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Effect of Hen's Age on the Level of Cystatin in the Chicken Egg White

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Abstract: Cystatins, protein-type cysteine proteinase inhibitors are widely distributed in animals and plants. It is generally assumed that these inhibitors are involved in the regulation of physiological and pathological processes caused by cysteine proteinases Cystatins. The study aimed to isolate and characterize the cystatin from various chicken in different ages between 20-80 weeks. We used acid treatment, affinity chromatography Sepharose-Papain-4B, and desalting G-25 column, followed gel chromatography G-100 column. We obtained that the chicken in age 50 weeks have a higher activity against papain and the yield of cystatin among the chicken in ages 20, 30, 40, 60, 65, 70 and 80 weeks. The purified cystatin was shown to be homogenous by polyacrylamide gel electrophoreses (SDS-PAGE) The molecular weight of first and second fractions were determined to be 40 000 and 13 000, respectively. The low molecular fraction was strongly inhibited papain (K_i = 0.0012 nM) and cathepsin B (K_i=2.554 nM).

Key words: chicken egg white, cystatin, hens age

Introduction

Cystatin, an inhibitor of sulphydryl protienases, was the first isolated from egg white by Fossum and Whitaker (1968). Cysteine proteinases are ultimately regulated by endogenous cysteine proteinase inhibitors, also named cystatins (Turk and Bode, 1991). Cystatin superfamily inhibitors have been subdivided into three families, the intracellular type lacking a signal peptide (Type I, cystatin A and B), commonly termed stefins, the abundant secreted, extracellular inhibitor, cystatin C (Type II), and the circulating kininogens (Type III), and non-inhibitory proteins, such as human histidine-rich glycoprotein and α2HS-glycoprotein (Rawling and Barrett, 1990). Members of the cystatin family are slightly larger than the stefins and contain 150 amino acid residues with molecular weight about 13000. They are nonglycosylated, single chain proteins, having two intermolecular disulphide bridges (Turk and Bode, 1991). The family consists mainly of variant species of cystatin C (Bezin *et al*., 1984), cystatin S and its variants (Isemura *et al*., 1987) and also cystatin D (Freije *et al*., 1991). A novel human cystatin gene was identified in a differential display comparison, aimed at the isolation of transcriptionally regulated genes involved in invasion and metastasis of breast cancer. It is named cystatin M, with 40% homology to human family II cystatins and similar overall structure (Sotiropoulou *et al*., 1997). Human cystatin C and its avian analogue chicken cystatin are most investigated members of the family II. Cystatin C is abundant in various tissues and body fluids. The highest levels have been determined in cerebrospinal fluid, seminal plasma (Lindahi *et al*., 1992). Quail cystatin, a new cysteine proteinase inhibitor of the cystatin superfamily, was purified from egg albumen of Japanese quail *Coturnix corturnix japonica*. It showed 90% sequence identity with chicken cystatin (Gerhartz *et al*., 1997). Two different cysteine proteinase inhibitors (Forms I and II) were isolated from Chum Salmon egg, and their molecular weights were found to be 16000 and 11000, respectively. They can be classified into new group of the cystatin superfamily (Mashita and Konagaya, 1991). Also the cystatin was isolated from duck egg white. The purified inhibitor that showed partial identity in the immunodiffusion test with chicken egg white cystatin had an apparent molecular mass of 9.3 kDa as determined by SDS/PAGE (Warwas *et al*., 1995). The greatest problem in utilizing egg cystatins for medical treatments is their high cost about 140 $ USA dollar for 1 mg pure cystatin (catalogue Sigma). Publication on cystains is less frequent in the literature, probably because of extremely low contents of cystatins in natural resources like eggs (Nakai, 2000). But other hand a few groups are still working in order to find methods of industrial recovering of cystatin from egg white. Six cysteine proteinase inhibitors were isolated from human urine by affinity chromatography on insolubilized carboxymethylpapain followed by ion-exchange chromatography and immunosorption. Physicochemical and immunochemical measurements identified one as cystatin A, one as cystatin B, one as cystatin C, one as cystatin S, and one as low molecular weight kininogen (Abrahmson *et al*., 1986). Cystatin C has been suggested to play a role in several other diseases associated with alterations of the proteolytic system, such as cancer (Kyhse-Andersen *et al*., 1994), inflammatory lung diseases (Buttle *et al*., 1991), periodontal disease (Skaleric *et al*., 1989), multiple sclerosis (Bollengier, 1987), renal failure (Bezin *et al*.,

1984), autoimmune diseases (Cattaneo *et al*., 1986), and HIV infection (Cattaneo *et al*., 1986).

