AN ABSTRACT OF THE THESIS OF

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David W. Barnes

Rainbow trout cystatin C cDNA has been isolated from trout liver. The full-length cystatin cDNA (674 bp) included the 5'untranslated region and the polyadenylation signal sequence AATAAA in the 3' region. Translation of the cDNA defines 132 amino acid residues. Comparison of the amino acid sequence with those of family 2 cystatins indicates that the 21 amino acids at the N-terminal end is a signal peptide necessary for cystatin secretion, and the remaining 111 amino acids represent mature cystatin. Four cysteine residues in the cystatin may form two disulfide bonds producing a molecule with the properties of a family 2 cystatin.

Trout cystatin C gene expression was analyzed by Northern blot. This gene is expressed at various levels in

all tissues examined. This difference may reflect differences in degree of regulation of cysteine proteinase activities. A high level of trout cystatin C expressed in trout hepatic tissue or cell cultures suggested that cystatin C expression might be related to tumorigenesis. Southern blot of trout genomic DNA showed that the copy number of the trout cystatin gene is probably one per haploid genome.

Trout cystatin C was expressed in *E. coli* at a yield of 3 - 5 mg/L culture, but no inhibitory activity was detected for the untreated recombinant protein. However, after refolding, recombinant cystatin C displayed inhibitory activity against papain. The dissociation constant of recombinant cystatin C against papain is 1.2×10^{-6} nM, similar to that of human cystatin C. Trout cystatin C was also expressed in yeast cells, but no inhibitory activity was detected either. No cystatin C was secreted in the yeast expression system using either the trout cystatin C secretion signal, or the yeast invertase secretion signal. The expression levels of trout cystatin C in our expression systems are still low for industrial requirements. Therefore, further investigation will be needed to construct more efficient expression systems and vectors for trout cystatin C heterologous production.

Rainbow Trout Cystatin C: Gene Expression, Heterologous Production and Characterization

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Fugen Li, Author

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CHAPTER 1

Cysteine Proteinase Inhibitor - Cystatin C

Cystatin Superfmily

The first cysteine proteinase inhibitor was discovered by Finkelstadt (1957) who reported a heat-stable inhibitor of cathepsin B and dipeptidy peptidase I in rat liver cytosol. Whitaker and co-workers discovered and partially characterized a protein from chicken egg-white that inhibited ficin and papain (Fossum and Whitaker, 1968; Sen and Whitaker, 1973). The name "cystatin" for the protein was proposed by Barrett (1981), because of its distinctive inhibitory properties of cysteine proteinases.

Chicken cystatin was purified with papain affinity chromatography, characterized and sequenced (Turk et al., 1983; Schwabe et al., 1984). The successful purification method for chicken cystatin was used to purify many other cysteine proteinase inhibitors from animals and plants. Therefore, many cysteine proteinase inhibitors have been

isolated, characterized and sequenced. Information about each cystatin provided a foundation for classification and evolutionary studies.

The proteins that can be shown statistically to have an evolutionary relationship and have similar properties to chicken cystatin form a distinct superfamily which is called the cystatin superfamily (Dayhoff et al., 1983). The known proteins of the cystatin superfamily can be seen to comprise three protein families (Dayhoff et al., 1983). Family 1, which may also be called the stefin family, contains the proteins that are distinguished within the superfamily by the lack of disulfide bonds and glycosylation, and by close sequence relationships to each other (Barrett et al., 1986). The most thoroughly studied inhibitors of this family are human cystatin A (Machleidt et el., 1983), human cystatin B (Ritonja et al., 1985), rat cystatin α (Takio et al., 1984) and rat cystatin β (Takio et al., 1983). Family 2, which may also be called the cystatin family, contains proteins that have two disulfide bonds and no glycosylation (Barrett et al., 1986). These include chicken cystatin (Schwabe et al., 1984), human cystatin C (Barrett et al., 1984), and human cystatin S (Isemura et al., 1984). Family 3 comprises the plasma kininogens, and may therefore also be called the kininogen family. Known members of this family, which contain 9 disulfide bonds, have been named "human α_1-thiol

proteinase inhibitor", "human α_2-thiol proteinase inhibitor", and "rat T-kininogen" (Barrett et al., 1986).

Muller-Esterl and Fritz (1986) proposed a model for the evolution of mammalian cysteine proteinase inhibitors on the basis of amino acid sequence homology. This model suggested that the variety of cysteine proteinase inhibitors has evolved from an ancestral stefin-type inhibitor (Family 1 cystatin) (Figure 1.1). From this prototype, the cystatin-like inhibitors have descended by acquiring a C-terminal extension. Gene triplication of the archetypal cystatin-like inhibitor then generated the kininogen heavy chain. Thus, three cystatin-(Family 2) rather than stefin-like (family 1) copies formed the building blocks of the human kininogen heavy chains (Muller-Esterl and Fritz, 1986; Salvesen et al., 1986).

Cystatin C Gene Expression and Functionality

Many cystatin C cDNAs or even whole genes have been isolated and sequenced providing information about cystatin C gene structure, function and regulation. Northern blot analysis indicated that chicken cystatin C gene is expressed in all tissues, but at variable levels. Very high concentration of chicken cystatin mRNA was found in chicken brain, but not in adult liver (Colella et al., 1989). Mouse

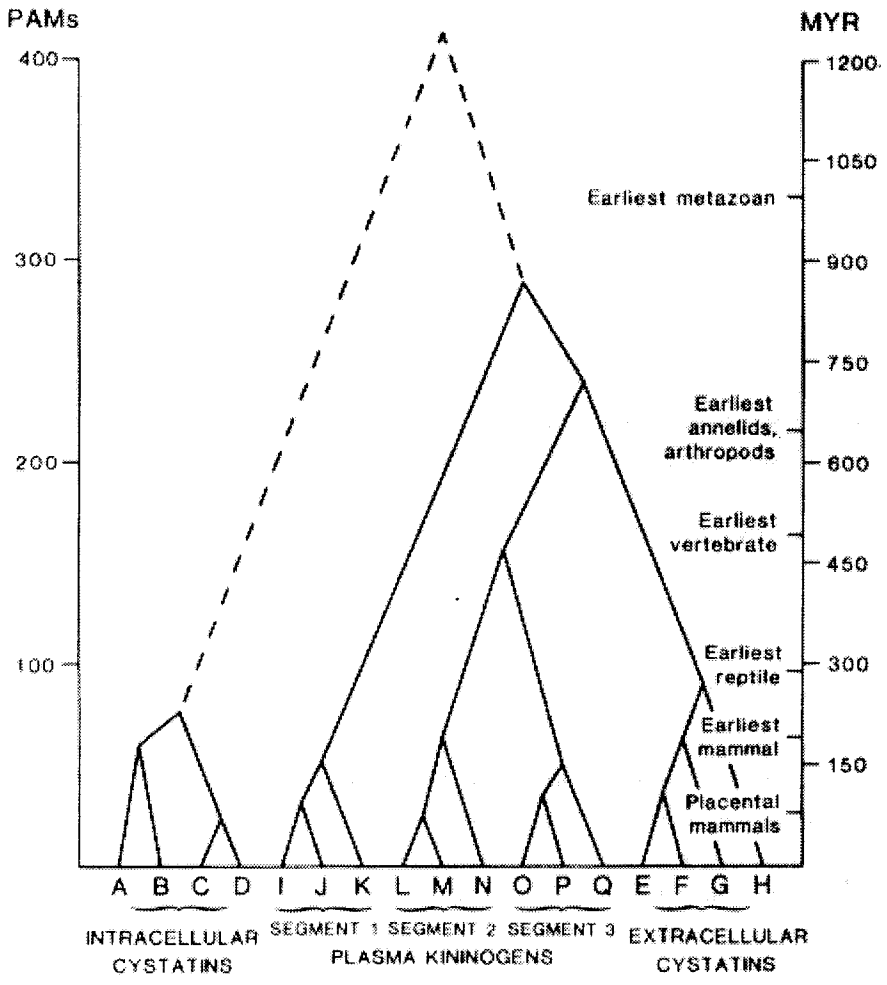


Figure 1.1. Dendrogram of the cystatin superfamily from Salvensen et al. (1986). The dendrogram has been calculated according to accepted point mutations (PAMs) methods. The extrapolation point is connected by broken lines to indicate that its position is uncertain. A second scale is indicated on the right hand side of the diagram in millions of years ago (MYR), which has been calibrated from the point of divergence of chicken cystatin from the other extracellular cystatins, representing early reptilian divergence. Several evolutionary events, dated from the fossil record, are included as guideline. The key to the sequences: A = human cystatin A; B = rat cystatin α; C = human cystatin B; D = rat cystatin β; E = human cystatin C; F = beef colostrum cystatin; G = human cystatin S; H = chicken cystatin; I = human kininogen segment 1; J = beef kininogen segment 1; K = rat kininogen segment 1; L = human kininogen segment 2; M = beef kininogen segment 2; N = rat kininogen segment 2; O = human kininogen segment 3; P = beef kininogen segment 3; Q = rat kininogen segment 3.

and rat cystatin C are expressed at high levels in the brain and low levels in the liver, an expression pattern similar to that observed in the chicken cystatin (Huh et al., 1995; Hakansson et al., 1996). The pattern of mouse and rat cystatin C expression is also similar to that of its human counterpart. This overall similarity of cystatin C gene expression between mammalian species indicates that mouse or rat may be suitable for generating an animal model to study the human genetic disease HCCAA (hereditary cystatin C amyloid angiopathy) (Huh et al., 1995). Trout cystatin C is also expressed in all tissues examined, highly in trout brain and developed eggs, but low in muscles (Li et al., 1998).

Human cystatin C (Abrahamson et al., 1990), chicken cystatin C (Colella and Bird, 1993) and mouse cystatin C (Huh et al., 1995) genes have been cloned and sequenced. All cystatin genes include three exons and introns. The 5'-flanking regions of the human cystatin C gene and chicken cystatin C gene share several features with those housekeeping genes. The 5'-flanking region of the mouse cystatin C gene, which contains a binding site for AP-1, is slightly different from those of other cystatin C genes. Therefore, mouse cystatin expression can be induced by TGF-β (Solem et al., 1990; Huh et al., 1995). Cystatin genes from

different organisms share the same functions but exhibit genetic diversity.

Cystatins are very strong inhibitors of cysteine proteinases. They are believed to protect cells from inappropriate endogenous or external proteolysis, and they could be involved in the control mechanisms responsible for intracellular or extracellular protein breakdown (Turk et al., 1986). Some cystatins, such as chicken cystatin and human cystatin C and stafin B, are able to block virus replication when added to infected cells in culture (Argos et al., 1984; Turk et al., 1986). Human cystatin C may protect against cancer invasion and metastasis, because human malignant melanoma cells releasing abundant cysteine proteinase inhibitors which strongly inhibit cathepsin B activity have minimal ability to invade or metastasize normal tissue (Turk et al., 1984; Sexton and Cox, 1997). The balance between cysteine proteinase and cystatin in the cells and tissues is very important to maintain cell survival, growth and function. Any imbalance can cause disease. In HCCAA, young adults are affected by massive, often fatal, cerebral hemorrhage. This disease is associated with an abnormally low concentration of cystatin C in cerebrospinal fluid and deposition of a cystatin C variant as amyloid (Cohen et al., 1983; Ghiso et al., 1986). Cystatin C is also associated with the filaments and bundles

of fibrils in the cytoplasm and nucleus (Zucker-Franklin et al., 1987). The presence of cystatin C gives evidence that astrocytes have some properties of monocytes, and they may possibly play a role in the development of some forms of amyloidosis. There may be a developmentally related expression of cystatin C, since astrocyte precursors do not express the gene at comparable levels (Solem et al., 1990).

Cystatin C Interaction with Cysteine Proteinase

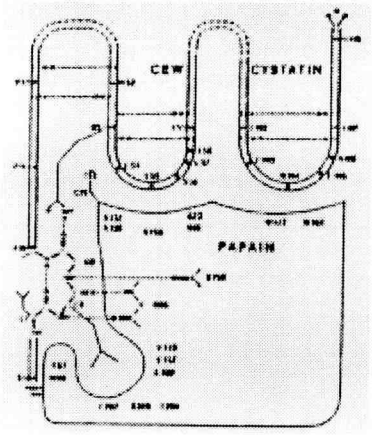
Cystatin C is the predominant family 2 cystatin in mammalian organisms. Most of the structure of the family 2 cystatins and the mechanism of interaction with cysteine proteinase have been studied with chicken cystatin C and human cystatin C. The crystal structures of chicken cystatin has been solved by X-ray diffraction (Figure 1.2). The reaction of chicken cystatin with papain has been studied in detail as a model for interactions between cystatins and cysteine proteinases. The inhibitor forms a tight, equilmolar complex with the cysteine proteinases, in which the reactive cysteine of the latter is inaccessible to substrates and to the thiol group reagents (Anastasi et al., 1983; Nicklin and Barrett, 1984; Lindahl et al., 1988; Bjork et al., 1989; Lindahl et al., 1992). Unlike the standard mechanism described for serine proteinase inhibitors

Figure 1.2. Ribbon-like representation of chicken egg white cystatin (Bode et al., 1990). α-Helical segments, extended β-strands and irregular intervening segments are shown as regularized helical and arrow-like elements and connecting laces. The view is approximately along the wedge-shaped edge placed at the left upper side, with amino terminus (from Gly9I onwards), first and second hairpin loop. The carboxyl terminus is at the bottom left, the appending helix on the right-hand side.

(Laskowski and Kato, 1980), which implies the formation of a stable complex and irreversible hydrolysis at the inhibitor active site, the reaction of cystatins with their cognate enzymes is reversible with apparently simple, bimolecular kinetics (Bode et al., 1990; Lalmanach et al., 1993).

The sequence homologies between the members of the cystatin superfamily indicate that they contain highly conserved amino acid residues or fragments, which may participate in inhibition (Ohkubo et al., 1984; Abrahamson et al., 1987; Lindahl et al., 1988). X-ray diffraction studies of the crystal structures of chicken cystatin and papain complex have shown that these are spatially contiguous and form a wedge-shaped edge highly complementary to the active site of papain (Figure 1.3). One of these conserved regions, the pentapeptide QVVAG, is the most frequently found variant of the QXVXG consensus sequence present in all inhibitory members of the superfamily and which is reported to be involved in inhibition (Ohkubo et al., 1984). Inhibition assays with the synthetic peptide (Teno et al., 1987) or recombinant cystatin having a single mutation at the QXVXG level (Jerala et al., 1990) have confirmed that this region is essential for inhibition of cysteine proteinases. Another critical region for inhibition lies at the N-terminal end of human cystatin C and chicken egg white cystatin (Abrahamson et al., 1987). It includes a

A.



B.

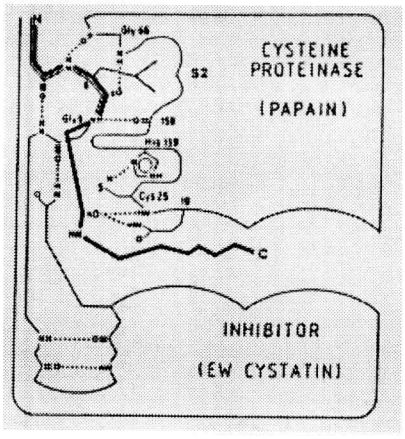


Figure 1.3. Scheme of the proposed model of the interaction of long-chain chicken egg white cystatin with papain (Bode et al., 1990). A). The intra- and the inter-molecular interaction of inhibitor segment Leu71-Ala101 (bottom left) are outlined in more detail. B). Only the main interaction features are outlined in more detail. Bold lines indicate the presumed arrangement of productively bound good peptide substrates.

conserved glycyl residue preceded by two additional residues which may interact with the enzyme in a substrate-like manner (Abrahamson et al., 1987; Grubb et al., 1990). Macheidt et al. (1989) have proposed a model illustrating the importance of the chicken cystatin N-terminal region for interaction with papain, but this model cannot be applied to other cystatin types, such as members of stefin and kininogen families, since their enzymatically cleaved or N-terminally truncated recombinant forms retain full inhibitory activity (Abe et al., 1988; Moreau et al., 1989). A third region, near the C-terminal end of chicken cystatin, contains a tryptophan involved residue in papain binding (Lindahl et al., 1988). This C-terminal region is also important in oryzacystatin (Abe et al., 1988), but its absence does not modify the inhibitory capacities of the isolated cystatin-like domain of human low molecular mass kininogen or rat thiostatin (Vogel et al., 1988; Moreau et al., 1989). In general, the crystal structure of chicken cystatin (Bode et al., 1988) suggests that the proteinase-binding site comprises three regions, the N-terminal region around Gly-9, the Gln-Leu-Val-Ser-Gly sequence at residues 53-57 and the C-terminal region around Trp-104 (Ohkubo et al., 1984; Abrahamson et al., 1987; Lindahl et al., 1988).

