Egg white cystatin isolation and its medical application

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Egg white contains numerous bioactive substances, which may be used for biomedical purposes. Cystatin is reversible, tight-binding competitive inhibitor of cysteine proteinases. Cystatin may be considered an alternative choice for the drug development therapy. A highly efficient method of isolation and purification should be found, and intensive studies are required to apply chicken cystatin in clinical treatment of diseases. In this study a new method of purification chicken cystatin from egg white by alcohol extraction was demonstrated to remove ovomucoid in pH 4.0, and the supernatant was subjected to affinity chromatography papain-Sepharose 4B column. The results of polyacrylamide gel electrophoresis indicate that the fraction with molecular mass (13 KDa) and specific activity 92 U/mg protein have a high affinity for cathepsin B and papain. The results show that the activities of cathepsin B and L decreased 2.8 and 2.0 fold in the rat serum with mammary carcinoma after injection the rat with chicken cystatin (400 μg/kg body weight). Positive reaction between cathepsin B and anti-chicken cystatin on the tumor cell surface was found using immunohistochemical staining. The results provide evidence that chicken cystatin inhibitor, which is one of the physiological proteinases inhibitors, isolated and purified from egg white, is able to inhibit the expression levels of cathepsin B and L in tumor cell invasion.

Keywords: Chicken cystatin, purification, cathepsin B and L, Mammary carcinoma

Introduction

Hen eggs are more and more widely used not only for direct consumption, but also as an important material in food production and in pharmaceutical and cosmetics industries (Kijowski et al., 2000). Biologically active substances contained in egg white, e.g. lysozyme, cystatin, avidin, conalbumin and ovomucin, have recently drawn much attention of researchers (Li-Chan et al., 1995). Today, the food industry is making use of a combination of factors to achieve food preservation (Wesierska *et al*., 2005). 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 "2HS-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). Quail cystatin, a new cysteine proteinase inhibitor of the cystatin superfamily, was purified from egg 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).
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 (Bottle *et al*., 1991), periodontal disease (Skaleric *et al*., 1989).

Materials and Methods

Biological Material:

The whole egg lay 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 from the homogenate of egg white diluted with an equal volume of 0.25% NaCl. The homogenate was brought to pH 4.0 with 3 M HCl, and then with 30 % alcohol, left for 1 h in room temperature. 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 inhibitor 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 column (Knauer) using Waters HPLC system 18 according to Butzow *et al*. (1987). Concentrated solution of 200 µg of protein was dissolved in 0.1% trifluroacetic acid and injected onto C (8x100 mm) HPLC column. A 18 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 ^{0}C until used. Protein concentrations were determined according to Bradford's method (1976) using bovine serum albumin as a standard.

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 standardized 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

Results and discussion

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 to 30 % alcohol the majority of non-inhibitory materials were removed by affinity chromatography on the Papain Sepharose 4B. Unbound and non-specifically bound proteins were chromatographs on a Sephadex G-100 column. The purity of the inhibitors were applied on SDS-PAGE electrophoreses (Figure 1). The result shows two papain inhibiting fractions, with molecular mass 25 kDa (fraction 1), and 13 kDa (fraction 2). The highest activity was achieved from fraction 2 than fraction 1. The activity of fraction 2 were 92.0 U/ml, while the inhibitory activity fraction 1 were 18.7 U/ml. The inhibitory activity against papain of fraction 2 was about 5 times higher than that of fraction 1. From the previous results the highest inhibitory activities against papain and cathepsin B were found in age 50 weeks of hens (Trziszka *et al.,* 2004). 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), antiviral (Cimerman *et al*., 1999)

The result indicates that the total inhibitory activity was increased significantly in found alcohol fraction in method isolation of cystatin in comparison to absence of alcohol fraction ($p < 0.0001$). The ratio increased 2.2-fold in found alcohol fraction (Table 1). A different concentration of isolate cystatin [0-100 µg/ml] was incubating with cathepsin B and papain. Flurogenic substrate used Z-Arg-Arg-AMC in phosphate buffer (pH 6.0) for cathepsin B and Z-Phe-Arg-AMC in acetate buffer (pH 5,5) for papain. The result from Figure 2 shows that the activity of cathepsin B and papain were decreased 57.5-fold (98 %) and 16-fold (94 %) respectively, in found 100 µg/ml chicken cystatin (Figure 2). All biological fluids investigated 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. 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. 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 (Heinekens *et al*., 1996; Cimerman *et al*., 1999). The incomparable levels between CB, CL and its natural inhibitors could

contribute to the uncontrolled proteolysis and thus the malignant progression of tongue, colorectal, stomach and breast cancer (Saleh *et al.,* 2003a; 2005; 2006).