Materials and Methods

Biological Material: The whole egg laid by hens of Tetra SL line in different ages between 20 - 80 weeks was received from commercial firm TASOMIK. The layers were kept in batteries and fed with standard feeding (2700 Kcal/kg feed and 16% of protein). The fresh eggs were broken and separated albumen from yolk. The albumen was initial material for investigations.

Purification of cystatin from egg white: The preparation of cystatin from egg white was purified (Siewinski, 1991) from the homogenate of egg white diluted with an equal volume of 0.25% NaCl. The homogenate was brought to pH 3.0 with 3 M HCl, and left for 1 h at 4°C, and then to pH 6.0 with 3 M NaOH, and left overnight. The precipitated ovomucin was removed by centrifugation at 14000rpm, for 1h. Then the supernatant was subjected to affinity chromatography papain-Sepharose 4B column (7.5 x 5 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.5). Then the column was washed with the same buffer. Proteins bound to papain-Sepharose 4B were eluted with 10 mM NaOH (pH 11.0), the fractions were pooled, adjusted to (pH 7.5) with 3 M HCl, and concentrated. The concentrated solution (CPI) was dialyzed against 10 mM Tris-HCl buffer (pH 7.5) containing 0.1 M sodium chloride, and was applied to Sephadex G-100 column (128x2.5 cm) equilibrated with the same buffer. The fractions were separated as two peaks corresponding to molecular weight values of proteins in the range of 13-41 kDa. The collected fractions of 13 kDa protein fractions were dialyzed against 10 mM Tris-HCl buffer (pH 8.0) and subsequently applied to DEAE-Sephacel column equilibrated with the same buffer. Elution of the column with linear gradient of sodium chloride (0.05 - 1.0 M) in the same buffer resulted in a single symmetrical peak of protein. The solution obtained from the peak fractions was concentrated for additional purification by reversed phase HPLC on a Nucleosil 100 C_{18} column (Knauer) using Waters HPLC system according to Butzow *et al.* (1987). Concentrated solution of 200 µg of protein was dissolved in 0.1% trifluroacetic acid and injected onto C_{18} (8x100 mm) HPLC column. A linear gradient of acetonitrile (0-60%) containing 0.1% trifuoroacetic acid was used to elute protein. The purity of the protein was checked by SDS-PAGE. Samples of the preparations were stored in lyophilized form at -20°C until used. Protein concentrations were determined according to Bradford's method (1976) using bovine serum albumin as a standard.

SDS-PAGE, (SDS - polyacrylamide gel electrophoresis): The SDS-PAGE was performed according to Laemmlil (1970) (under reducing and non reducing conditions using 12.5% gel in the presence of SDS. Bovine serum albumin, Bovine liver catalase, Chymotrypsin and Cytochrome C were used as standards for molecular weight calculation. 12.5% gels were used in the presence of SDS with 0.025 M. Tris-glycine buffer (pH 8.6) as electrode buffer. The gel were polymerised with ammonium persulphate and TEMED, 10 µg samples were layered on top of the gel, and a constant voltage was applied until attacking dye (bromophenol blue) reached the lower end of the gel. The gels were fixed in 25% tichloroacetic acid for 2h, rinsed in distilled water and stained in a 0.25% aqueous solution of Coomassie brilliant blue G-250 in 10% acetic acid containing 40% methanol. Destaining was performed in a 7% acetic acid solution in distilled water, containing 7% methanol for 2h.

Kinetics of inhibition of chicken cystatin: The buffers were the same as those described in Assays of Enzymes and Inhibitors. Inhibitor (variable concentrations) and substrate (5 µM Z-Phe-Arg-AMC and Z-Arg-Arg-AMC) were dissolved in 1.97 ml buffer contained in a spectrophotometer cuvette thermostated at 37°C. Constant concentrations of cathepsin B (100 PM) and papain (50 PM) were used throughout. All experiments were done under pseudo-first order conditions with inhibitor concentrations at least 10-fold higher than the enzyme concentrations. The progress curve were monitored at excitation and emission wavelengths of 370 and 440 nm, respectively, using a Perkin Elmer LS-51spectrofluorimeter connected with microcomputer. Software program was used to analysis the data.