The cystatins are competitive reversible inhibitors, generally displaying broad specificity and high-affinity

binding to target cysteine proteinases (Table 1). The values of K_i for all cysteine proteinases are in the nM range. All mammalian cysteine proteinases belonging to the papain superfamily are inhibited to some extent by the human cystatins, as is also the case for a number of cysteine proteinases of plant origin. Cystatin C is the most general inhibitor, in that it binds tightly to all investigated enzymes, whereas cystatin D seems to be more specific, based on its poor inhibition of cathepsin B. One of the two functional cystatin domains of the kininogens has inhibitory activity against calpains, a feature not shared by the family 1 and 2 cystatins.

Heterologous Production of Cystatins

Escherichia coli is the most successful host for heterologous protein expression. Many cystatins, such as human cystatin C (Abrahamson et al., 1988; Dalboge et al., 1989), rat and mouse cystatin C (Hakansson et al., 1996), and chicken cystatin C (Auerswald et al., 1989 and 1991) have been expressed successfully in *E. coli* system. Cystatin C is a secreted protein and contains two disulfide bonds. Human cystatin C, rat and mouse cystatin C have been successfully expressed and secreted using the OmpA signal peptide in *E. coli*. Those cystatins have full inhibitory

Table 1. Dissociation Constants of Complexes between Human Cystatin and Cysteine Proteinases*

	K_i (nM)				
Cystatin	Papain	Cathepsin B	Cathepsin H	Cathepsin L	Cathepsin S
A	0.019	8.2	0.31	1.3	0.05
B	0.12	73	0.58	0.23	0.07
C	0.000011	0.25	0.28	<0.005	0.008
D	1.2	>1000	8.5	25	0.24
S	108	--	--	--	--
SN	0.016	19	--	--	--
SA	0.32	--	--	--	--
L-kininogen	0.015	600	0.72	0.017	--

* Abrahamson, M. 1994. Cystatins. Methods Enzymol. 244: 685-700.

activity against papain. The yield of human cystatin C reaches 1000 μg/ml (Dalboge et al., 1989), but the yields of mouse and rat cystatin C are 2 and 1 mg/L culture medium, respectively (Hasansson et al., 1996). Cystatins expressed with the same vector and host cell strain exhibit very different expression levels. This implies that each protein has its own characteristic expression properties.

Chicken egg white cystatin was successfully expressed and secreted using the ompA signal peptide in *E. coli* (Auerwald et al., 1991). Fusion chicken egg white cystatin protein has also been successfully produced in *E. coli*. However, the fusion cystatin did not have inhibitory activity, because it could not form the correct structure in bacterial cells. After partial refolding by passage through a Sephadex G-25 column, 37% of the fusion cystatin was found to be active by titration with papain (Auerwald et al., 1989). The properties of the cystatin produced by the bacterial expression system corresponded to those of natural cystatin. Secretion expression systems may have advantages over the fusion system, but often produce the heterologous protein at a low level.

The yeast *Saccharomyces cerevisiae* has also gained popularity as a host for heterologous gene expression and protein secretion. This interest is due both to the ease and favorable economics of yeast fermentation, developed over

years of industrial experience, and to the rapid progress made in the molecular genetics of the organism (Smith et al., 1985). Many proteins including hepatitis B virus surface antigen (Valenzuela et al. 1982), interferon (Hitzeman et al., 1983) and human erythropoietin (Elliott et la., 1989), have been successfully expressed and secreted in yeast. However, no information on cystatin expression in yeast was available. This project has explored the fish cystatin expression in yeast. Although the results were not satisfactory compared with other proteins that have been successfully expressed in yeast, they provide information for further study of cystatin expression in yeast.

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CHAPTER 2

Gene Expression and Characterization of Rainbow Trout (*Oncorhynchus mykiss*) Cystatin C

Abstract

Cystatin C is one of a family of cysteine proteinase inhibitors of cathepsins and other cysteine proteinases. Among warm-blooded vertebrates, small functional regions of cystatin amino acid sequences are well conserved among species, but major portions of cystatin amino acid sequences vary evolutionarily. Although considerable attention has been given to mammalian and avian cystatins, little data exist on cystatins from other vertebrates. A cDNA clone for trout cystatin C was isolated from a lambda gt11 cDNA library of rainbow trout (*Oncorhynchus mykiss*) liver. An apparently full-length cDNA clone of 674 bp encoding 132 amino acid residues was obtained. Sequence analysis indicated that trout cystatin C contains an N-terminal signal sequence extension of 21 amino acids and a mature sequence of 111 amino acid residues, with amino acid residues conserved in functional regions relative to mammalian and avian cystatin C. Using cloned cDNA as a probe, we investigated expression of the cystatin C gene in trout tissues, several cell lines of trout liver or liver tumor, and cell cultures of liver tumor origin. Cystatin C mRNA was in high abundance in trout embryo tissue, tumor-derived liver cell line and some normal adult tissues. Southern hybridization analysis indicated one copy of the

trout cystatin C gene per haploid genome, and sequence comparisons indicated considerable divergence in large portions of the coding region of the trout cystatin C gene relative to a variety of species.

Introduction

Cystatins, a superfamily of cysteine proteinase inhibitors, are widely distributed in animal tissues and body fluids. The superfamily is divided into three groups on the basis of molecular structure (Barrett et al., 1986). Family I cystatins lack disulfide bonds; human cystatin A (Machleidt, et al., 1983) and B (Ritonja et al., 1985) and rat cystatins α (Takio et al., 1983) and β (Takio et al., 1984) are typical examples. Family II cystatins contain two disulfide bonds as exemplified by human cystatin C (Abrahamson et al., 1987), human cystatin S (Isemura et al., 1986), chicken cystatin (Colella et al., 1989), mouse cystatin C (Solem et al., 1990) and rat cystatin (Cole et al., 1989). Both families are also characterized by molecular weights between M_r 10,000 and M_r 20,000. Family III cystatins are kininogens (Abe et al., 1987).

A major role of cystatins is the inhibition of proteolysis by cysteine proteinases such as cathepsins B, H, and L (Rawling and Barrett, 1990; Koide and Noso, 1994), and

a function in inhibition of proteolytically dependent tumor metastasis has been suggested (Sexton and Cox, 1997). Cystatins may also contribute to defense against viral proteinases that are required for virus replication (Argos et al., 1984). Genetic abnormalities in cystatins are associated with neurological diseases in humans (Ghiso et al., 1986; Abrahamson et al., 1987), and developmental regulation in neural tissue precursor cells has been demonstrated (Solem et al., 1990).

Although considerable attention has been given to mammalian and avian cystatins, little data exist on cystatins from other vertebrates. We pursued molecular cloning of fish cystatin C (family II cystatin) as a means for exploring cystatin biological function, regulation, and genetic diversity. We are also exploring the potential of the protein in the seafood industry as an inhibitor of proteolytic degradation of fish muscle. This paper reports the molecular cloning of cystatin C cDNA from rainbow trout.

We found significant amino acid sequence identity in the conservative regions between trout cystatin C and mammalian cystatins, especially those in family II. In addition, we examined the relative levels of cystatin C mRNA in adult and embryonic trout tissues. We also examined the levels of cystatin mRNA in cell lines established from trout liver tumors and normal liver, and in cell cultures derived

from aflatoxin-induced hepatic tumors. These data may form the basis for further studies on the expression and regulation of cystatins and the biological roles of cystatin for fish tumorigenesis, growth and development.

Materials and Methods

Materials: TRI REAGENT was purchased from Molecular Research Center, Inc, (Cincinnati, OH). Reverse transcription system kit, Lambda gt 11 system, subcloning plasmid and Lambda DNA packaging system were obtained from Promega (Madison, WI). PCR reagent kit was obtained from Perkin-Elmer Cetus (Norwalk, CT). Geneclean kit was from Bio 101 (La Jolla, CA). cDNA synthesis kit was from Pharmacia Biotech (Uppsala, Sweden). BioMag mRNA purification kit was purchased from PerSeptive Diagnostics Inc. (Cambridge, MA). Random primed DNA labeling kit was obtained from Boehringer Mannheim Biochemica (Indianapolis, IN). Temperature cycler was from Ericomp Inc. (San Diego, CA). α-^{32}P dATP, α-^{32}P dCTP were purchased from DuPont Company, Biotechnology System (Wilmington, DE).

Powdered cell culture media formulations (DMEM, L-15, F-12), 2-mercaptoethanol, L-glutamine, and non-essential amino acids were purchased from Gibco (Grand Island, NY); sodium bicarbonate was from J.T. Baker (Phillipsburg, NJ);

HEPES was from Research Organics (Cleveland, OH); antibiotics and insulin were from Sigma (St. Louis, MO); bFGF was from R & D Systems (Minneapolis, MN); EGF was from UBI (Lake Placid, NY); zebrafish embryo extract was prepared as described (Sun et al., 1995); trout embryo extract was prepared as described (Collodi and Barnes, 1990; Bradford et al., 1994a, 1994b); trout serum was prepared as described (Collodi et al., 1992); fetal calf serum was from Hyclone (Logan, UT).

Construction of rainbow trout liver cDNA library: Total RNA was extracted from rainbow trout liver by the TRI-REAGENT method (Chomezynski, 1993). Poly(A^+) RNA was purified with a BioMag mRNA purification kit. After the synthesis of single-stranded cDNA by reverse transcriptase with total poly(A^+) RNA as a template, double-stranded cDNA was synthesized according to the method of Gubler and Hoffman (1983) using *E. coli* polymerase I. Flush ends of the cDNA were generated with T4 DNA polymerase and ligated with EcoRI/NotI adapters, and inserted into the phage vector λgt 11. The DNA was then packaged into bacteriophage particles using a lambda packaging system (Promega) and grown on *E. coli* strain LE392.

Library screening: Recombinant plaques were transferred onto nitrocellulose (Schleicher & Schuell, Keene, NH). The filters were dried in a vacuum oven at 80°C

for 2 hours and prehybridized with a solution containing 6 X SSC, 5 X Denhardt's reagent, 0.5% SDS, 50% formamide and 100 μg/ml denatured sonicated calf thymus DNA at 42°C for 4 hours. After the prehybridization, the filters were hybridized with random-labeled partial cystatin cDNA generated by PCR (Li et al., 1997) in the same solution used for the prehybridization at 42°C for 20 hours. The filters were finally washed in 0.25 X SSC, 0.1% SDS at 65°C and exposed to hyperfilm with intensifying screen at -80°C. The positive plaques selected on the first screening were further screened under the same conditions used in the first screening.

DNA sequencing: DNA from positive recombinant phages was isolated according to the method described by Sambrook et al. (1989). All DNA from positive recombinants was cut with EcoRI to check the size of the inserts. Two primers from λgt 11 arms were used for DNA sequencing. The chosen clone was digested with EcoRI and the insert was subcloned into the EcoRI site of pGEM-3z (Promega) and sequenced again.

Northern blot: Total RNA was extracted from tissues of rainbow trout and trout eggs (25 days after fertilization), and from cell cultures. Ten μg of total RNA from tissue samples and 20 μg from cell culture samples were denatured

and electrophoresed in a formaldehyde-containing agarose gel. After electrophoresis, the RNA was transferred onto a nylon membrane (INC Biomedical Inc. OH) and hybridized with random-labeled cystatin cDNA at 42°C in a solution containing 5 X Denhardt's, 5 X SSC, 50 mM sodium phosphate (pH 6.5), 0.1% SDS, 50% formamide, and 100 µg/ml heat-denatured sonicated calf thymus DNA. The filter was washed with 1 X SSC containing 0.1% SDS at room temperature for 30 min, with 0.25 X SSC containing 0.1% SDS at room temperature for 15 min and then exposed to hyperfilm at -80°C. After the films were developed the filter was stained for ribosomal RNA with methylene blue.

Southern blot: Trout genomic DNA was isolated from liver tissue according to the method of Sambrook et al. (1989). About 20 µg of the DNA was digested with restriction enzymes, electrophoresed on 1% agarose gel, denatured and transferred onto nitrocellulose membrane and hybridized with random-labeled cystatin C cDNA at 42°C in the same solution used for the library screening. The filters were washed with 0.25 X SSC containing 0.1% SDS at 65°C and then exposed to hyperfilm at -80°C.

Cell lines: The RTH-149 cell line, derived from a rainbow trout hepatoma, was cultured in LDF medium containing 5% fetal calf serum (Lannan et al., 1984). The

RTL-W1 cell line, from the normal liver of a four year old male rainbow trout, was grown in L-15 medium with 5% fetal bovine serum (Lee et al., 1993). RTH cells were obtained from Dr. John Fryer, Department of Microbiology, Oregon State University. RTL-W1 cells were a gift from Dr. Niels Bols, University of Waterloo, and were obtained from Dr. Christopher Bayne, Department of Zoology, Oregon State University.

Cell culture medium for primary cell culture: Growth medium consisted of DLF medium (67.5% Dulbecco's modified Eagle's medium, 25% Leibovitz's L-15, 7.5% Ham's F-12) containing sodium bicarbonate (6 mM), HEPES (15 mM), penicillin-G (200 U/ml), streptomycin sulfate (200 μg/ml), ampicillin (25 μg/ml), bovine insulin (10 μg/ml), 2-mercaptoethanol (55 μM), L-glutamine (1 mM), non-essential amino acids (50 μM), human recombinant b-FGF (10 ng/ml), murine EGF (50 ng/ml), zebrafish embryo extract (5%), trout embryo extract (25 μg protein/ml), trout serum (0.25%), and fetal calf serum (5%).

Primary cell cultures for trout liver tumors: Rainbow trout were initiated as embryos by a single 30 min bath immersion in 80 ppm aflatoxin B1. This treatment protocol elicits primarily cholangiocellular and mixed hepatocellular/cholangiocellular carcinomas in the trout

model (Hendricks et al., 1984). Fish (0.6-0.8 kg wet weight) were killed at two years of age and sprayed with 70% ethanol. Grossly visible hepatic tumors were removed by dissection and placed in 6-cm plastic petri dishes containing 1 ml DLF medium. Tissues were washed several times in medium and then minced in 1 ml trypsin/EDTA (0.2%/1 mM in phosphate-buffed saline) using a #10 scalpel blade. Trypsin was inactivated by adding an equal volume of fetal calf serum and the minceate was transferred into a sterile 15 ml centrifuge tube. Contents were diluted to 10 ml with DLF medium and gently centrifuged for 5 minutes. Supernatants were aspirated, pellets (consisting of cells and small pieces of tissue) were resuspended in 5 ml complete growth medium (described above), and seeded into 25 cm^2 tissue culture flasks. Flasks were incubated under air atmosphere at 16°C; Culture medium was replaced with fresh medium weekly for three months.

Results

Isolation and characterization of a cDNA clone for trout cystatin

From 1.2 X 10^4 independent cDNA clones of a trout liver λgt 11 cDNA library, four clones were hybridized with ^{32}P-labeled partial trout cystatin cDNA which was generated by

RT-PCR as we have described previously (Li et al., 1997). The positive clones were designated as cst1, cst2, cst3 and cst4. All clones on λgt 11 were sequenced initially with two primers from λgt 11 arms and sequences were aligned for comparison with the cystatin sequences in the GenBank. Cst2, about 700 bp, appeared to contain a full-length trout cystatin cDNA. Cst3 and cst4, about 500 bp, were partial cystatin cDNAs. Cst1, about 1000 bp, appeared to be composed of a partial cystatin cDNA at the 3' region and a different sequence at the 5' region. Possibly two cDNA fragments were ligated together during cDNA library construction.

