied to a C-4 µBondasphere column (7.8 x 300 mm, Nihon Waters, Japan) equilibrated with 0.1% trifluoroacetic acid. The active fractions were eluted by a linear gradient of 0–50% acetonitrile (A). These active fractions were applied to a DEAE-Hitachi gel column (7.8 x 100 mm, Hitachi, Japan) (B) and then separated by a C-18 µBondasphere column (3.6 x 200 mm, Nihon Waters, Japan) (C). The bracket indicates the fractions pooled from the chromatogram. The major component of the eluent was collected and used for further experiments. Polyacrylamide gel electrophoresis of purified ECI is shown. SDS-PAGE (15% gel) was performed after reduction of the sample (D). The gel was stained with Coomassie Brilliant Blue R-250.

**Fig. 2.** Amino acid sequence of the salmon egg cysteine protease inhibitor. The identified residues are indicated by arrows. The amino acid sequences of S-pyridylethylated protein and peptides digested by lysyl endopeptidase (K1–K3) and Staphylococcus V8 protease (V1–V3), shown by bars, were analyzed by automated Edman degradation. The solid and dashed lines of the bar show identified and unidentified residues, respectively, by the sequence analysis.
The thyroglobulin-like motifs showed that approximately 30–50% of the residues are identical between them, and 5 cysteine residues comprising the motif are highly conserved. On the other hand, the amino acid sequence of the salmon ECI completely differs from known protease inhibitors. Therefore, these findings indicate that the salmon ECI is a novel class of protease inhibitor containing the thyroglobulin-like cysteine-rich motif.

Papain inhibitory activities of proteins containing the thyroglobulin-like motif, mouse entactin and thyroglobulin, were examined. No apparent inhibitory activity against papain was observed. Thus, the function of the thyroglobulin-like motif of these homologous proteins remains unknown. Further work is under way to elucidate the inhibition mechanism, structure, and physiological function of ECI.

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