Inhibitory activity against papain: The inhibitory activity was referred to as total cysteine protease inhibitor activity (CPIs) (Barrett and Kirschke, 1981). The active papain was obtained as determined by active site titration with E-64 100 µl of 6 nm final concentration. Papain was preincubated for 5min at 37°C with increasing amounts (10-100 µl) of the CPIs sample in presence of 1.3 mM EDTA, 2.5 mM DTT and 0.05% Brij-35 in acetate buffer (pH 5.5). After the addition of 100 µl of 200 µM substrate Z-Phe-Arg-AMC, the reaction mixture was incubated for 30 min at 37°C. The reaction was stopped by addition of 2 ml of 1 mM iodoacetic acid. Fluorescence at 370 nm excitation and 440 nm emission wavelength was determined against blank, which was prepared by adding iodoacetic acid before the substrate. Fluorescence readings were standarized with the reaction product 7-AMC (Barrett, 1980). A control assay in which CPIs sample was replaced with the reaction buffer to determine the uninhibited papain activity was prepared. One inhibitory unit against papain equals one activity unit of papain and represents the amount of the inhibitor that totally inhibits papain activity

Table 1: Results of a typical purification of chicken cystatin

Purification step	Age of hens (weeks)							
Homogenates	20	30	40	50	60	65	70	80
Volume [ml]	1000	1000	1000	1000	1000	1000	1000	1000
Total protein [mg]	626	827	870	626	962	425	412	559
Active material [mEU]	72.9	75.6	77.9	99.9	82.8	26.3	12.8	56.8
Specific activity [mEU/ml]	0.08	0.09	0.09	0.16	0.09	0.06	0.03	0.1
Yield [%]	100	100	100	100	100	100	100	100
Purification fold	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Sepharese-4B-papain								
Volume [ml]	200	200	200	200	200	200	200	200
Total protein [mg]	16.8	22.3	26.8	41.2	36.2	10.2	8.7	10.6
Active material [mEU]	57.1	65.6	67.9	87.8	77.5	18.3	9.3	7.9
Specific activity [mEU/ml]	3.9	2.9	2.5	2.1	2.5	1.8	1.07	0.7
Yield [%]	78.3	85.2	87.2	87.9	93.7	69.2	77.0	13.6
Purification fold	49.1	32.6	28.1	13.1	24.9	30.0	35.0	7.3
Sephadex G-100 column (age 50 weeks)			Fraction 1			Fraction 2		
Volume [ml]			10			10		
Total protein [mg]			25			15		
Active material [mEU]			28.7			62.9		
Specific activity [mEU/ml]			1.12			4.13		
Yield [%]			28.2			62.5		
Purification fold			7.0			25.8		

in the assay. This amount is determined by extrapolation of the titration curve to zero papain activity.

Inhibitory activity against Cathepsin B: Purified human liver Cathepsin B (EC 3.4.22.1) was used. The active concentration of cathepsin was determined by stoichiometric titration with E-64. The clear supernatants (samples) were incubated with 1.3 µM cathepsin B in activation 0.4 M sodium phosphate buffer (pH 6.0), containing 4 mM EDTA, 2.5 mM DTT for 30 min at 37°C as described in more detail for papain. The residual activity was measured as described above for papain assay, and compared to the uninhibited control assay were CPIs sample was replaced by sodium phosphate buffer. One inhibitory unit against Cathepsin B equals one unit of Cathepsin B activity.

Statistical analysis: The levels of variables in the hen's age compared with the cystatin levels were analyzed statistically. The 0.05 level of probability was assumed as significant.

Results

The inhibitors were isolated from chicken egg white in different ages, from 20 weeks to 80 weeks as described under "Experimental Procedures." Initially, the supernatants were exposed to acidic pH, and then the majority of non-inhibitory materials were removed by affinity chromatography on the Papain Sepharose 4B. Unbound and non-specifically bound proteins were eluted with a high salt buffer, with 10 mM NaOH was used for elution of papain-inhibiting proteins, followed immediately by readjustment of the pH to 7.5 and desalting by G-25 column gel chromatography. Following concentration procedure samples were examined to define the total inhibitory activity against papain and cathepsin B.

Table 1 show that the total yield and inhibitory activity were significant related with hen ages. The total yield and inhibitory activity were decreased 6 fold and 8-fold in age 80 weeks in comparison with hen in age 20 weeks, also the highest total inhibitory activity was found in ages between 40-50 weeks in comparison with other ages. The total inhibitory activity in age 40 and 50 weeks were increased 9.5 fold and 12 fold in comparison with hen in age 80 weeks. Also the total yield increased 7- fold in age 40 and 50 weeks in comparison with hen in age 80 weeks. We found that the hen in age 50 weeks have highest total inhibitory activity than the hen in age 40 weeks.