Cst2 was subcloned into pGEM-3z and sequenced again. Figure 2.1 shows the complete nucleotide sequence of cst2, which contained 674 bp and the polyadenylation signal AATAAA in the 3' region, but lacked a poly(A) tail. Predicted translation of the cDNA indicates a protein of 132 amino acids (Figure 2.1). Comparison of this amino acid sequence with that of previously described family II cystatins is shown in Figure 2. The precursor trout cystatin C contains a putative hydrophobic signal peptide of 21 amino acid residues and a mature sequence of 111 amino acid residues. The cleavage site for the signal peptide is proposed to be between the Ala residue shown at position -1 and the Gly residue shown at position +1 (Figure 2.1).

```
CAAAGATATCTAACGGGAAAATGATCATGGAATGGAAAATCGTCGTTCCTTTGTTCGCCG 60
                    M  I  M  E  W  K  I  V  V  P  L  F  A  V

TGGCCTTTACGGTGGCGAACGCCGGTTTGATCGGAGGCCCCATGGACGCAAATATGAACG 120
  A  F  T  V  A  N  A  G  L  I  G  G  P  M  D  A  N  M  N  D
                   -1 +1                          10

ACCAAGGAACGAGAGACGCCCTGCAGTTCGCGGTGGTCGAACACAACAAGAAAACAAACG 180
  Q  G  T  R  D  A  L  Q  F  A  V  V  E  H  N  K  K  T  N  D
                   20                          30

ACATGTTTGTCAGGCAGGTGGCCAAGGTTGTCAATGCACAGAAGCAGGTGGTATCTGGGA 240
  M  F  V  R  Q  V  A  K  V  V  N  A  Q  K  Q  V  V  S  G  M
                   40                          50

TGAAGTACATCTTCACAGTGCAGATGGGCAGGACCCCATGCAGGAAGGGAGGTGTTGAGA 300
  K  Y  I  F  T  V  Q  M  G  R  T  P  C  R  K  G  G  V  E  K
                   60                          70

AGGTCTGCTCCGTGCACAAGGACCCACAGATGGCTGTGCCCTACAAGTGCACCTTCGAGG 360
  V  C  S  V  H  K  D  P  Q  M  A  V  P  Y  K  C  T  F  E  V
                   80                          90

TGTGGAGCCGCCCCTGGATGAGCGATATCCAGATGGTCAAGAACCAGTGTGAAAGTTAAG 420
  W  S  R  P  W  M  S  D  I  Q  M  V  K  N  Q  C  E  S
                  100                         110

ACCCAGTGAAGAGAACTTCAATCAATGTCTAGTCTACCCAATAACTACTATTATCTAGTA 480

CTAGTGTTATTTGTTAGTCTCACCAATGCAGTTCAACCTCCTTGTCTAGGATGTATTCAG 540

AGAATCCCACTAATAAAAGATGTTCAAACTTATTGCATGCCCACACTAATATAAGCACTT 600

AATGCAAACATTGCTGTCTTGAGAATGTAGTATTAAAATGATGCAACAGTTAACTAAATA 660

AATGTTTTGGAACA 674
```

Figure 2.1. Complete nucleotide sequence of rainbow trout cystatin C cDNA and the deduced amino acid sequence. The putative signal peptide appears before the amino-terminal glycine residue 1. The polyadenylation signal is underlined.

Comparison of the deduced amino acid sequence of trout cystatin C with the family II cystatins showed high sequence identities in the conservative regions [consensus sequences of reactive sites: Gly(4), Gln-X-Val-X-Gly(48-52), and Ile(Val)-Pro-Trp(96-98)] (Figure 2.2) and identified 4 cysteine residues which probably form two disulfide linkages as is true for other family II cystatins. The percentage of amino acid sequence identities of trout cystatin C are as follows: 96% with salmon cystatin (Koide and Noso, 1994), 43% with chicken cystatin (Colella et al., 1989), 35% with mouse (Solem et al, 1990), rat (Cole et al., 1989) and human (Abrahamson et al., 1987) cystatins.

Expression of trout cystatin gene in various tissues and cell lines

The trout cystatin C cDNA was used as a hybridization probe to evaluate the expression of cystatin C mRNA. Cystatin C mRNA was present in all trout tissues studied, but showed variation in steady-state level (Figure 2.3). The highest concentrations of cystatin C mRNA were found in trout brain and fertilized eggs (25 days old). The size of trout cystatin C mRNA was about 750 bp in all positive samples. A mRNA signal in whole zebrafish mRNA was not detected (Figure 2.3), and cystatin C mRNA also was not detected in Northern blots of zebrafish embryo cell cultures

```
Trout            GLIGGPMDANMNDQGTRDALQFAVVEHNKKTNDMFVRQVA   40
Salmon           ****************************************
Chicken     SEDRSR*L*A*VPVDE**E*LQR*****MA*Y*RAS**KYSSR*V
Mouse C   ATPKQGPRML*A*EE*DA*EE*V*R**D***S*Y**GS**AYHSRAI
Rat C     GTSRPPPR*L*A*QE*DASEE*VQR**D***S*Y**GS**AYHSRAI
Human C   SSPGKPPR*V******SVEEE*V*R**D***G*Y**AS***YHSRAL

Trout            KVVNAQKQVVSGMKYIFTVQMGRTPCRK GGVEKVCSVHKD  80
Salmon           **************************** *****I******
Chicken          R*IS*KR*L***I***LQ*ER***T*P*SS*DLQS*EF*DE
Mouse C          Q**R*R**L*A*VN*FLD*E****T*T*SQTNLTD*PF*DQ
Rat C            Q**R*R**L*A*IN*YLD*E****T*T*SQTNLTN*PF*DQ
Human C          Q**R*R**I*A*VN*FLD*EL***T*T*TQPNLDN*PF*DQ

Trout            PQMAVPYKCTFEVWSIPWMSDIQMVKNQCES           111
Salmon           ********************G*K********
Chicken          *E**KYTT***V*Y****LNQ*KLLESK*Q
Mouse C          *HLMRKAL*S*QIY*V**KGTHSLT*FS*NA
Rat C            *HLMRKAL*S*QIY*V**KGTHTLT*SS*NA
Human C          *HLKRKAF*S*QIYAV*SQGTMTLS*ST*QDA
```

Figure 2.2. Comparison of the deduced amino acid sequence of rainbow trout cystatin with those of chum salmon, chicken, mouse, rat and human family II cystatins.
*Represents residues identical to those of trout cystatin C.

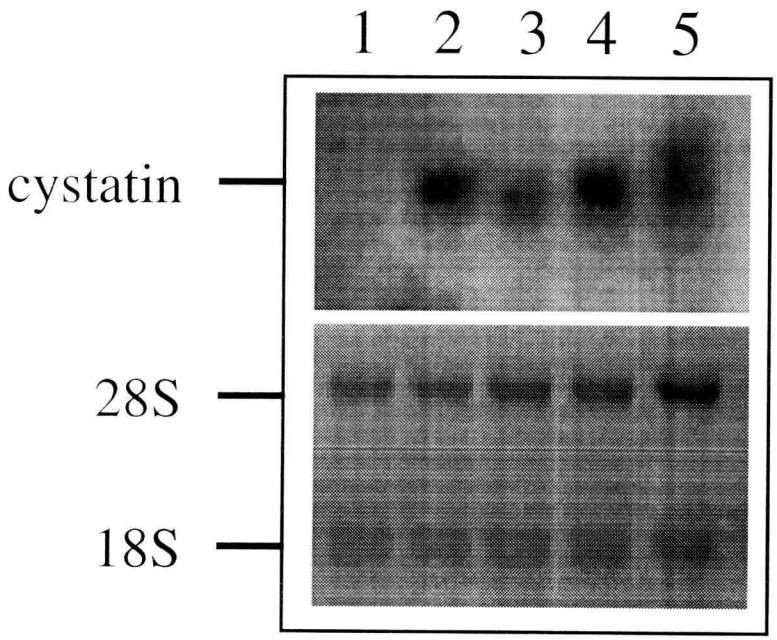

Figure 2.3. Northern blot analysis of trout cystatin C mRNA expression in trout tissues. Total RNA (10 µg) from whole zebrafish (lane 1); trout liver (lane 2), trout muscle (lane 3); trout brain (lane 4) and 25 day-old eggs (lane 5) was hybridized with radiolabeled trout cystatin C cDNA.

using labeled trout cystatin cDNA for hybridization (data not shown here).

Cystatin C mRNA levels were different among trout liver-derived cell lines and cell cultures (Figure 2.4). Two primary cell cultures derived from different tumors from the same aflatoxin-treated fish showed quantitative differences in cystatin C mRNA levels. The trout hepatoma-derived RTH-149 cell line and one of two tumor primary cultures showed high levels of cystatin C mRNA, comparable to or greater than mRNA levels in normal trout liver. However, the normal trout liver-derived RTL-W1 cell line did not express cystatin C mRNA.

Gene for trout cystatin C and sequence relationships among fish species

Genomic DNA of rainbow trout was digested with EcoRI and HindIII and transferred onto a nitrocellulose membrane that was hybridized with ^{32}P-labeled cDNA. EcoRI and HindIII digestion showed a single major band, although minor bands were detected in the HindIII digestion (Figure 2.5). From these results, we suggest that the copy number of the trout cystatin gene is probably one per haploid genome.

Figure 2.6 shows a comparison of cystatin C nucleic acid sequence of trout and salmon with cystatin C nucleic acid sequences of zebrafish and carp, the only other fish

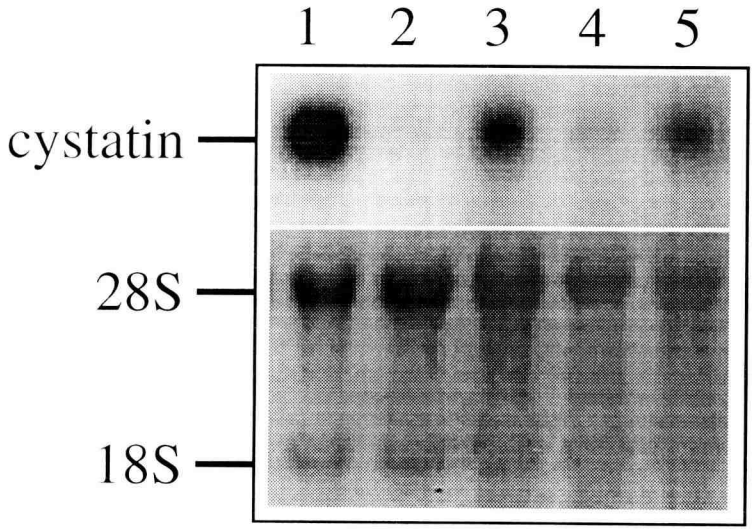

Figure 2.4. Cystatin expression in trout liver-derived cell cultures. Twenty μg total RNA of each sample was loaded for the Northern blot. Lanes are: 1). RTH-149; 2). RTL-W1; 3). primary cell culture from tumor 1; 4). primary cell culture from tumor 2. 5). normal liver. Radiolabeled trout cystatin cDNA was used as a probe for hybridization. Ribosomal RNA stained with methylene blue is shown below.

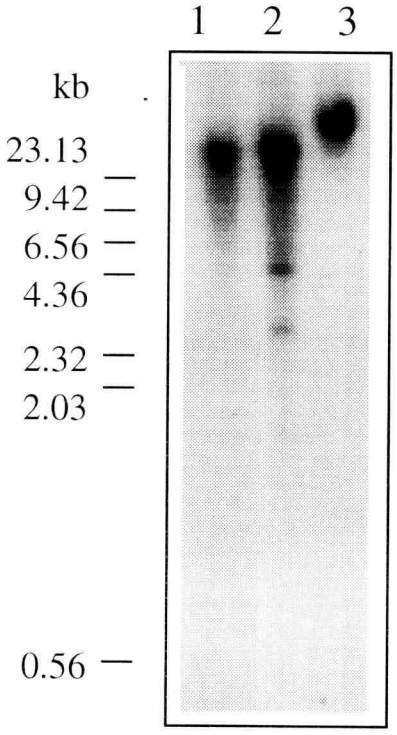

Figure 2.5. Southern blot analysis of rainbow trout genomic DNA for cystatin C-related sequences. Total DNA (20 μg) was digested with EcoRI (lane 1), HindIII (lane 2), or was undigested (lane 3). The blot was hybridized with radiolabeled trout cystatin C cDNA. HindIII-digested lambda DNA is shown as molecular marker.

```
trout       GCCAAGGTTG TCAATGCACA GAAGCAGGTG GTATCTGGGA TGAAGTACAT
salmon      ********** ********** ***A****** ********** **********
zebrafish   T****A**CA C***GCTC** A*****A**T **TG****** *T**A*****
carp        T****A**AA ****G*TT** AC*A*AA**T *CCG****C* ****A*****

trout       CTTCACAGTG CAGATGGGCA GGACCCCATG CAGGAAGGGA GGTGTTGAGA
salmon      ********** ********** ********** ********** **********
zebrafish   ******C*** G*TG***C** *A***A*T** ***A*****T **A******G
carp        ******T*** A******AAG TAG**T*C** **AA*****T **A***A***

trout       AGGTCTGCTC CGTGCACAAG GACCCACAGA TGGCTGTGCC CTACAAGTGC
salmon      **A******* *********A *****G**** ********** **********
zebrafish   **C*G**TG* TA*T**TG** A*****G*A* *A**ACA*GT *A*GG*A***
carp        CCA*G**TG* ***T*CGA** A*T**CAGT* *T*AACA*GT *ATTC*****

trout       ACCTTCGAGG TGTGGAGCCG CCC.CTGGAT GAGCGATATC CAGATGGTCA
salmon      ********** ********** ***.****** ********** **********
zebrafish   *AAA*A*T** *C****C*AA G**TA***GA **ATTT***T A**G*CACTG
carp        *AAA*AAC** *C*******A G**.A***T* A*A*TCCT*G A*AG*CACTG

trout       AGAACCAGTG TGAAAGTTAA G......... ...ACCCAGT GAAGAGAACT
salmon      ********** ********** *CCCTTAGTT AAG******* *G********
zebrafish   *A***TCC** CCTG...**G AG...ATGCT CTGTTGT*** TTGAGCTTT*
carp        *A***ACC** CATG...**G AGCTTGTGAC GAGTGCAT** TGGGGTGTT*

trout       TC.AATCAAT GTCTAGTCTA CCCAATAACT ACTATTAT
salmon      **C******* ********** ********** ********
zebrafish   CGAC*CTCTA AGAGCAATAT A*TC...CT* *A****GC
carp        TAAC**TCAA GCAGCAATA* AATTTA.CT* GC*G**GG
```

Figure 2.6. Nucleotide sequences for published portions of fish cystatin cDNA aligned by the CLUSTALW network program on a HP UNIX workstation. Nucleotide residues identical to those of trout cystatin C are represented as "*". Alignment gaps are represented as ".".

species for which sequences have been published (Tsai et al., 1996; Gong et al., 1997). Greater identity was seen between zebrafish and carp than between the salmonids and either zebrafish or carp, consistent with the evolutionary relationships among these fish species. Sequence identity between zebrafish and trout is 52%, while identity between zebrafish and carp cystatin is 65%. Mouse cystatin cDNA shows 44% identity with trout cystatin cDNA.

Discussion

This chapter describes the isolation of the cDNA for trout cystatin C from a trout liver cDNA library. The deduced mature trout cystatin C sequence consists of 111 amino acid residues and has strong identity with family II cystatins. We also identified a region coding for a putative signal sequence in trout cystatin C cDNA, suggesting that this protein exists predominantly in the extracellular space, as do most members of the cystatin family II. A greater sequence identity of trout cystatin C was seen with salmon and chicken cystatins than with mouse, rat and human cystatins; this is reasonable based on the phylogenetic distances among the vertebrates. X-ray crystallography of chicken cystatin has suggested that the conserved amino acid sequences that exist between trout

cystatin C and that of chicken and other species at Gly(4), Gln-X-Val-X-Gly(48-52) and Ile(Val)-Pro-Trp(96-98) are probably essential for the interaction between chicken cystatin and papain (Machleidt et al., 1993).

Cystatin C has inhibitory activity against lysosomal cysteine proteinases such as cathepsin B, H, and L (Barrett, 1987; Turk and Bode, 1993). Proteinases and their inhibitors are found in elevated levels in areas of the body with high protein turnover (Cole et al., 1989). The rainbow trout cystatin C was expressed in all tissues examined, but at various levels in different tissues (Figure 2.3). Differences in cystatin C expression level among tissues may reflect the regulation of cysteine proteinase activities that occurs during growth and morphological changes (Colella et al., 1989). The strongest signal was seen in developing eggs, and high cystatin C activity may play a part in embryogenesis and regulation of early embryonic growth (Yamashita and Konagaya, 1991). The precise function of this protein is not well known, and further work on regulation and expression of cystatin may help to define its role in trout.

Although small functional portions of the cystatin C molecule are evolutionarily conserved among a variety of species, the genomic sequences in the majority of the coding region show considerable divergence. This is consistent with

our failure to detect either trout cystatin C mRNA in Northern blots using mouse cystatin C sequences as a probe or zebrafish cystatin C mRNA in Northern blots using trout cystatin C sequences as a probe. In these circumstances, isolation of a useful trout cystatin C probe required use of polymerase chain reaction with degenerate primers derived from the evolutionarily conserved regions of the gene. Southern blot analysis of trout DNA using trout cystatin cDNA as a probe suggested a single copy of the cystatin C gene per haploid trout genome. The salmonid genome is characterized by tetraploidy at many loci, and detection of a single cystatin C gene suggests that the gene has either remained highly conserved in multiple copies in this species, or that superfluous copies have been deleted or diverged markedly since the tetraploidization occurred.