Table 1 Effect of alcohol fraction on the total inhibitory activity against papain. Inhibition was expressed as (U/mg protein).

Fractions	Total inhibitory activity	Ratio alcohol/without
Alcohol fraction	92.6***	2.2
Without alcohol	42.8	

The significant was calculated at ($p \leq 0.05$)

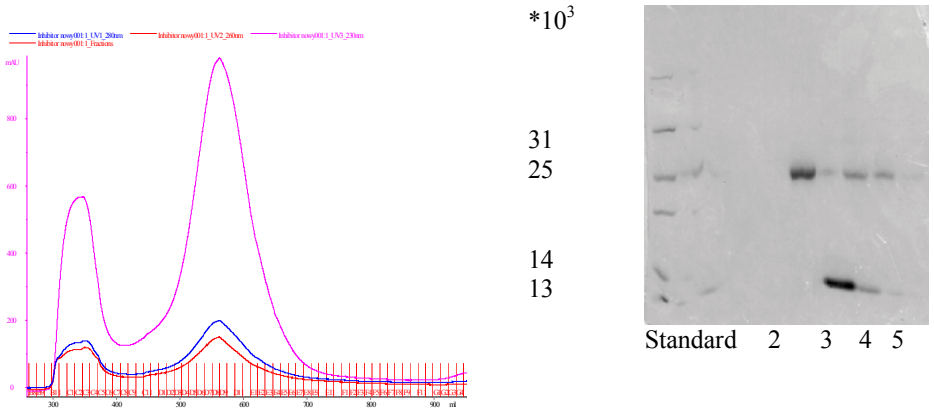


Figure1 SDS-PAGE of homogenized chicken egg white (hen's age: 50 weeks) on polyacrylamide 12 % gel as described in Materials and Methods. Gel was stained with Coomassie brilliant blue. The sample and marker proteins were incubated at 100°C for 5 min in SDS with 2-mercaptoethanol. 20µg/lane of samples were applied on the gel. (1)) Standard proteins; (2, 3) peak no. 1 and 2 after G-100 column; (4, 5) a whole egg white after Sepharose-papain –4B column

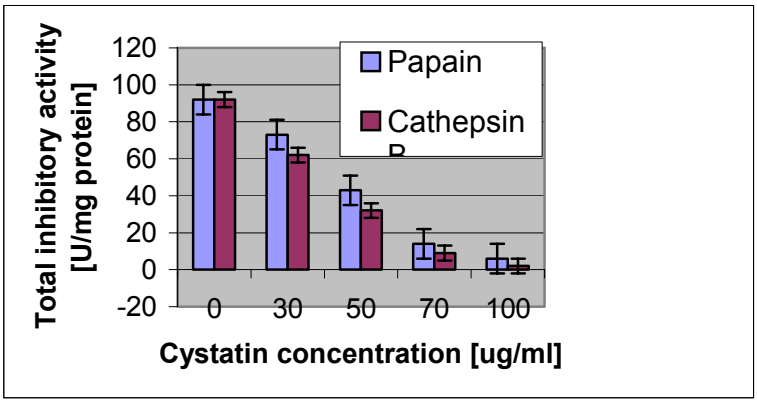


Figure 2. Inhibition of cathepsin B (CB) and papain by different isolation of cystatin from hens. Inhibition was expressed as (U/mg protein)