The concentrated, inhibitory sample in age 50 weeks was chromatographer on a Sephadex G-100 column. The purity of the inhibitors were applied on SDS-PAGE electrophoreses (Fig. 1). The result shows two papain inhibiting fractions, with molecular mass 40 kDa (fraction 1), and 13 kDa (fraction 2). The highest activity was achieved from fraction 2 than fraction 1. The activity and total protein of fraction 2 were 62.9 mEU/ml and 15 mg/ml respectively, while the inhibitory activity and total protein of fraction 1 were 28.7 mEU/ml and 25 mg/ml,

Table 2: Inhibition of cathepsin B and papain by different isolation of cystatin from hens in various ages

Variants chicken (weeks)	Inhibition of expressed as (mEU/mg protein)	
	Papain activity	Cathepsin B activity
20	26.26	1715
30	30.45	17.78
40	35.31	19.60
50	38.20	24.80
60	28.12	18.90
65	20.45	10.94
70	14.15	4.90
80	12.67	4.65

(10 µg) of isolate cystatin used as in Material and Methods incubate with cathepsin and papain. Fluorogenic substrate used Z-Arg-Arg-AMC in phosphate buffer (pH 6.0), and Z-Phe-Arg-AMC in acetate buffer (pH 5,5) for cathepsin and papain. The significant was calculated at (p£0.05)

respectively. The specific inhibitory activity against papain of fraction 2 was about 4 times higher than that of fraction 1. After homogenization and filtration one term egg white yielded 1000 ml (30 eggs). Fig. 2 shows that the yield of cystatin significantly related with hen ages. The yield of cystatin in age 20 weeks was 15 mg protein, while in age 80 weeks 8 mg protein. The highest yield was obtained from hen in ages between 50 to 60 weeks in comparison with other ages. The total yield in age 50 weeks was 40 mg protein, while in age 60 weeks 34 mg protein but with lowest inhibitory activity in comparison with hen in age 50 weeks. . Table 2 shows that the total inhibitory activities against papain and cathepsin B were increased significantly with the age of hens from 20 to 50 weeks, and this activity was decreased significantly with increasing the age of hens to 80 weeks ($p \leq 0.05$). The total inhibitory activities against papain and cathepsin B in age 20 weeks against papain and cathepsin B were 26.3 and 17.2 mEU/min/mg protein, respectively. While the total inhibitory activities against papain and cathepsin B in age 50 weeks against papain and cathepsin B were 38.2 and 24.8 mEU/min/mg protein, respectively. In other hand, show the total inhibitory activities in age 80 weeks against papain and cathepsin B was decreased to 12.7 and 4.6 mEU/min/mg protein, respectively.

In this experiment we used different concentrations of purified cystatin which obtained from hens in age 50 weeks between (0-8 nM) to find the optimal concentration of cystatin which can inhibit the papain and cathepsin B as in experimental procedure. The reaction was started by addition of fluorogenic substrate Z-Arg-Arg-AMC for cathepsin B and Z-Phe-Arg-AMC for papain. The liberated AMC was measured using a spectrophotometer, excitation and emission

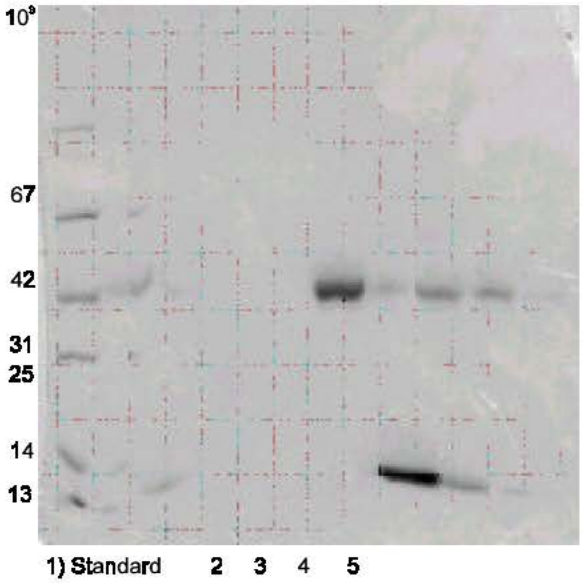

Fig. 1: SDS-PAGE of homogenized chicken egg white (hen's age 50 weeks) on polyacrylamide 12% ges

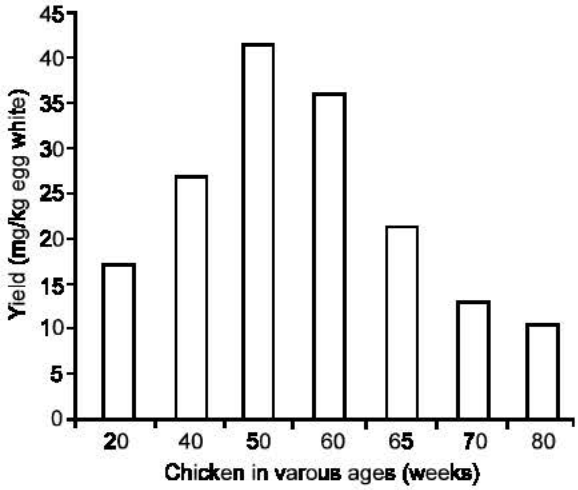

Fig. 2: Yield of cystatin in the chicken egg white in the various ages. The results expressed as mg/kg egg white)