No cystatin C mRNA was detected in the RTL-W1 (Lee et al., 1993) cell line, although cystatin C mRNA was easily detected in mRNA from trout liver tissue. It is possible that RTL-W1 cells have lost some characteristics of liver tissue, but cystatin C mRNA was strongly expressed in the hepatoma-derived (Lannan et al., 1984) RTH-149 cell line. In addition, two tumor primary cell cultures expressed cystatin mRNA. These results raise the possibility that the RTH and RTL-W1 cell lines may have been derived from different cell types within the liver. They also suggest that expression of

this proteinase inhibitor may be important to the low metastatic behavior of hepatic tumors in the trout model.

A potential value may exist for the use of cystatin C in the seafood industry to control rapid texture softening of fish muscle as a result of proteolysis by cysteine proteinases. In surimi production, proteolytic degradation of muscle protein historically has been controlled through the use of crude preparations such as bovine plasma protein, egg white or potato powder as proteinase inhibitors (Morrissey et al., 1993; Seymour et al., 1994). However, these preparations also have undesirable effects (Akazawa et al., 1993; Morrissey et al., 1993; Porter et al., 1993; Reppond and Babbitt, 1993). Recombinant trout cystatin C may provide an alternative inhibitor of the proteinase associated with surimi production.

Acknowledgment

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CHAPTER 3

Rainbow Trout Cystatin C: Expression in *E. Coli* and *Saccharomyces cerevisiae*

Abstract

Trout cystatin C has certain biological properties that are significant to the surimi industry. To study the relationship between protein structure and function of fish cystatin C with the aim of exploring the possibility of its commercial value in the surimi industry, we established yeast and bacterial systems for heterologous expression of cloned trout cystatin C cDNA. Trout cystatin C was successfully synthesized in yeast and bacterial cells, but not secreted into yeast media even with trout signal and yeast invertase signal sequences. Cystatin expressed in cytoplasm of yeast or bacterial cells does not have inhibitory activity. Expression level was 3.0 - 5.0 mg/l in bacterial cells, but was relatively low in yeast cells. Recombinant trout cystatin C from bacterial cells purified by Ni-NTA chromatography gained inhibitory activity after refolding by dialysis against refolding buffer. Only about 20% of recombinant cystatin C folded to assume the correct tertiary structure necessary for inhibitory activity.

Introduction

The cystatin superfamily comprises a group of proteinase inhibitors which are widely distributed in animal

tissues and which form tight and reversible complexes with cysteine proteinases such as cathepsins B, H, L, and S (Nicklin and Barrett, 1984; Freije et al., 1993). Fish cystatin has also been detected in many tissues. Particularly high levels of cystatin have been found in developed eggs and abnormal tissues. The cystatins are most likely involved in embryogenesis and the regulation of early embryonic growth, in the regulation of pathological processes, and in protection against microorganisms (Freije et al., 1993; Yamashita and Konagaya, 1991; Li et at., 1998).

Many cystatins have been cloned and studied including human cystatin C (Abrahamson et al., 1987), human cystatin S (Isemura et al., 1986), chicken cystatin (Colella et al., 1989), mouse cystatin C (Solem et al., 1990) and rat cystatin (Cole et al., 1989). Recently fish cystatins have been cloned from rainbow trout (Li et al., 1995), chum salmon (Yamashita et al., 1996), carp (Tsai et al., 1996) and zebrafish (Gong et al., 1997). Amino acid sequences among mammalian cystatins are very divergent (Koide and Noso, 1994). The homologies between fish cystatins are also low except between trout and salmon, representing closely related species (Li et al., 1998). Therefore, studies on all cystatins may help to understand cystatin evolution.

In order to study structure and function of cystatins, protein must be produced and purified. Since most cystatin cDNAs are available, recombinant cystatin could be produced in bacterial, yeast, or mammalian expression systems. Human cystatin C (Abrahamson et al., 1988; Dalboge et al., 1989), rat cystatin C (Hakansson et al., 1996), mouse cystatin C (Hakansson et al., 1996) and chicken cystatin C (Auerswald et al., 1989; Auerswald et al., 1991) have been expressed successfully in the *E. coli* system. However, no information on expression of fish cystatin is available. This paper describes the construction of yeast and bacterial expression vectors and trout cystatin C expression in both systems. Although fish mRNA has been detected in yeast using yeast expression vectors and fish cystatin was synthesized in yeast cells, no recombinant cystatin C was secreted into yeast media using cystatin or yeast invertase signal sequence. Recombinant fish cystatin has also been successfully expressed in *E. coli* cells. No inhibitory activity of recombinant cystatin from yeast and bacterial cells was found, perhaps because the cystatin did not form disulfide bonds and assume the correct tertiary structure. After recombinant cystatin C refolding, the protein displayed inhibitory activity against papain.

Materials and Methods

Materials

Oligonucleotides were synthesized on an Applied Biosystems 392 DNA synthesizer from Applied Biosystem (Foster City, CA). DNA polymerase and other reagents for polymerase chain reaction (PCR) amplification of DNA were from the AmpliTag kit from Perkin-Elmer Cetus (Norwalk, CT). Restriction endonucleases and DNA-modifying enzymes were purchased from Strategene (La Jolla, CA). Papain, mersalyl acid (2-[3-(hydroxymercuri)-2-methoxypropyl]carbamoylphenoxy -acetic acid), fast Garnet GBC base (4-amino-2,3-dimethylazobenzene), polyxyethylene 23 lauryl ester (Brij 35, 30% w/v), IPTG (isopropyl-β-D-thiogalactoside), antibiotics and amino acids were obtained from Sigma (St. Louis, MO). Yeast extract and peptone were from Gibco (Grand Island, NY).

Strains and Plasmids

Escherichia coli DH5α (*rec A⁻, F⁻, endA1, gyrA96, thi-1, hsdR17,SUP44, relA1*) was used for plasmid transformation and preparation. Yeast strain INVSC2 (MATα, his3-D200, ura3-167) (Invitrogen, Carlsbad, CA) was used as the host for synthesis of trout cystatin C. Yeast expression vector pYES2 (Invitrogen, Carlsbad, CA) contains Gal1 promoter,

Ura^+ and Amp^+ selective makers, and yeast 2 μm and *E. coli* origins of replication. The Gal1 promoter is inducible with galactose. Bacterial strain BL21 (F^- omp^T[lon] hsdSB with DE3, a λ prophage carrying the T7 RNA polymerase gene) was purchased from New England Biolabs (Cambridge, MA). Bacterial expression vector pRSETB (Invitrogen, Carlsbad, CA) contains T7 promoter, 6xHis tag for metal binding and Amp^+ selective maker.

Construction of a *S. cerevisiae* Expression Vector

Since the yeast cells permitted considerable flexibility in the use of the secretion signal sequence (Kaiser et al., 1987) and were capable of recognizing and processing the secretion of trout cystatin, two different designs for synthesis of trout cystatin C in yeast were established as follows:

(1). Construction of recombinant plasmid for expressing trout cystatin C in yeast with trout cystatin secretion signal sequence. The full length trout cystatin C cDNA in pGEM-3z was isolated by EcoRI digestion and then inserted into pYES2 downstream from the Gal1 promoter. The orientation of the insert in pYES2 was determined by restriction endonuclease analysis. The expression plasmid was named pYTCST (Figure 3.1).

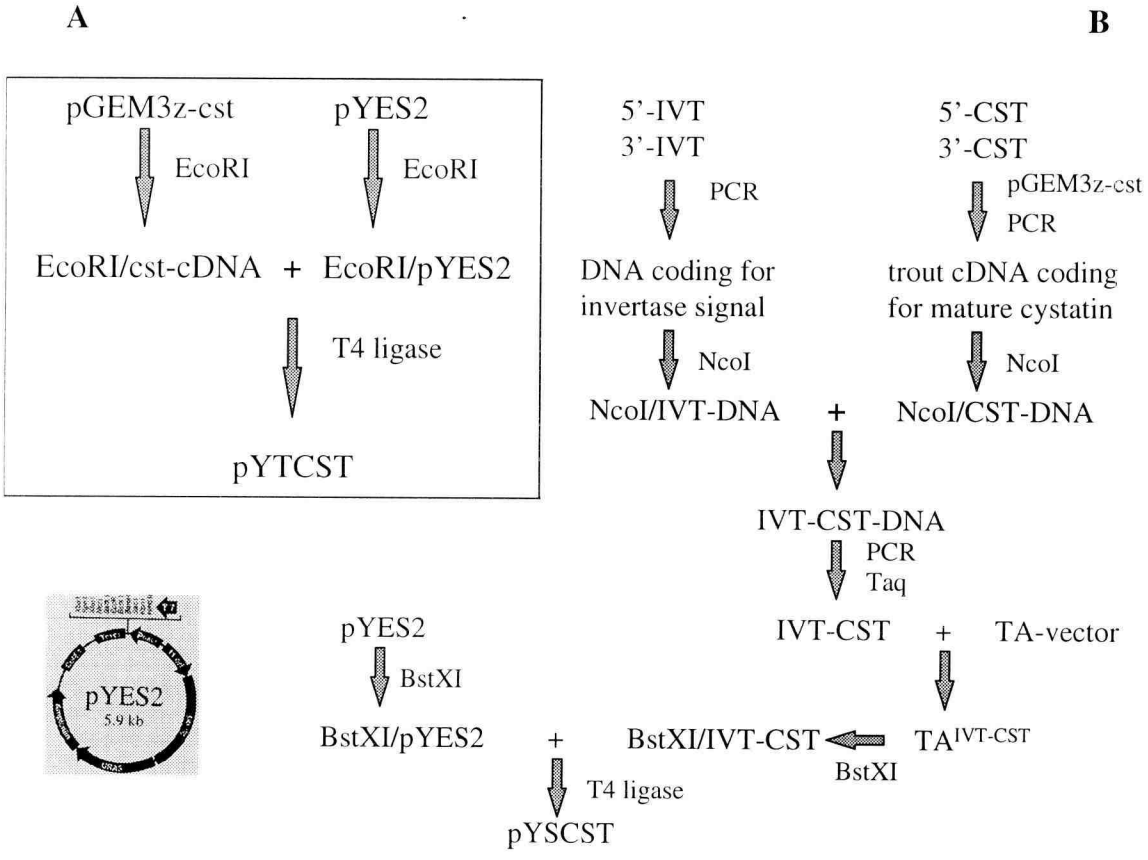


Figure 3.1. Construction of yeast plasmids for expression of recombinant trout cystatin C. (A). Construction of plasmid pYTCST for expression of trout cystatin C in yeast with trout cystatin secretion signal sequence. (B). Construction of plasmid pYSCST for expression of trout cystatin C in yeast with yeast invertase secretion signal sequence.

(2). Construction of recombinant plasmid for expressing trout cystatin C in yeast with yeast invertase secretion signal sequence. Two synthesized oligonucleotide primers were used to make the invertase signal coding region. 5'-IVT added with EcoRI site is: CCGAATTCATGATGCTTTTGCAAGCTTTCCTT -TTCCTTTT. 3'-IVT added with NcoI site is: CATGCCATGGCAGATA TTTTGGCTGCAAAACCAGCCAAAAGGAAAAGG. The invertase signal coding region made by two primers with *Taq* polymerase was digested with NcoI. PCR was used to obtain trout cystatin C cDNA which excluded the leader sequence. This cDNA was also digested with NcoI. The invertase signal coding region was ligated with trout cystatin C cDNA. Then the hybridized DNA was amplified by PCR and cloned into a TA cloning vector (Invitrogen, Carlsbad, CA). TA cloning vector with hybrid DNA was purified and digested with BstXI. Expression vector pYES2 was opened with BstXI digestion. Finally, the BstXI-digested hybrid DNA and linearized pYES2 were mixed and ligated with T4 DNA ligase. The recircularized plasmid was named pYSCST (Figure 3.1). DNA sequencing confirmed that no mutations were present.

Growth Media and Conditions for Yeast

E. coli DH5α was grown in LB medium (1% trypton, 0.5% yeast extract, 1% NaCl, pH 7.5). Ampicillin (100 μg/ml) was added for selective pressure to screen and maintain plasmid-

containing bacterial colonies. YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose, pH 7.0) was used for yeast INVSC2 growth and transformation. Yeast selective (Ura^+) minimal medium contained 0.67% yeast nitrogen base without amino acids and ammonium sulfate, 2% dextrose, 0.002% histidine (for minimal plates, plus 2% agar). For expression of trout cystatin C in yeast, the cells were inoculated in a 2 ml selective minimal medium and grown for 2 days at 30°C on a shaker with a speed of 250 rpm. Then cell culture was transferred to 100 ml selective minimal medium in a 250 ml flask, and the same incubation conditions were continued until OD_{600} reached 1.00. Yeast cells were isolated by centrifugation and transferred to fresh medium with 2% galactose in place of glucose for induction. After 4 hours, the broth and yeast cells were separated by centrifuging the culture at 3000 x *g*.

Yeast Transformation

Yeast INVSC2 cells were transformed with the recombinant plasmid pYTCST and pYSCST carrying the trout cystatin C cDNA using the lithium acetate method (Ito et al., 1983; Becker and Lundblad, 1994). The transformed yeast cells were plated onto selective minimal medium plates to select Ura^+ colonies. The plasmid DNAs of colonies growing

on the selective plates were isolated and confirmed by restriction endonucleases analysis.

Total RNA from Yeast Cells

Ten milliliter of yeast cells grew until OD_{600} reached 1.0 and were centrifuged at 1500 x g. The yeast pellet was washed with 1 ml ice-cold water and centrifuged again. The washed yeast cells were resuspended in 400 µl TES (10 mM Tris.Cl, pH 7.5, 10 mM EDTA, 0.5% SDS) and 400 µl acid phenol and vortexed vigorously for 10 sec. The solution was incubated at 65°C for 45 min, placed on ice for 5 min, and then centrifuged at 4°C for 5 min. The aqueous phase was collected (Collart and Oliviero, 1994). The following steps are the regular steps for a phenol extraction method described in detail by Collart and Oliviero (1994).

Northern Blot to Detect Trout Cystatin C mRNA in Yeast Cells

Ten microgram of total yeast RNA was denatured and electrophoresed in a formaldehyde-containing agarose gel. The procedure is described in chapter 2.

Protein Extraction from Yeast Cells

Yeast cells were grown according to the method described above and centrifuged. The cell pellet was suspended in three volumes of glass bead disruption buffer

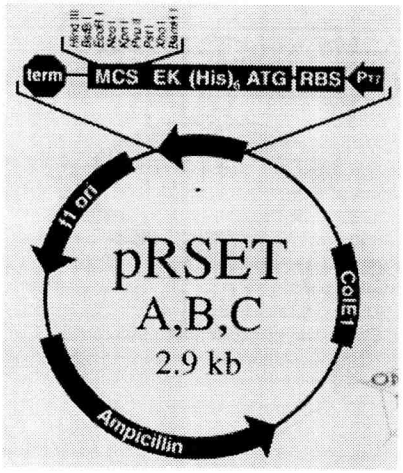
(20 mM Tris.Cl, pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 1 mM DTT, 0.3 M ammonium sulfate). Four volumes of chilled, acid-washed glass beads (0.45 to 0.55 mm) were added to the suspension. The suspension was vortexed at maximum speed for 30 to 60 seconds at 4°C and placed on ice for 1 to 2 min; this was repeated 3 to 5 times. Cell breakage was inspected under a microscope. The mixture was briefly centrifuged and the supernatant was collected for SDS/polyacrylamide gel electrophoresis (SDS-PAGE) analysis and inhibitory assay (Dunn and Wobbe, 1994).

Construction of an *E. coli* expression Vector

Vector pRSETB was opened with NcoI and Hind III digestion. Trout cystatin C cDNA (excluding the leader sequence) was isolated from pGEM-3z digested with NcoI and Hind III. The linearized pRSETB and cystatin cDNA were mixed and ligated with T4 DNA ligase. The insert was confirmed by size and restriction endonuclease analysis. The expression plasmid was named pRCST (Figure 3.2).

Growth Media and conditions for *E. coli*

For expression of trout cystatin C in *E. coli* cells, the cells were inoculated in a 10 ml LB broth with 100 µg/ml ampicillin and grown overnight at 37°C on a shaker with a speed of 250 rpm. The cell culture was transferred to a 1000



pGEM3z-cst

NcoI
Hind III

NcoI-XbaI/CST

pRSET-B

NcoI
Hind III

NcoI-XbaI/pRSET-B

\+

T4 ligase

pRCST

Figure 3.2. Construction of bacterial plasmid for expression of recombinant trout cystatin C.

ml fresh LB broth with the same concentration of ampicillin as above in a 2.5 liter flask, and the same incubating conditions continued until OD_{600} reached 0.8. A final concentration of 1 mM IPTG was added into the bacterial culture to induce the expression of cystatin C for 4 hours. The bacterial cells were collected for protein purification by centrifuging the culture at 4000 x g for 10 min.

Cystatin C Purification from *E. coli* Cells

Denaturing conditions were chosen to purify the 6xHis-tagged proteins (The QIAexpressionist, 1992). The bacterial cells were suspended in Buffer A (6 M GuHCl, 0.1 M Sodium phosphate, 0.01 M Tris/HCl, pH 8.0) at 5 ml per gram wet cells and stirred for 1 hour at room temperature. The supernatant was collected by centrifuging lysate at 10,000 x g for 15 min at 4°C and loaded on a Ni-NTA column previously equilibrated in Buffer A at a flow rate of 10 - 15 ml/hr. At first, the column was washed with 10 column volumes of Buffer A, followed by Buffer B (8 M urea, 0.1 M Sodium phosphate, 0.01 M Tris/HCl, pH 8.0) until the A_{280} of the flow-through was less than 0.01, and finally with Buffer C (8 M urea, 0.1 M Sodium phosphate, 0.01 M Tris/HCl, pH 6.3) until the A_{280} was less than 0.01 again. Then, the recombinant protein was eluted by 30 ml Buffer D (8 M urea, 0.1 M Sodium phosphate, 0.01 M Tris/HCl, pH 5.9), followed

by 30 ml Buffer E (8 M urea, 0.1 M Sodium phosphate, 0.01 M Tris/HCl, pH 4.5). Three milliliter fractions were collected from the elution and analyzed by SDS-PAGE. The fractions with target protein were pooled and refolded in a folding buffer (see below) to recover inhibitory activity. Recombinant cystatin C in the refolding solution was re-purified with a new Ni-NTA column under non-denaturing conditions. The salt concentration in refolding solution was adjusted to 50 mM Sodium phosphate and 100 mM NaCl and pH 8.0. The treated solution was loaded twice on the new Ni-NTA column previously equilibrated in Buffer F (50 mM Sodium phosphate, 100 mM NaCl, 10% glycerol, pH 8.0). The column was washed with 20 column volumes of Buffer F until the A_{280} was less than 0.01. and followed with Buffer G (50 mM Sodium phosphate, 100 mM NaCl, 10% glycerol, pH 6.0) until the flow-through A_{280} was less than 0.01 again. Finally, the protein was eluted with 30 ml gradient of 0 - 0.5 M imidazole in Buffer G. One milliliter fractions were collected and analyzed on SDS-PAGE. Inhibitory activity of those fractions against papain was measured.

Recombinant cystatin C refolding

Recombinant cystatin C purified after the first column was dialyzed against 1 liter folding buffer (1 M urea, 0.05 M Tris/HCl, 0.005% Tween - 80, 1 mM reduced glutathione,

0.015 mM oxidized glutathione, pH 8.0) at 4°C for 30 hours. Afterwards, a equal volume of water was added to the folding buffer dialysis continued overnight. How recombinant cystatin C folded was determined by measuring inhibitory activity against papain.

Inhibitory Activity Assay against Papain

The inhibitory activity against papain as a model for cysteine proteinase was measured according to the method of Barrett and Kirschre (1981) and Izquierdo-Pulido et al. (1994) using a-N-benzoyl-DL-arginine-2-naphthylaminde (BANA) as the synthetic substrate (Barrett, 1972). Papain stock solution (4.5 μg/μl) was prepared in water and diluted with 4% Brij 35 solution to a working concentration of papain at 45 ng/μl. A solution containing 10 μl of papain (45 ng/μl) and 25 μl of recombinant cystatin C was added to 300 μl of 100 mM phosphate buffer (pH 6.0) containing 1.33 mM EDTA and 2.7 mM cysteine. The mixture was preincubated for 5 min at 40°C to activate papain and allow interaction between papain and cystatin C, and 10 μl of 40 mg/ml BANA in dimethylsulfoxide was added and vortexed to start the reaction at 40°C for 10 min. The reaction was terminated by adding 400 μl of a mixture of Fast Garnet GBC base (4-amino-2,3-dimethylazobenzene), sodium nitrite, and Mersalyl acid.

After allowing 10 min for color development, the residual papain activity was measured by A_{520} due to a red azo dye produced by β-naphthylamide liberated and coupled with Fast Garnet. A reagent blank was prepared by adding the enzyme after color development and a positive control was carried out by adding inhibitor buffer in place of inhibitor.

Results

Trout Cystatin C Expressed in Yeast Cells

1. High Level of cystatin C mRNA in yeast cells

Total RNA was extracted from yeast cells carrying pYES2, pYTCST or pYSCST with galactose induction or without galactose induction and used for Northern blot. Radiolabeled trout cystatin C cDNA was used to detect cystatin mRNA transcribed in yeast cells. Cystatin C mRNA levels were significantly different between yeast cells with different expression vectors, and after induction or non-induction by galactose (Figure 3.3). No cystatin C mRNA was detected in yeast cells containing pYES2 vector alone with or without induction. Yeast cells containing pYTCST and pYSCST had much higher level of cystatin C mRNA with galactose induction than without induction. Galactose induced the transcription

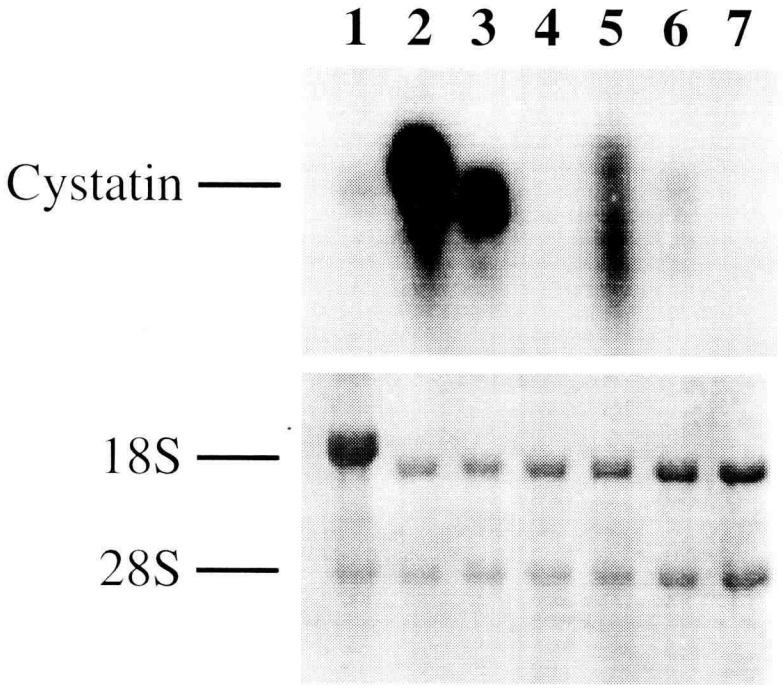


Figure 3.3. Northern blot analysis of trout cystatin C mRNA expression in trout liver and yeast cells. Twenty μg total RNA of trout liver and 10 μg total RNA of each sample from yeast cells transformed with different expression vectors induced or not induced by galactose were loaded on each lanes: 1). Trout liver; 2). Yeast cells with pYTCST induced by galactose; 3). Yeast cells with pYSCST induced by galactose; 4). Yeast cells with pYES2 induced by galactose; 5). Yeast cells containing pYTCST without galactose induction; 6). Yeast cells containing pYSCST without galactose induction; 7). Yeast cells containing pYES2 without galactose induction. Radiolabeled trout cystatin cDNA was used as a probe for hybridization. Ribosomal RNA stained with methylene blue is shown below.

of cystatin C under the Gal1 promoter of the expression vector. The size of cystatin c mRNA is different with pYTCST and pYSCST, because of the different size of cystatin c cDNA insert in the expression vectors. Since 5′ and 3′ untranslated regions have been excluded from the cystatin C insert in pYSCST, the size of cystatin C mRNA from pYSCST is smaller than that from pYTCST in which cystatin C insert still includes 5′and 3′ untranslated regions.

2. Cystatin C expressed in cytoplasm of yeast

Trout cystatin has been expressed in yeast cells containing pYTCST and pYSCST after galactose induction (Figure 3.4). However, this cystatin did not show inhibitory activity against papain using the inhibitory assay and papain staining method (Garcia-Carreno et al., 1993; data not shown here), most likely because cystatin expressed in the cytoplasm of yeast cells did not fold properly to assume a correct structure. Based on the property of expression vectors, cystatin should be secreted into the broth. But no cystatin was detected from concentrated broth on SDS-PAGE and no inhibitory activity of yeast broth against papain was observed.

Trout Cystatin C Expression in *E. coli* cells

1. Cystatin C production in cytoplasm of bacterial cells

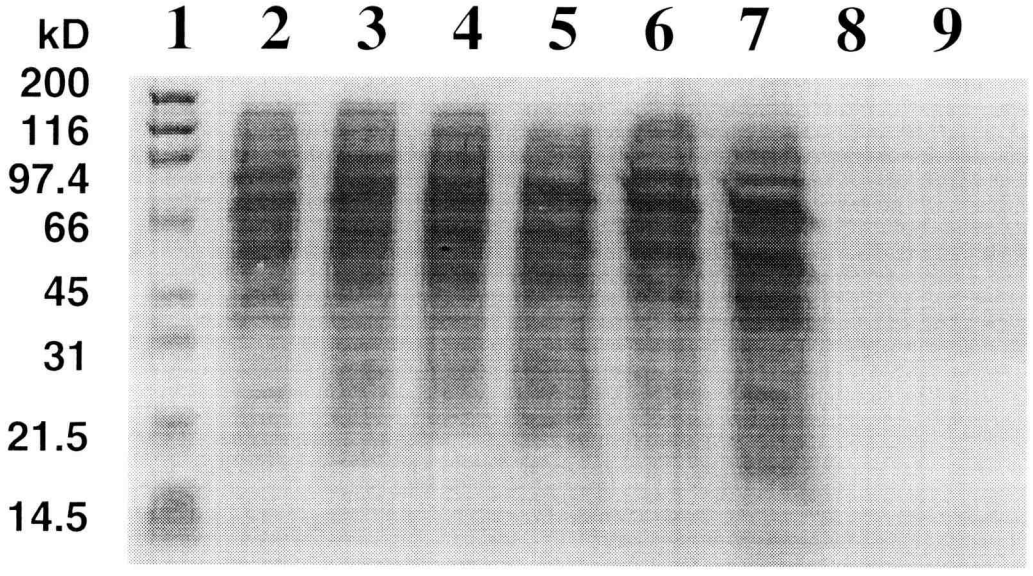


Figure 3.4. SDS-PAGE analysis for proteins extracted from yeast cells containing different expression vectors with or without galactose induction. Ten μg total protein from yeast cell extractions or 10 μl concentrated culture broth were loaded on each lane. Lanes are: 1). Broad range protein molecular Marker; 2). Yeast cells containing pYES2 without galactose induction; 3). Yeast cells containing pYES2 with galactose induction; 4). Yeast cells containing pYTCST without galactose induction; 5). Yeast cells containing pYTCST with galactose induction; 6). Yeast cells containing pYSCST without galactose induction; 7). Yeast cells containing PYSCST with galactose induction; 8). Concentrated yeast broth from yeast containing pYTCST with galactose induction; 9). Concentrated yeast broth from yeast containing pYSCST with galactose induction.

Crude protein extracts were prepared from bacterial cells carrying pRCST. Target protein was briefly purified on Ni-NTA resin and analyzed by SDS-PAGE (Figure 3.5). Two major bands were observed on SDS gel. One band represents monomers of recombinant cystatin and the other is cystatin dimer. The size of cystatin monomers should be about 15 kD, but migrated at about 18 kD because most proteins with 6xHis tags run more slowly on SDS gels than the equivalent untagged proteins. The recombinant cystatin includes 40 amino acids related to protein expression, purification and characterization, but not to inhibitory activity. As no secretion signal amino acid sequence is attached at the 5' region of the recombinant cystatin, no recombinant cystatin was found in the culture broth. No inhibitory activity of recombinant cystatin was observed.

2. Recombinant cystatin C purification and recovery of inhibitory activity of recombinant cystatin C

high level of proteins can be expressed in bacterial cells and protein-containing inclusion bodies may be formed in the cytoplasm. It might be expected that cystatin extracted from inclusion bodies of bacterial cells will not have inhibitory activity. Refolding of the protein may recover inhibitory activity. Therefore, denaturing conditions were used to extract recombinant cystatin from bacterial cells. The extract was passed through a Ni-NTA

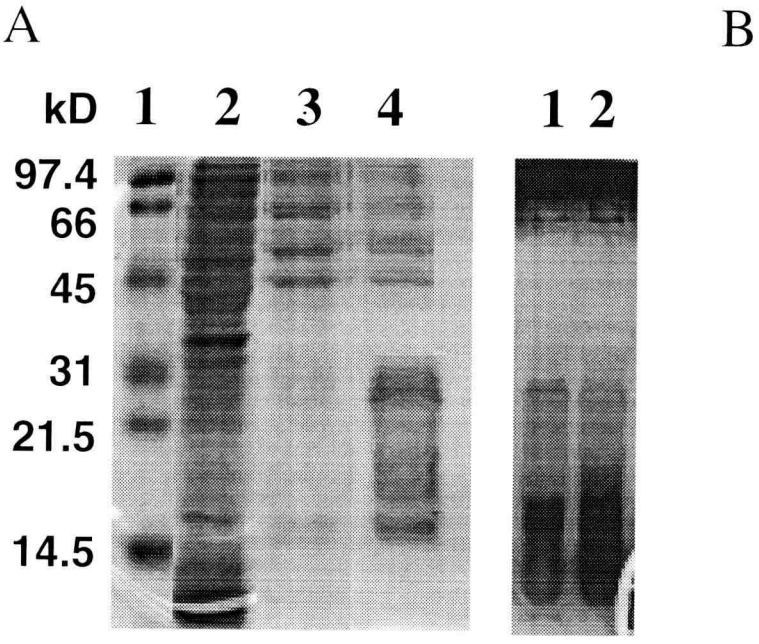


Figure 3.5. SDS-PAGE gel and silver staining gel for analysis of recombinant cystatin C. (A). SDS-PAGE analysis of recombinant cystatin C before and after brief purification on Ni-NTA resins. Twenty μg of protein was loaded on each lane. Lanes are: 1). Low molecular weight marker; 2). Total protein from bacterial cells with IPTG induction; 3). Purified protein from bacterial cells without IPTG induction; 4). Purified protein from bacterial cells with IPTG induction. (B). Silver Staining SDS-PAGE for analysis of purified and refolded recombinant cystatin C. Ten μg of protein from different fraction was loaded on each lane.

column, and the elution profile is shown in Figure 3.6. Proteins from each fraction were analyzed on SDS-PAGE and the positive fractions were pooled. Since denaturing conditions were used to purify the recombinant protein, no inhibitory activity against papain was found in any fraction.

In order to recover the inhibitory activity, recombinant cystatin was dialyzed against protein folding buffer. As the salt concentration in the protein solution gradually reduced, recombinant cystatin formed disulfide bonds as protein folding progressed and inhibitory activity gradually increased. After dialysis, the protein solution was applied to another Ni-NTA column under non-denaturing conditions for re-purification of recombinant cystatin. The elution profile is shown in Figure 3.7. The peak containing recombinant cystatin was sharp. The fractions with inhibitory activity were analyzed on SDS-PAGE and major protein was the cystatin monomer (Figure 3.5B).

Discussion

Yeast *Saccharomyces cerevisiae* has been successfully used in producing foreign proteins including hepatitis B virus surface antigen (Valenzuela et al., 1982), interferon (Hitzeman et al., 1983), human erythropoietin (Elliott et

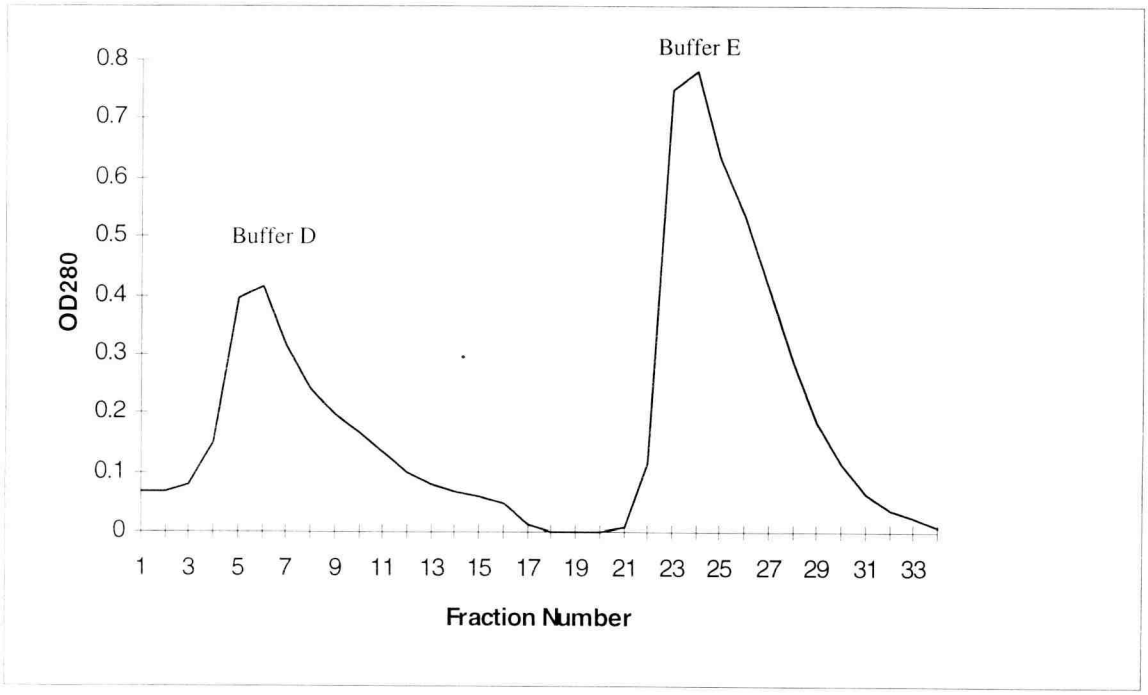


Figure 3.6. Elution profiles of Ni-NTA chromatography for total bacterial protein. The first fraction peak eluted by buffer D contained most recombinant cystatin C monomers. The second fraction peak eluted by buffer E contained most recombinant cystatin C dimers.