wavelengths at 370 nm, and 440 nm, respectively. From the results shown in (Fig. 3) found that the concentration of the inhibitor, which normalized the enzyme activities of cathepsin B, and papain in the experimental samples approximately to the 5 nM. In this concentration about 90% of cathepsin B and papain activities were inhibited. The inhibition constants presented in Table 3 and Fig. 4 is derived from inhibition of the enzymatic activities of papain and cathepsin B measured at equilibrium with the fluorogenic substrate Z-Phe-Arg-AMC for papain and Z-Arg-Arg-AMC for cathepsin B in different inhibitor

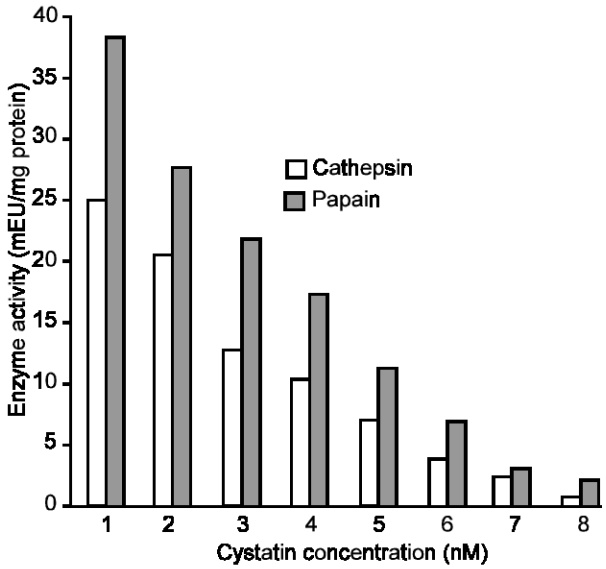

Fig 3: Inhibition of cathepsin B and papain activities by different concentrations of chicken cystatin (hens in age 50 weeks). The result was expressed as mEU/mg protein). The reaction was started by addition of fluorogenic substrate Z-Arg-Arg-AMC for cathepsin B and Z-Phe-Arg-AMC for papain. The liberated AMC was measured using a spectrophotometer, excitation and emission wavelengths at 370 nm, and 440 nm, respectively.

concentrations. Inhibits cathepsin B was (K_i = 2.554) and (k_i = 0.0012) for papain.

Discussion

Endogenous cysteine proteinase inhibitors presumably regulate lysosomal cysteine endopeptidases, such as cathepsins B, and L, *in vivo* (Kastelic *et al.*, 1994). Cathepsin B overexpression is common to many malignant tumors (Schwartz, 1995). There is a considerable interest in cysteine proteinase inhibitors because of their biological function and for medical reasons (Cox *et al.*, 1999). The possibility of cystatin application in medical, and biological treatment has been reported in the literature, such as antimicrobial (Bjorck *et al.*, 1989, 1990), antiviral (Ebina and Tsukada, 1991) and insecticidal effects (Koiwa *et al.*, 1998). In this study, we investigate to relate between the hens age and the total inhibitory activity from egg white. The result shows that the total yield and inhibitory activity were significant related with hen ages. The total yield and inhibitory activity were decreased 6 fold and 8-fold in age 80 weeks in comparison with hen in age 20 weeks, also the highest total inhibitory activity was found in ages between 40-50 weeks in comparison with other ages, and also shows that the yield of cystatin significantly related with hen ages. All biological fluids investigated