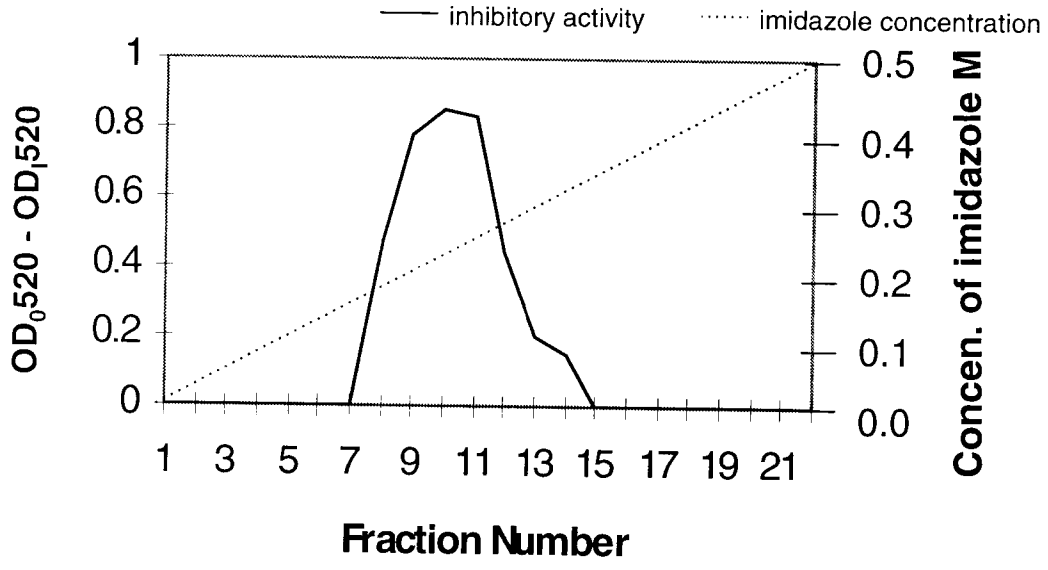


Figure 3.7. Elution profiles of Ni-NTA chromatography for refolded recombinant cystatin C after first column. The refolded cystatin was eluted with an imidazole gradient concentration. Only one peak of eluted protein was strongly inhibitory against papain.

al., 1989), and many other kinds of proteins. Since trout cystatin C is a secreted protein, we used yeast as a host for expression of cloned trout cystatin C cDNA with the aim of establishing a suitable expression system for production of high levels of trout cystatin C and studies of relationship between the structure and function of cystatin. Two recombinant plasmids for producing trout cystatin C in yeast were constructed, pYTCST and pYSCST. Although yeast cells containing both types of expression vectors successfully synthesized cystatin C, no cystatin was secreted into the yeast medium. This implies that yeast cells may not be able to secrete trout cystatin C or could secrete only very low levels of cystatin below the level of detection of the inhibitory assay.

Northern blot of yeast total RNA indicated that no cystatin mRNA was detected in yeast cells with the control pYES2 vector, so the host does not have a similar gene to trout cystatin C. A high level of cystatin mRNA was transcribed upon galactose induction in yeast cells containing pYTCST or pYSCST. However, cystatin protein did not appear on SDS-PAGE (Figure 3.4). Cystatin mRNA in yeast cells may not be stable and may be degraded by the host very rapidly. Also, cystatin C may be toxic to yeast cells. Further experiments are needed to obtain more detailed information.

Since a low level of cystatin C was expressed with yeast expression systems and no efficient method could be used to purify the recombinant cystatin C from the yeast, *E. coli* system was chosen as an alternative way to express trout cystatin C. Many proteins including human cystatin (Abrahamson et al., 1988) and chicken cystatin (Auerswald et al., 1989) have been successfully expressed at high level in bacterial cells. The 6xHis tag of expression plasmid pRCST attached to the recombinant protein helps purification of the target protein. The expression vector was transformed into BL21 host cells. In this system the T7 polymerase which is induced by IPTG binds T7 promoter to transcribe the recombinant cystatin C mRNA. Recombinant cystatin was synthesized at a relatively high level, about 3.0 - 5.0 mg/l, but the percentage of cystatin C in total protein is dependent on growth and induction conditions.

The purified recombinant cystatin C does not have inhibitory activity even under non-denaturing conditions, because the correct disulfide bonds do not form in the cytoplasm of the bacterial cells. In order to recover the inhibitory activity, recombinant cystatin C must refold in low salt buffer containing reduced/oxidized glutathione to facilitate disulfide bond formation and exchange. Protein refolding must take place slowly and in dilute solution to avoid the formation of insoluble aggregates, and should be

carried out at a redox potential which is close to the equilibrium of Cys-SH and Cys-S-S-Cys. Disulfide bridges then only become stabilized if they are trapped in a strong and correct tertiary structure. Refolding is generally carried out by step-wise dilution of denaturants in a dialysis procedure. Zinc finger proteins have been successfully refolded following all of the above steps (Jaenicke and Rudolph, 1991). When purified recombinant cystatin C in 8 M urea buffer was dialyzed against 1 M urea buffer for 30 hours, 20% of total recombinant cystatin refolded to gain inhibitory activity by titration with papain (see mehtods in the next chapter). This result is close to those reported for recombinant chicken cystatin C (Auerswald et., 1989).

Because of the potential value of trout cystatin C as a food additive to inhibit cysteine proteinases during surimi processing (Morrissey et al., 1993; Seymour et al., 1994), one of our aims in expression of cloned trout cystatin C cDNA in yeast or bacteria is to explore the possibility of producing large quantities of active trout cystatin C using yeast or bacterial fermentation. Neither the yeast or bacterial system in this study has not produced sufficient levels of cystatin C for industrial production. Further investigation is necessary to increase expression and secretion levels of trout cystatin C.

Although the present yeast or bacterial system has not been optimized for synthesis of trout cystatin, the successful expression of trout cystatin C in the bacterial system provides a suitable tool for systematic study of the relationship between structure and function of fish cystatin by means of site-directed mutagenesis, and the characterization of fish cystatin.

Acknowledgment

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CHAPTER 4

Enzymological Studies on Recombinant Trout Cystatin C Produced from *Escherichia coli*

Abstract

Recombinant cystatin C expressed in *E. coli* gained inhibitory activity after refolding. The properties of recombinant trout cystatin C are silimar to natural human cystatin C and chicken egg-white cystatin C, as well as recombinant rat and mouse cystatin C. $K_{i(app)}$ of recombinant cystatin C for papain is 0.22 nM. K_i is 1.2×10^{-6} nM, indicating that recombinant cystatin C bound very tightly with papain. Although the recombinant trout cystatin C contained an amino acid leader sequence not related to cystatin, the inhibitory activity was not affected by this sequence. Recombinant cystatin C titration by papain showed only 20% of the total protein as active cystatin. Changing refolding conditions may increase the percentage of active cystatin.

Introduction

Cystatins comprise a superfamily of sequentially homologous proteins, which form equimolar, tight and reversible complexes with papain-like cysteine proteinase. Chicken cystatin is the member of the cystatin family which has been most thoroughly characterized, since it can be purified in reasonably large amounts. The reaction of

chicken cystatin with papain has been studied in some detail as a model for other reactions between cystatins and cysteine proteinases. Many studies showed that the inhibition of papain by chicken cystatin is best described as a simple, reversible bimolecular reaction, leading to formation of an inhibitor-proteinase complex with a dissociation equilibrium constant of around 60 fM (Bjork et al., 1989). This has been used as a model for other mammalian cystatin enzymological studies.

With the development of protein expression systems, many mammalian cystatins have been successfully expressed in bacterial cells using expression vectors. Sufficient protein can be produced with bacterial or other expression systems. Enzymological studies for many cystatins such as human cystatin C (Abrahamson et al., 1988), human cystatin D (Freije, et al., 1993), mouse cystatin C and rat cystatin C (Hakansson et al., 1996) have been carried out. Inhibitory activity of all recombinant cystatins for papain is the same as that of chicken cystatin (Auerswald et al., 1991). The properties of those cystatins expressed in bacterial cells remains the same as that of the native protein.

This paper describes the characterization of recombinant trout cystatin C expressed in bacterial cells. Although recombinant cystatin C contained T7 leader sequences and other amino acid sequences, it has full

inhibitory activity after refolding. The $K_{i(app)}$ of the recombinant cystatin C for papain is 0.22 nM. K_i is 1 fM, similar to human cystatin (Lindahl et al., 1992) and chicken cystatin (Nicklin and Barrett, 1984).

Materials and Methods

Material

L-*trans*-Epoxysuccinyl-leucylamido(4-guanidino)butane (E-64), papain (EC3.4.22.2), 2-[3-(hydroxymercuri)-2-methoxypropyl]carbamoylphenoxyacetic acid (mersalyl acid), fast Garnet GBC base (4-amino-2,3-dimethylazobenzene), polyxyethylene 23 lauryl ester (Brij 35, 30% w/v), α-N-benzoyl-DL-arginine-2-naphthylaminde (BANA), dithiothreitol (DTT), dimethylsulfoxide(DMSO),benzyloxycarbonylphenylalanyl-arginylaminomethylcoumarin (Z-Phe-Arg-NMec) and aminomethylcoumarin were purchased from Sigma (St. Louis, MO). Uvikon 810/820 spectrophotometer is from Kontron spectrophotometer (Redwood City, CA). Perkin-Elmer LS-3 spectrofluorimeter is from Perkin-Elmer (Norwalk, CT).

Reagents for papain activity

Buffer/activator: 88 mM KH_2PO_4-12 mM Na_2HPO_4-1.33 mM disodium EDTA (pH 6.0) contains freshly made 2.7 mM cysteine by addition of the free base.

Color reagent: Mersalyl-Brij solution was prepared with 2.43 g Mersalyl acid dissolved in 60 ml 0.5 M NaOH. The solution was made to about 450 ml with water and 0.3 g disodium EDTA was added into the solution. In order to dissolve the EDTA, the pH of the solution was adjusted to 4.0 by slow addition of 1 M HCl with stirring. Finally, the solution was made to 500 ml with water and another 500 ml of 4% (w/v) Brij 35 was added. The solution was stored at 4°C (Barrett and Kirschke, 1981).

The terminal reagent: Fast Garnet is preferably prepared freshly by diazotization of the base. The base stock solution was prepared with 225 mg 4-amino-2,3-dimethylazobenzene dissolved in 50 ml ethanol. This solution was added with 30 ml of 1 M HCl, diluted to 100 ml with water and stored at 4°C. One milliliter of base stock solution in a test tube was stood in a beaker of ice and water, mixed with 0.1 ml of 0.2 M $NaNO_2$ for 5 min, and diluted to 100 ml with Mersalyl-Brij reagent. The complete color reagent is stable at 4°C for 1 day (Barrett and Kirschke, 1981).

Measurement of inhibitory activity against papain

The detailed procedure for measuring the inhibitory activity of recombinant cystatin C against papain was described in chapter 3.

Active-site titration of papain with E-64

This was performed as described by Barrett & Kirschke (1981). Working solutions of E-64 at 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 μM concentration were prepared as required from a 1.0 mM stock solution.

To 300 μl of buffer/activator was added 16 μl of papain solution (250 ng/μl) and 50 μl of 0.0, 0.25, 0.5, 0.75, 1.00, 1.25, 1.5 μM E-64. The mixture was preincubated for 5 min at 40°C, and 10 μl of 40 mg/ml BANA in dimethyl sulfoxide wad added and vortexed to start the reaction. The mixture was incubated at 40°C precisely for 10 min for activity assays. Then 400 μl of the terminal reagent was added for the termination of reaction and color development. Residual papain activity was measured by the absorbance at 520 nm. The results were plotted against inhibitor concentration (Barrett and Kirschre, 1981; Izquierdo-Pulido et al., 1994).

Inhibitory-site titration of recombinant cystatin C with papain

Since the active papain concentration was determined by E-64 titration, the concentration of recombinant cystatin C with inhibitory activity could be measured by papain titration (Kos et a., 1986). To 300 μl of buffer/activator

was added 20 µl of papain solution (25 ng/µl) and 5, 10, 15, 20, 25, 30 µl of recombinant cystatin C. The volume of the mixture was made up to 400 µl with water. The solution was preincubated for 5 min at 40°C, and 10 µl of 40 mg/ml BANA in dimethyl sulfoxide was added and vortexed to start the reaction. The mixture was incubated at 40°C precisely for 10 min for activity assays. Then 400 µl of the terminal reagent was added for the termination of reaction and color development. Residual papain activity was measured by the absorbance at 520 nm. The results were plotted against the volume of recombinant cystatin C (Barrett and Kirschre, 1981; Izquierdo-Pulido et al., 1994).

Reagent for determination of K_i value

Substrate solution: Stock solution of Z-Phe-Arg-NMc was made by dissolution to 1.0 mM (6.49 mg/10 ml water) and stored at 4°C. The stock solutions were diluted to 200 µM with water before use (Abrahamson, 1994).

Assay buffer concentrate: 0.4 M sodium phosphate buffer, pH 6.0, containing 4 mM EDTA was made. Powdered dithiothreitol (DTT) was added to 4 mM before use.

Solution for dilutions: 0.01% (v/v) Brij 35 in water.

Solution for fluorimeter calibration: A stock solution of 0.25 mM aminomethylcoumarin dissolved in dimethyl

sulfoxide was kept at 4°C. The stock solution was diluted to 0.2 μM with water to obtain the calibration solution (Abrahamson, 1994).

Papain solution: Papain solution was diluted to approximately 5 nM. The exact enzyme concentration is not critical for the evaluation of results. However, the final enzyme concentration in the reaction solution should be around K_m (0.05 nM for papain) (Abrahamson, 1994).

Recombinant cystatin C: The exact concentration of active cystatin was made with water at 16, 24, 32, and 40 nM. The final working concentration in the reaction solution was 0.16, 0.24, 0.32, and 0.40 nM, which is around 5 times papain K_m (Allison and Purich, 1979)

Determination of K_i value for recombinant cystatin C against papain

Fluorimeter excitation and emission wavelengths were set to 360 and 460 nm, respectively. The fluorimeter was calibrated with 0.2 μM aminomethylcoumarin to let the maximal response correspond to 2% hydrolysis of the assay substrate. A mixture containing 2040 μl 0.01% Brij 35 solution, 750 μl assay buffer concentration, and 30 μl papain solution was incubated at 40°C for 5 min to activate papain. After that, the mixture was added into the fluorimeter cuvette and 150 μl of substrate solution was

added. When a steady-state rate of substrate hydrolysis (υ_o) was recorded, 30 µl of recombinant cystatin C solution was added and the progress curve was recorded until a new steady-state rate (υ_i) could be determined (Abrahamson, 1994).

The linear equation derived by Henderson for tight binding inhibitors was used to evaluate the results from experiments with different dilutions of the cystatin samples. The relative steady-state rates of substrate hydrolysis before and after addition of cystatin samples were then used to determine the apparent K_i value [$K_{i(app)}$] as the slope from the plot of $[I]/(1- \upsilon_i/ \upsilon_o)$ against υ_o/ υ_i. This value was then used to calculate the substrate independent K_i from the equation $K_{i(app)} = K_i (1 + [S]/K_m)$ (Nicklin and Barrett, 1984; Abrahamson, 1994).

Results

Active Papain Concentration Determined by E-64 Titration

E-64 at a concentration of 0.5 mM has no effect on serine proteinases and the metalloproteinases. However, E-64 at very low concentration can rapidly inactivate cysteine proteinases such as cathepsins B, H and L, and papain (Barrett et al., 1982). For papain, the rate of inactivation

by E-64 is high and the binding of papain and E-64 is between them is irreversible. The complex of E-64 and papain is in a 1:1 molar ratio.

A solution of papain (250 ng/μl) was treated with a series of increasing amounts of E-64 (see the Materials and Methods section), and assayed for activity. Papain activity declined almost linearly with increasing amount of E-64 (Figure 4.1). The intercept obscissa was taken to give the molar concentration of E-64 (1.3 μM) which can completely inactivate total papain in the reaction solution. Based on the E-64 concentration, we calculate the concentration of active papain to be 4.3 μM under these conditions.

Active Recombinant Cystatin C Concentration Determined by Papain Titration

The stoichiometry of complex formation between papain and chicken cystatin in a 1:1 ratio has been established by covalent cross-linking of the complex (Nicklin and Barrett, 1984). Cystatin C binds papain with a pM - fM range of K_i (Nicklin and Barrett, 1984; Abrahamson et al., 1988; Hakansson et al., 1996).

Papain solution was treated with increasing amounts of recombinant cystatin C and assayed for residual activity. The activity of papain declined almost linearly with

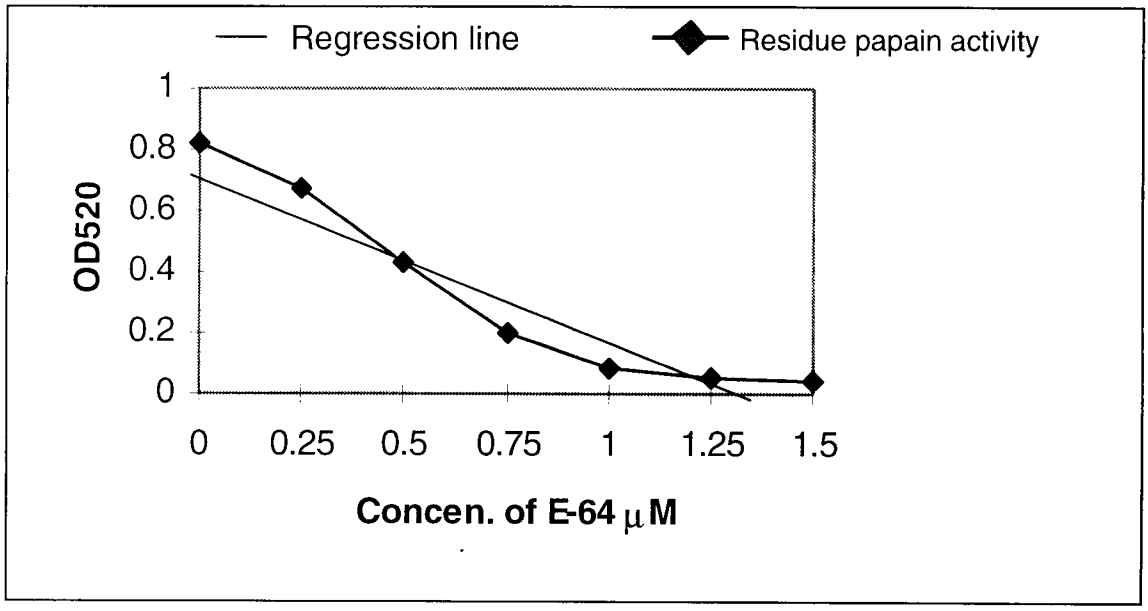

Figure 4.1. Titration of papain with E-64. For each point, 16 μl of papain solution (250 ng/μl) was allowed to react with 50 μl of 0, 0.25, 0.5 … 1.5 μM E-64. The residual papain activity was measured at 520 nm. From this plot it can be calculated that the enzyme solution had 4.3 μM of active papain.

increasing amount of recombinant cystatin C. A graph of the activity of residual papain versus the amount of recombinant cystatin C (Figure 4.2) allows determination the amount of cystatin C which can completely inactivate total papain in the reaction solution. Based on the amount of recombinant cystatin C, we calculate a concentration of 0.34 μM.

Determination of K_i of Papain and Recombinant Cystatin C

In order to determine the K_i of papain with recombinant cystatin C, the initial velocity of substrate hydrolysis by papain was measured before and after addition of cystatin. The initial velocity decreased after cystatin was added, with decreasing rate of initial velocity dependent on the amount of cystatin added in the reaction solution. When a greater amount of cystatin was added, the initial velocity decreased more rapidly (Figure 4.3).

Using the equation $\upsilon_o/\ \upsilon_I = 1 + [I]/K_{i(app)}$ (Nicklin and Barrett, 1984), we can plot $(\upsilon_o/\ \upsilon_I - 1)$ against [I]. The line passes through the origin with a gradient of $1/K_{i(app)}$ (Figure 4.4). From this plot $K_{i(app)}$ is 0.22 nM. From the equation $K_{i(app)} = K_i\ (1 + [S]/K_m)$, K_i can be calculated. K_i of the recombinant cystatin C was 1.2×10^{-6} nM which is in the same range as human cystatin C (Lindahl et al., 1992). Thus recombinant cystatin C has characteristics similar to

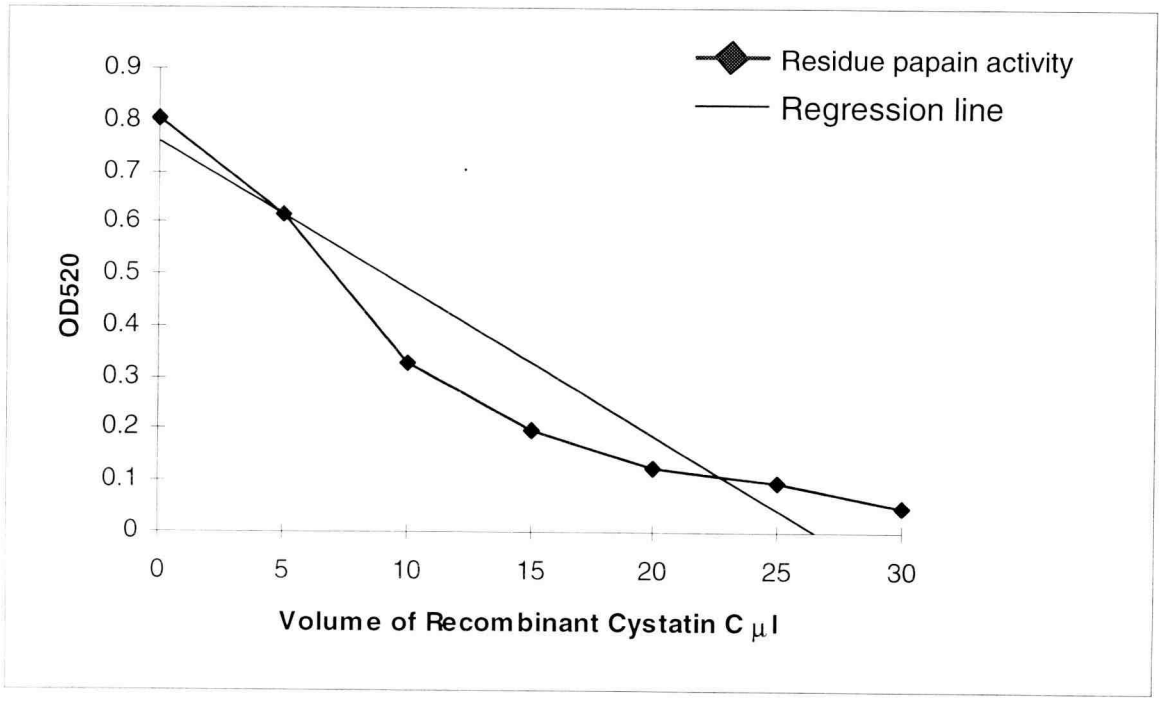

Figure 4.2. Titration of recombinant cystatin C with papain. For each point, 20 µl of papain solution (25 ng/µl) was allowed to react with 0 to 30 µl of recombinant cystatin C. From this plot it can be calculated that the inhibitor solution of the active recombinant trout cystatin C was 0.34 µM.

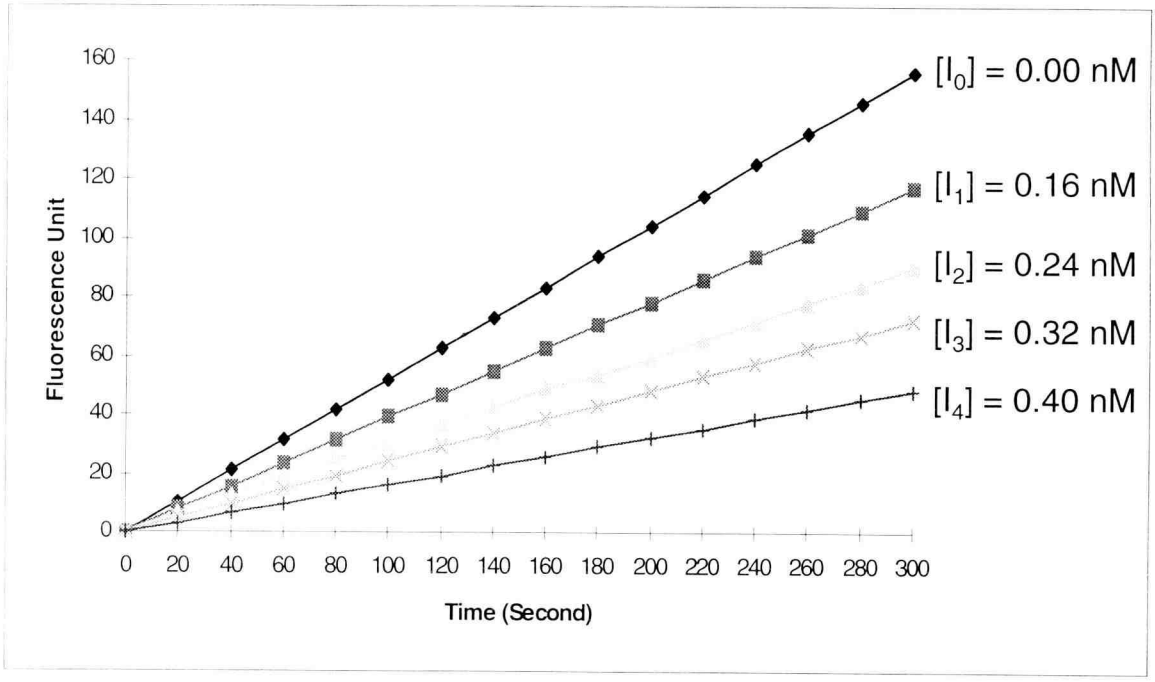

Figure 4.3. Plot of fluorescent product units versus reaction time for papain-catalyzed reaction. Different concentrations of recombinant cystatin C were allowed to react with 0.05 nM papain. The recombinant cystatin concentrations (top to bottom) are 0, 0.16, 0.24, 0.32, 0.40 nM, respectively.

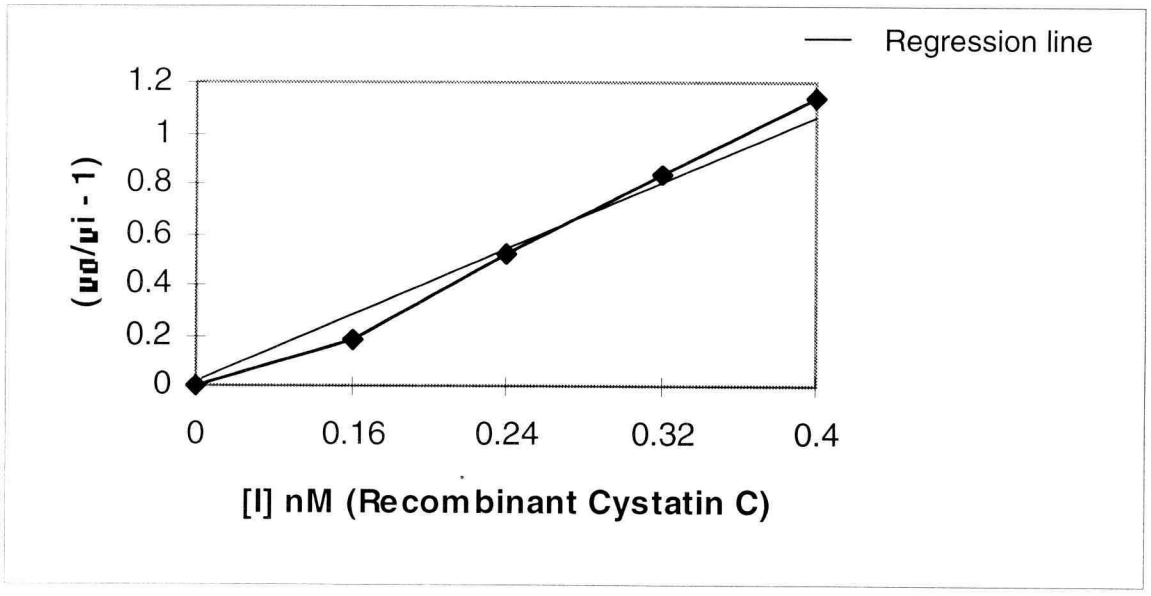

Figure 4.4. Plot of (υ_o/ υ_I - 1) versus [I] (recombinant cystatin C concentration). From this plot, it can be calculated that $K_{i(app)}$ is 0.22 nM.

natural cystatin, even though the recombinant cystatin C contained additional amino acid sequences at its N-terminus.

Discussion

For these studies of enzyme kinetics, the concentration of the active enzyme was determined. Papain from comercial sources may not be 100% active. E-64 is a class-specific inhibitor for the cysteine proteinases. E-64 irreversibly binds papain quickly in a 1:1 molar ratio. Therefore, E-64 is an ideal compound to be used for active-site titration of the papain-related cysteine proteinases (Barrett et la., 1982).

Recombinant trout cystatin C expressed from *E. coli* must be titrated because the cystatin does not demonstrate inhibitory activity before refolding. Estimation of the percentage of correct tertiary structure after protein refolding depends on the methods and conditions used. Since K_i values for cystatin C from variety of sources with papain have been found to be less than 5 pM, the concentration of free papain in equilibrium with excess cystatin should be negligible (Abrahamson et al., 1987). This expectation was confirmed by titration curves of papain with recombinant trout cystatin C (Figure 2; Barrett et al., 1982).

Since cystatin is a tightly-binding inhibitor of papain, K_i should be in the pM range. Based on the $K_{i(app)}$ and K_i of recombinant trout cystatin C with papain, we concluded that this cystatin has the same characteristics as human cystatin C and chicken cystatin C. Although additional amino acid sequences are contained at the N-terminus of recombinant trout cystatin C to facilitate cystatin expression and purification, they do not appear to affect inhibitory activity. However, trout cystatin C is a secretory protein which includes two disulfide bonds, and the protein expressed in bacterial cells does not have any inhibitory activity. Using dialysis methods to refold recombinant trout cystatin C, we converted 20% of the protein to a correct tertiary structure with inhibitory activity. Alternative methods may be explored to improve protein refolding. One such approach would be construction of an expression system which could secrete recombinant cystatin C.

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CHAPTER 5

Conclusion and Future Perspectives

Rainbow trout cystatin C cDNA has been isolated from trout liver. Analysis of this cDNA indicated high homology to other fish cystatin cDNAs. Translation of the cDNA predicted 132 amino acid residues. Amino acid sequence comparison demonstrated that 21 amino acids at the N-terminal end comprise a signal peptide that leads to cystatin secretion, and 111 amino acids are the mature cystatin. Four cysteine residues in trout cystatin probably form two intramolecular disulfide bonds indicating that this trout cystatin belongs to the cystatin family 2.

We have analyzed trout cystatin C expression in different tissues. The results indicated that trout cystatin C was expressed in all tissues examined, but at different levels. Although trout cystatin C expression behaves as a housekeeping gene, its expression may still be regulated in some tissues or embryonal developmental stages. An approach to address this question would be to isolate and sequence the entire trout cystatin C gene, using trout cystatin C cDNA as a probe. Analysis of the trout cystatin C promoter may suggest which regulatory factors may affect expression

of this gene. Deletion and mutation methods also might be pursued to dissect the regulatory function of the trout cystatin C promoter region.

Trout cystatin C was expressed highly in developed eggs and hepatic tumor cells or cell lines. This means trout cystatin C may influence fish embryogenesis and tumorigenesis. It has been shown that human cystatin C can inhibit proteolytically-dependent tumor metastasis (Sexton and Cox, 1997). However, The role played trout cystatin C in those functions remains unclear. Additional analysis of more tissues and cell samples would be required to address these questions.