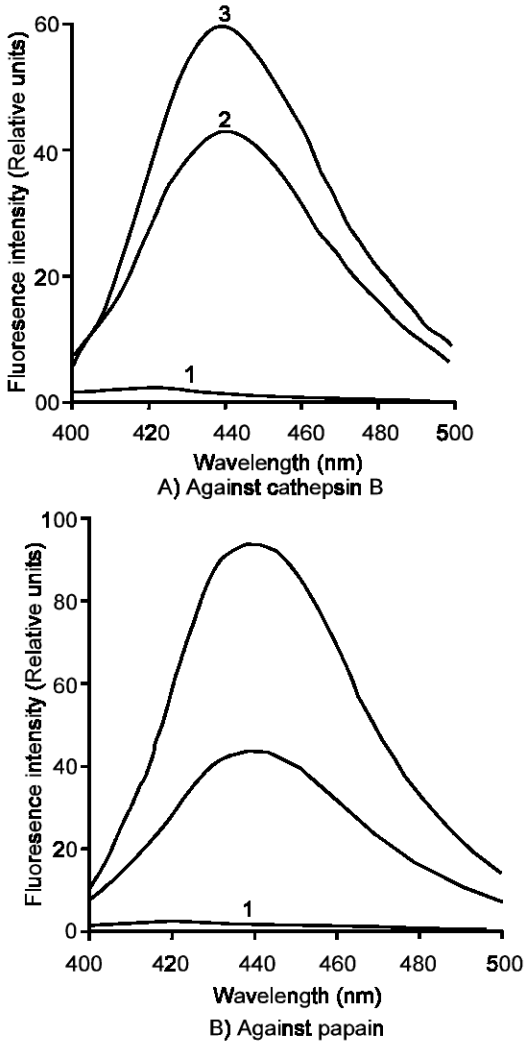

Fig 4: Fluorescence intensity of the cathepsin B and papain inhibition by chicken egg white cystatin. The fluorescence excitation was measured at 370 nm, and the fluorescence band at 440 nm. 1) Negative control. 2) 1 mM of papain or cathepsin B incubated with 80 mg of inhibitor in acetate buffer pH 5.5 and phosphate buffer pH 6.0, respectively. 3) 1mM of papain and 1.3 mM of cathepsin B incubated in acetate buffer and phosphate buffer without inhibitor, respecitively.. The reaction has been started after added 20 mM of substrate (Z-Ph-Arg-AMC) for papain and (Z-Arg-Arg-AMC) for cathepsin B

showed cysteine proteinase inhibiting activity, but it is not known whether the above-mentioned inhibitors account for major part of this activity; neither is it known whether their relative concentrations vary between different biological sources, and if they have the capacity to play important physiological roles as cysteine proteinase inhibitors (Kyhse-Andersen *et al.*, 1994). The hens in age 50 weeks have highest total inhibitory activity among other ages. Inspired by the results above,

Table 3: Inhibition constants Ki for chicken egg white

Inhibitor	Activity Ki		
	%	Cathepsin B	Papain
Chicken egg white age 47 weeks	75	2.554	0.0012
Chicken cystatin (Auerswald *et al*., 1991)	62	2.900	0.002

Ki values were determined from inhibition of the enzymatic activities of papain and cathepsin B measured at equilibrium with the fluorogenic substrate Z-Phe-Arg-AMC for papain and Z-Arg-Arg-AMC for cathepsin B in different inhibitor concentrations; activity, inhibitory amount expressed as percentage of protein concentration

we tried to purify the cystatin from chicken egg white in age 50 weeks. The procedure used for purification of egg white cystatin represents a modified protocol employed by Saleh *et al*. (2001). Purification of cystatin, following affinity chromatography, Sephadex G-100 gel filtration column, G-25 column chromatography, Inspired by the results above, we tried to purify the cystatin from chicken egg white in age 50 weeks. Purification of cystatin, following affinity chromatography, Sephadex G-100 gel filtration column, G-25 column chromatography, the results have shown existence two fractions, contained a high molecular mass 40 kDa, and low molecular mass 13 kDa. The specific inhibitory activity against papain of fraction 2 was about 4 times higher than that of fraction 1. As a result a low molecular mass inhibitor was isolated. Purified inhibitor was found to be homogenous on SDS -PAGE gel electrophoresis. The molecular mass was estimated to be about 13 kDa. On basis of the molecular weight, high specificity for (cathepsin B and papain), stability to heat and pH, N-terminal sequence of the purified cystatin Met-Val-Gly-Glu-Luc-Arg-asp-Luc, and the inhibition constant. The inhibitor could be classified as member of family II of the cystatin superfamily, as a cystatin, about 90% of the total papain inhibitory activity was due to low molecular fractions that contained cystatin. It thus seems likely that cystatin is most important in the regulation of both cysteine proteinase activities in the chicken egg white.

We found that the total inhibitory activities against papain and cathepsin B were increased significantly with the age of hens from 20 to 50 weeks, and this activity was decreased significantly with increasing the age of hens to 80 weeks ($p \leq 0.05$). Also the cystatin isolated from chicken egg white in age 50 weeks inhibited cathepsin B activity about 80%, which is the highest inhibition degree among cystatin from other ages. Similar results for egg white cystatins were reported by Gburek *et al*. (1995) and Warwas *et al*. (1995). Kobayashi and Terao, (1994) reported that human amniotic fluid has anti-metastasis factor of molecular mass about 60-70 kDa identified as typsin proteinase inhibitor. Also, Gutowicz *et al*. (1996) Isolated cysteine proteinase inhibitor from human urine with molecular mass 76 kDa, but these was not fully characterized. We found that the concentration of the inhibitor, which normalized the enzyme activities of cathepsin B, and papain in the experimental samples approximately to the 5 nM. In this concentration about 90% of cathepsin B and papain activities were inhibited. All cystatins inhibit the majority of cysteine proteinases of the papain superfamily, including plant enzymes, papain, ficin, actinidin, and the lysosomal cysteine proteinases cathepsins B, H, L, S, and C. Inhibitory spectra of cystatins, however, are variable for cysteine proteinases. Inhibition of cathepsin B tends to be weaker than that of papain and cathepsins H, L, except cystatin C, which is a strong inhibitor of cathepsin B (Bezin, 1984; Sueyoshi *et al*., 1985; Kobayashi and Terao, 1994; Cimerman *et al*., 1999).