Although trout cystatin C was expressed in the *E. coli* system, we did not obtain high yields of active protein. We also were not successful in producing secreted trout cystatin C using the yeast invertase signal sequence or the cystatin signal sequence. Changing growth conditions may increase cystatin C production, but yields must be greatly increased to meet the goal of the commercial utilization of trout cystatin for the sumiri industry. Further investigations into other expression systems and vectors are necessary to increase the expression levels and secretion level of trout cystatin C.

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APPENDIX

Appendix

Mouse Cystatin C Expression Regulated by Transforming Growth Factor Beta

Introduction

The cystatins are a group of potent cysteine-proteinase inhibitors. There are at least three distinct types in the cystatin superfamily, each type consisting of several proteins (Rawlings and Barrett, 1990). Cystatin C belongs to the family 2 cystatins and consists of 120 amino acids, with two intrachain disulfide bonds (Barrett et al., 1986).

The mouse cystatin C gene has been cloned, sequenced and located to chromosome 2 (Huh et al., 1995). This clone contains a 6.1 kb genomic DNA which includes 0.9 kb of 5'-flanking and 1.7 kb of 3'-flanking regions. The gene has three exons interrupted by two intron sequences. Several features of the putative promoter region are characteristic of so-called housekeeping genes: no TATA or CAAT boxes, GC-richness and two copies of a Sp1-binding motif, GGGCGG, which are present in the 5'-flanking region within 300 bp upstream from the initiation codon. However, in contrast to many housekeeping genes, the mouse cystatin C gene contains a hexa-nucleotide TGTTCT, which is a core sequence of the

androgen-responsive element (*ARE*), and a 21-nucleotide, 5'-AGACTAGCAGCTGACTGAAGC, which includes two potential binding sites for the transcription factor, AP-1, in the promoter region.

It has been shown that two adjacent AP-1-like binding sites act synergistically to confer inducibility beyond that observed for a single AP-1 consensus sequence (Friling et al., 1992). A strong induction expression of the cystatin C gene by TGF-β in serum free mouse embryo (SFME) cells has been reported (Solem et al., 1990). The presence of the two AP-1-like binding sites in the mouse cystatin C gene promoter indicates that transcription factor AP-1 may play a role in cystatin C gene expression. There is evidence that induction of gene expression by TGF-β is mediated by transcription factor AP-1 (Kim et al., 1990). This paper briefly describes the regulation of the mouse cystatin C gene by TGF-β to induce a reporter gene (luciferase) in order to explain how TGF-β induces mouse cystatin C gene expression in SFME cells.

Methods

SFME cell culture: The Balb/c serum-free mouse embryo (SFME) cell line was cultured in FD basal nutrient medium (a

1:1 mixture of Dulbeco's modified Eagle medium and Ham's F-12 medium) supplemented with penicillin (200 U/ml), streptomycin (200 μg/ml), ampicillin (25 μg/ml), sodium bicarbonate (1.2 g/L), HEPES (15 mM), bovine insulin (10 μg/ml), transferrin (25 μg/ml), murine epidermal growth factor (50 ng/ml), and human high density lipoprotein (10 μg/ml) in culture vessels precoated with bovine fibronectin. The detailed procedures for culture of SFME cells have been described in Loo et al. (1987, 1989a, 1989b).

Generation of the mouse cystatin C 5'-flanking region by PCR (Polymerase Chain Reaction): Two primers were designed based on the mouse cystatin C 5'-flanking region. 5'-primer including a NheI restriction site is GGGCTAGCAGATCTACCTACTTC. 3'-primer including a XhoI restriction site is GGCTCGAGAGCTCCAACAAGACT. Mouse genomic DNA isolated from mouse liver was used for amplification of cystatin C 5'-flanking region by *Taq* polymerase. The predicted size of the PCR product was 940 bp. The PCR product was directly ligated into the TA cloning vector (Invitrogen, Carlsbad, CA).

Construction of mouse cystatin C promoter vector for expression of reporter gene: the TA cloning vector with mouse cystatin C promoter insert was purified and digested with NheI and XhoI. The reporter vector pGL2-Enhancer (pGL2E) (Promega, Madison, WI) was opened with NheI and XhoI

digestion. Finally, the NheI/XhoI digested mouse cystatin C promoter DNA and linearized pGL2E were mixed and ligated with T4 DNA ligase. The recircularied plasmid was called pGL2MC (Figure A.1). DNA sequencing confirmed that no mutations were present in the cystatin C promoter region.

Transient transfection into SFME cells: Calcium phosphate-mediated transfection was carried out as described (Helmrich et al., 1988). Fifteen μg plasmid DNA in 50 mM $CaCl_2$ (pH 7.2) was sheared with a 25 gauge needle and dropped into a solution of 50 mM HEPES (n-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid) (pH 7.2), 0.25 M sodium chloride, 1.8 mM sodium phosphate (pH 7.2). Approximately 10^7 cells/75 cm^2 flask were transfected with the plasmid-containing solution. The cells were isolated and replated into 6 cm-diameter dishes, and the cells were treated with or without TGF-β (10 ng/ml) and grown for 11, 26, 52 and 73 hours. Finally, the cells were processed for a luciferase assay.

Luciferase Assay: The SFME cells were rinsed out with 2 ml phosphate-buffered saline and lysed by incubating for 15 min with 1 ml cell lysis buffer (25 mM Tris-HCl, pH 7.5, 10 mM EDTA, 15% sucrose, 2 mg/ml lysozyme). The cells then were scraped from the plates, and debris was removed by a brief centrifugation. Luciferase activity of the cell lysate was

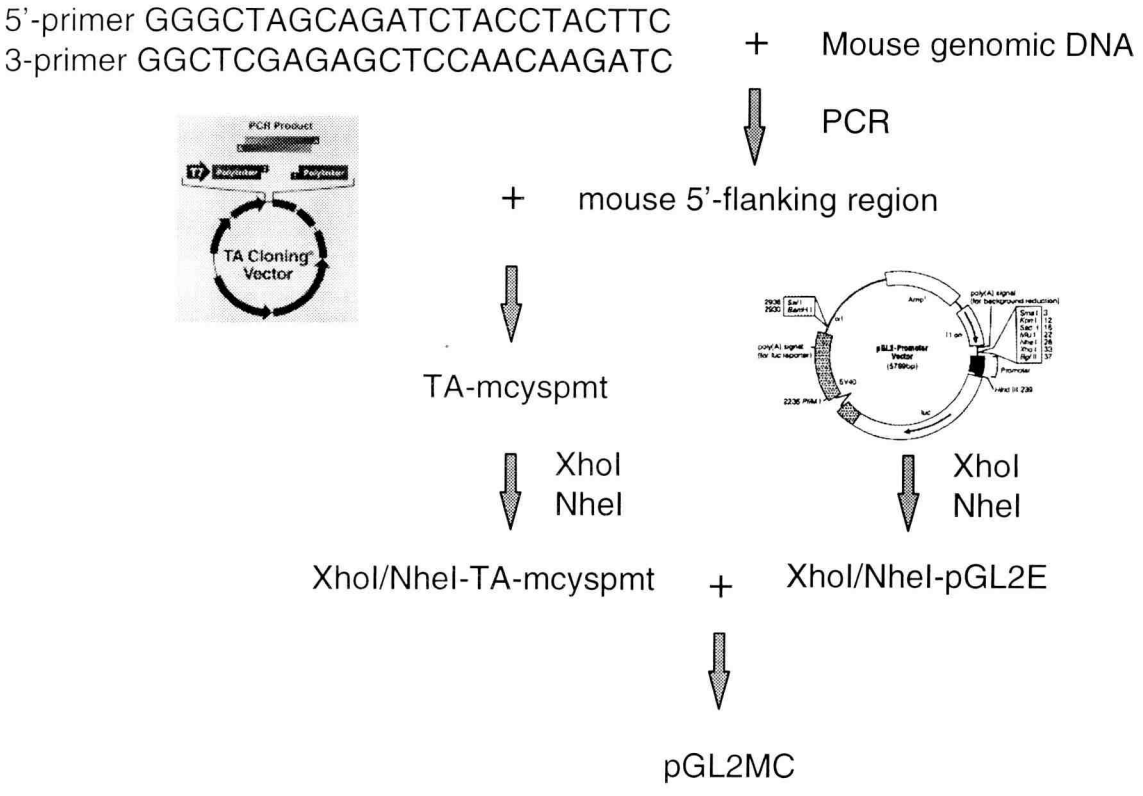

Figure A.1. Construction of plasmid for transient expression of luciferase regulated by mouse cystatin C promoter in SFME cells.

assessed in a LKB luminometer (model 1250) using a commercial assay kit (Enhanced Luciferase Assay Kit 1800K; Analytical Luminescence Laboratory; Ann Arbor, MI) performed according to the vendor's instructions.

Results

The expected size of the PCR product based on the length of mouse cystatin C genomic DNA sequence between the two primers is 940 bp. A prominent band of this size was seen on agarose gel after amplification of the mouse cystatin C 5'-flanking region by PCR (Figure A.2). This PCR product was ligated on TA cloning vector, and inserted into the pGL2E vector. DNA sequencing based on pGL2 primers confirmed that no mutations were present in the mouse cystatin C promoter region generated by PCR (data not shown here).

SFME cells were transfected with pGL2E or pGL2MC and grown with or without TGF-β for different time periods. Results of luciferase assay for transfected cells were shown in figure A.3. Luciferase activity was very low in pGL2E transfected cells, and no difference existed between TGF-β treatment and no TGF-β treatment. However, Luciferase activity was relatively high in pGL2MC cells treated with TGF-β after 26 hours transfection. Activity decreased at

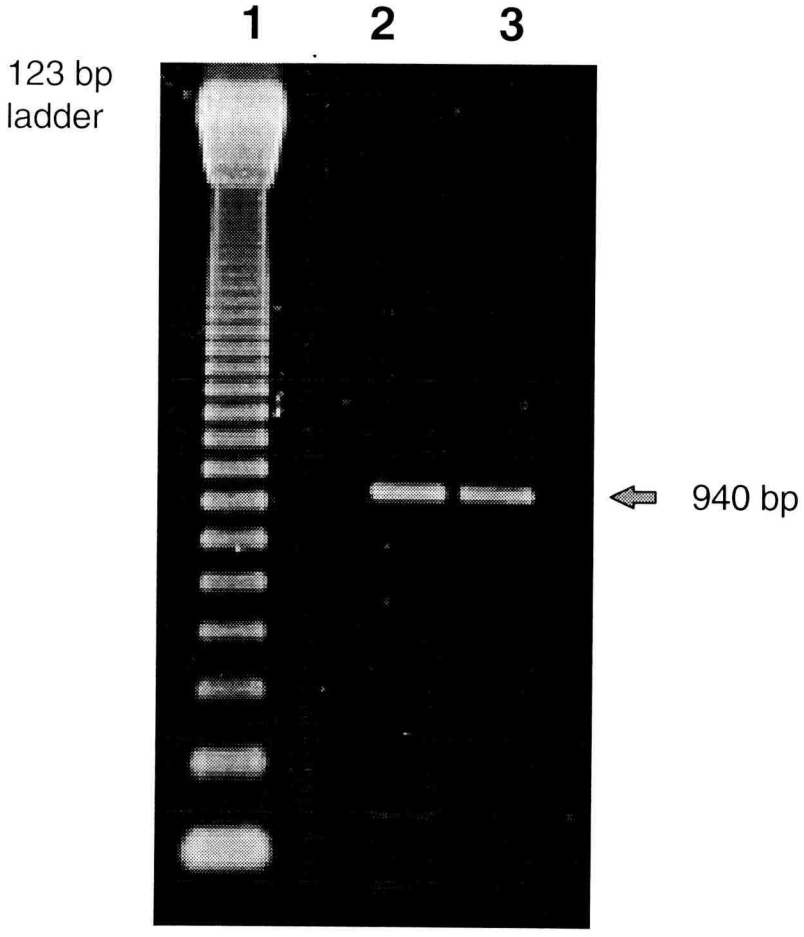

Figure A.2. Analysis of mouse cystatin C PCR products on 1% agarose gel. Lanes are: 1). 123 bp ladder for molecular marker; 2). Mouse cystatin C promoter DNA from 10 µl PCR reaction; 3). Mouse cystatin C promoter DNA from 5 µl PCR reaction.

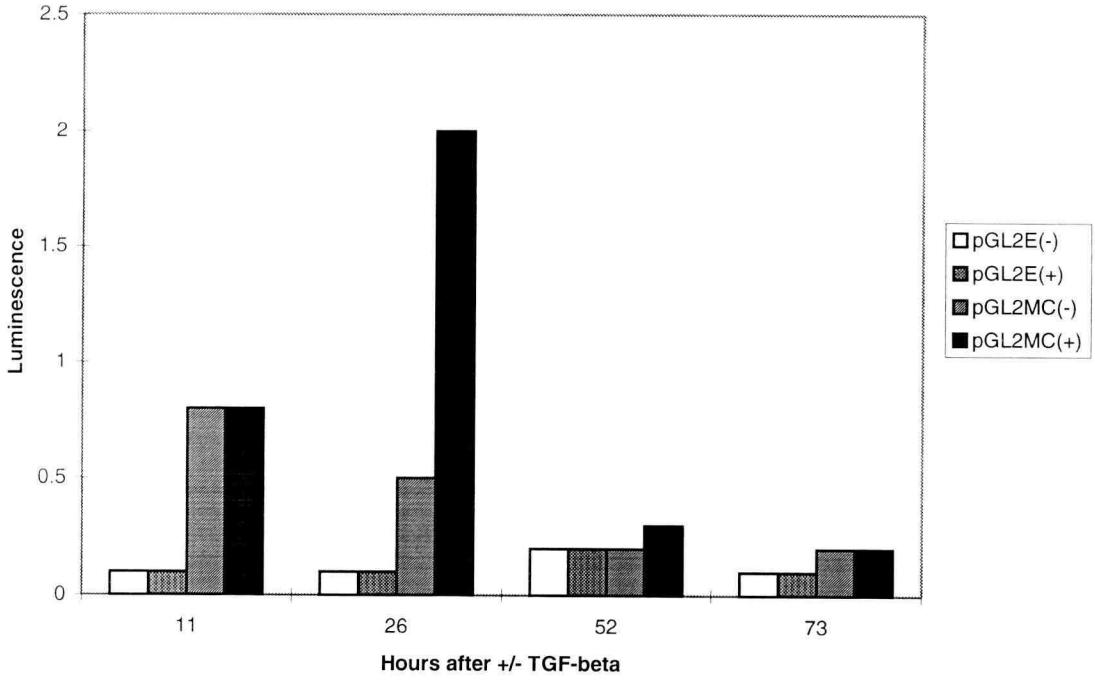

Figure A.3. Relative luciferase activities expressed in SFME cells from constructs with +/- TGF-β. pGL2E(-): pGL2E transfected cells without TGF-β treatment; pGL2E(+): pGL2E transfected cells with TGF-β treatment; pGL2MC(-): pGL2MC transfected cells without TGF-β treatment; pGL2MC(+): pGL2MC transfected cells with TGF-β treatment.

later times. Luciferase activity was low and not time-dependent in pGL2MC transfected cells without TGF-β treatment. These results indicate that TGF-β regulated the mouse cystatin C promoter to induce the transcription of luciferase reporter gene.

Discussion

Reporter gene transcription regulated by the mouse cystatin C 5'-flanking region was induced by TGF-β in pGL2MC transfected SFME cells. Analysis of the mouse cystatin C gene indicated that the 5'-flanking region contains two AP-1-like binding sequences which are immediately upstream from the first Sp1-binding site (Huh et al., 1995). The presence of the AP-1-like binding site in the mouse cystatin C promoter suggests that cystatin C induction by TGF-β may be mediated by the AP-1 complex. However, further experiments need to be done to support the hypothesis. One possible experiment is to delete the two AP-1-like binding sites in the mouse cystatin C gene promoter to evaluate the effect on the inducible response of the reporter gene by TGF-β. Another experiment is to check whether Fos and Jun expression are induced by TGF-β in SFME cells.

In recent years evidence has accumulated suggesting that transcriptional regulatory proteins such as Jun and Fos, the protein products of c-*jun* and c-*fos* protooncogenes, play a role in coupling extracellular signals to gene expression in the nucleus by interacting with AP-1 binding sites in target genes (Friling et al., 1992). The inducible transcription enhancer AP-1 binding site, observed in the promoter region of several genes, was shown to be the DNA binding site for Jun-Fos heterodimeric complexes (Chung et al., 1996). The rapid and transient induction of expression of Jun- and Fos-related proteins by a great variety of extracellular stimulatory agents enables these proteins to modulate their AP-1 DNA binding activities and to mediate specific alternations in gene transcription in response to environmental stimuli (Curran and Franza, 1988). Our hypothesis for mouse cystatin C expression induced by TGF-β is that TGF-β induces Fos and Jun expression, and Fos and Jun bind AP-1-like sites in the mouse cystatin C promoter to induce cystatin C expression.

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