References

Abrahmson, M., A.J. Barrett, G. Salvesen and A. Grubb, 1986. Isolation of six cysteine proteinase inhibitors from human urine. J. Biol. Chem., 261: 11282-11289.

Auerswald, A., G. Genenger, R. Mentele, S. Lenzen, I. Assalg-machleidt, L. Mitschang, H. Oschklant and H. Fritz, 1991. Purification and characterization of a chicken egg white cystatin variant expressed in an *Escherichia coli* pIN-III-ompA system. Eur. J. Biochem., 200: 131-138.

Barrett, A.J. and H. Kirschke, 1981. Cathepsin B, cathepsin H, and cathepsin L. Methods Enzymol. 80: 535-561.

Barrett, A.J, 1980. Fluorimetric assays for cathepsin B and cathepsin H with methylcoumarylamide substrates. Biochem. J., 187: 909-912.

Bezin, J., T. Popovie, V. Turk, U. Brochart and W. Machleidt, 1984. Human cystatin a new protein inhibitor of cysteine proteinases. Biochem. Biophys. Res. Commun., 118: 103-109.

Bollengier, F., 1987. Cystatin C, alias post-γ-globulin: A marker for multiple sclerosis? J. Clin. Chem. Clin. Biochem., 25: 589 - 593.

Bjorck, L., P. Akesson, M. Bohus, J. Trojnar, M. Abrahamson, I. Olafsson and A. Grubb, 1989. Bacterial growth blocked by a synthetic peptide based on the structure of human protienase inhibitor. Nature, 337: 387-388.

Bjorck, L., A. Grubb and L. Kjellen, 1990. Cystatin C, a proteinases inhibitor, blocks replication of herpes virus. J. Virol., 64: 385-386.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Buttle, D.J., M. Abrahamson, D. Burnett, J.S. Mort, A.J. Barrett, P.M. Dando and S.L. Hill, 1991. Human sputum cathepsin B degrades proteoglycan, is inhibited by alfa 2-macroglobulin and is modulated by neutrophil elastase cleavage of cathepsin B precursor and cystatin C. Biochem. J., 276: 325-331.

Butzow, R., M.L. Huhtala, H. Bohn, I. Virtanen and M. Seppala, 1987. Purification and charactrization of placenta protein 5. Biochem. Biophys. Res. Commun., 150: 483 - 490.

Cattaneo, A., J.L. Sansot, D. Prevot, C. Blanc, Y. Manuel and A. Colle, 1986. Cystatin C (post γ globulin) in serum from patients with autoimmune diseases. In: Cysteine proteinases and their inhibitors. Turk V (ed.), pp: 507-516 Walter de Gruyter: Berlin, New York.

Cimerman, N., M.T. Prebanda, B. Turk, T. Popovic, I. Dolenc and V. Turk, 1999. Interaction of cystatin C variants with papain and human cathepsins B, H and L. J. Enzyme Inhibit., 14: 167-174.

Cox, J.L., P.S. Sexton, T.J. Green and N.A. Darmani, 1999. Inhibition of B16 melanoma metastasis by overexpression of the cysteine proteinase inhibitor cystatin C. Melanoma Res., 9: 369 - 374.

Ebina, T. and K. Tsukada, 1991. Protease inhibitors prevent the development of human rotavirus-induced diarrhea in sucking mice. Microbiol. and Immunol., 35: 583-588.

Fossum, K. J.R. and Whitaker, 1968. Ficin and papain inhibitor from chicken egg white. Arch Biochem. Biophys, 125: 367-375.

Freije, J.P., M. Abrahamson, I. Olafsson, G. Velasco, A. Grubb and C. Lopez-Otin, 1991. Structure and expression of the gene encoding cystatin D, a novel human cysteine proteinase inhibitor. J. Biol. Chem., 266: 20538 - 20543.

Gburek, J., J. Osada, M. Siekierka and M. Warwas, 1995. Clearance of chicken cystatin from the rat circulation. Comp. Biochem. Physiol., 112B: 697-701.

Gerhartz, B., R.A. Engh, R. Menttele, C. Eckerskom, R. Torquato, J. Wittmann, H.J. Kolb, W. Macheidt, H. Fritz and E.A. Auerswald, 1997. Quail cystatin: Isolation and charactrization of a new member of the cystatin family and its hypothetical interaction with cathepsin B. FEBS. Lett., 412: 551-558.

Gutowicz, J., K. Michalak, A. Pola, I. derdowska and M. Siewinski, 1996. Fluorescence labelling of thiol proteinase inhibitors extracted from urine of colorectal cancer patients. Current Topics in Biophys., 20: 144-148.

Isemura, S., E. Saitoh and L. Sanada, 1987. Charaterization and amino acid sequence of a new acidic cysteine proteinase inhibitor (cystatin SA) structurally closely related to cystatin S, from human saliva. J. Biochem., 102: 693-704.

Kastelic, L., B. Turk, N. Kopitar-Jerala, A. Stolfa, S. Rainer, V. Turk and T.T. Lah, 1994. Stefin B, the major molecular weight inhibitor in ovarian carcinoma. Cancer Lett., 82: 81-88.

Kobayashi, H. and T. Terao, 1994. Isolation and characterization of the anti-metastatic factor from human amniotic fluid. Chiba international symposium on cancer. Suzuki M., and Hiwasa T. (eds.) Reprinted from Proteases Involved in Cancer. Chiba. Japan, pp: 117-120.

Koiwa, H., R.F. Shade, K. Zhu-salzman, L. Subramanian, L.L. Murdock, S.S. Nielsen, R.A. Bressan and P.M. Hasegawa, 1998. Phage display selection can differentiate insecticide activity of soybean cystatins. Plant J., 14: 371-379.

Kyhse-Andersen, J., C. Schmidt, G. Nordin, B. Anderssen, P. Nilsson, V. Lindstrom and A. Grubb, 1994. Serum cystatin C, determined by rapid, automated particle-enhanced turbidimetric method, is a better marker than serum creatinine for glomerular filtration rate. Clin. Chem., 40: 1921-1926.

Laemmlil, U.K, 1970. Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature, 227: 680 - 685.

Lindahi, P., M. Nycander, K. Ylienjaryi, E. Pol and I. Bjork, 1992. Characterization by rapid-kinetic and equilibrium methods of the reaction between N-terminally truncated forms of chicken cystatin and the cysteine proteinases papain and actinidin. Biochem. J., 286: 165-171.

Mashita, M. and S. Konagaya,, 1991. Cysteine proteinase inhibitor in egg of *Chum salmon*. J. Biochem. Tokyo, 110: 762-766.

Nakai, S., 2000. Molecular modification of egg proteins for functional improvement. In: Sim, J.S. & Nakai, S. (eds) egg nutrition and biotechnology, pp: 205-217, CAB International: wallingford, UK.

Rawling, N.D. and A.J. Barrett, 1990. Evolution of proteins of the cystatin superfamily. Biochem. Biophys. Res. Commun., 118: 103-109.

Saleh, Y., P. Ziolkowski, M. Siewinski, J. Milach, P. Marszlik and J. Rybka, 2001. The combined treatment of transplantable solid mammary carcinoma in Wistar rats by use of photodynamic therapy and cysteine proteinase inhibitor. *in vivo*, 15: 351-358.

Schwartz, M.K., 1995. Tissue cathepsin as tumor markers. Clin. Chim. Acta, 237: 67-78.

Siewinski, M., 1991. Method of purification of thiol proteinase inhibitors from human urine. Cancer Biochem. Biophys., 12: 33-44.

Skaleric, U., J. Baknik, V. Curin, T. Lah and V. Turk, 1989. Immunochemical quantitation of cysteine proteinase inhibitor cystatin C of inflamed gingival. Arch. Oral. Biol., 25: 589-593.

Sotiropoulou, G., A. Anisowicz and R. Sager, 1997. Identification, cloning, and characterization of cystatin M, a novel cysteine proteinase inhibitor, down-regulated in breast cancer. J. Biol. Chem., 272: 903-910.

Sueyoshi, T., K. Enjyoji, T. Shimada, H. Kato, S. Iwanaga, Y. Bando, E. Kominami and N. katunima, 1985. A new function of kininogens as thiol proteinase inhibitors: Inhibition of papain and cathepsins B, H, and L by bovine rat and human plasma kininogens. FEBS. Lett., 182: 193 - 195.

Turk, V. and W. Bode, 1991. The cystatins: protein inhibitors of cysteine proteinases. FEBS. Lett., 285: 213-219.

Warwas, M., J. Gburek, J. Osada and K. Garab, 1995. Purification and characterization of cystatin from duck egg white. Acta. Biochimica. Polonica, 42: 351-356.

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