CB and CL activities were measured in all animals with breast cancer homogenate tissues and in non-treatment animal tissues (control). The mean activities of CB and CL were much higher in tumor homogenate tissues in comparison with those in the control homogenates and the differences are highly significant ($p< 0.0001$) before treatment. The activities of CB and CL were significantly decreased in tumor tissues ($p<0.0001$) after the animals were treated with cystatin. CB and CL activities decreased (2.8-fold and 2.0-fold) respectively, in comparison with untreated animals (Table 2). While the level of endogenous protease inhibitor activity increased (3.2-fold) in the mammary tissue compared to this in controls after the rat treated with cystatin. After treatment the levels of CB, CL and endogenous protease inhibitor reduces to that of control values. Chicken cystatin was able to effectively inhibit patients' CB and CL activities in sera of breast cancer *in vitro* (Thomssen *et al*., 1995; Saleh et al., 2003b). Evidence results suggested that the cystatin isolated from egg *in vivo* could regulate the level of CB and CL to normal values in serum rat with mammary cancer progression (Saleh *et al*., 2001; 2003a; Nakabayashi *et al.,* 2005). Our findings show that CB and CL stimulation of initiating metastatic events, including elevated cell invasion and migration, are prevented by cystatin treatment. Our findings also suggest that the future development of cystatin mimetic hold the potential to improve the therapeutic response of human breast cancers.

Table 2 Values of activities of cathepsin and endogenous inhibitor after treated the rat with chicken cystatin. The activities are expressed as mEU/mg protein in homogenates tissue of rat with mammary carcinoma.

	BEFORE TREATMENT Control [n=30]	 Tumor [n=30]	AFTER TREATMENT Tumor [[n=30]
Cathepsin B Median Mean±SD [Range]	 31.2 31.4±15.5 [11.5-57.7]	 74.2*** 74.3±38.9 [38.7-97.4]	 26.4*** 26.5±8.8 [8.7-33.4]
Cathepsin L Median Mean±SD [Range]	 15.4 15.5±5.6 [2.5-20.2]	 40.7*** 40.8±24.6 [13.4-53.5]	 20.2*** 20.3±6.9 [5.1-24.5]
Endogenous CPI Median Mean±SD [Range]	 25.3 25.5±7.70 [3.2-30.4]	 9.7*** 9.8±3.4 [1.8-12.4]	 29.6*** 29.7±0.8 [3.8-33.7]
Significance [P]	P≤ 0.0001		NS

*** *The 0.05 level of probability was taken as significant between tumor tissues before and after treated the rat with chicken cystatin in comparison to control groups (healthy).*
NS; No significance between control groups and tumor groups after treated the rat with chicken cystatin

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References:

BARRETT, A.J., and KIRSCHKE, H. (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.
BJORCK, L., AKESSON, P., BOHUS, M., TROJNAR, J., ABRAHAMSON, M., OLAFSSON, I., GRUBB, A. (1989) Bacterial growth blocked by a synthetic peptide based on the structure of human proteinase inhibitor. *Nature* **337**: 387-388
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.
BOTTLE, DJ., ABRAHMSON, M., BURNETT, D,, MORT, JS., BARRETT, AJ., DANDO, PM., and HILL, SL. (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., HUHTALA, M.L., BOHN, H., VIRTANEN, I., SEPPALA, M. (1987) Purification and characterization of placenta protein 5. *Biochem Biophys Res Commun* **150**: 483 - 490.
CIMERMAN, N., PREBANDA, M.T., TURK, B., POPOVIC, T., DOLENC, I., and TURK, V. (1999) Interaction of cystatin C variants with papain and human cathepsins B, H, and L. *J. Enzyme Inhibit* **14**: 167 - 174.
COX, J.L., SEXTON, P.S., GREEN T.J., and DARMANI, N.A. (1999) Inhibition of B16 melanoma metastasis by overexpression of the cysteine proteinase inhibitor cystatin C. *Melanoma Res* **9**: 369 - 374.
HEINEKENS, Y.M.C., VEEMAN, E.C.I., and NIEUW, A.A.V. (1996) Cystatins in health and disease. *Biol Chem* **377**: 71-86.
GERHARTZ, B., ENGH, R.A., MENTELE, R., ECKERSKOM, C., TORQUATO, R., WITTAMAN, J., KOLB, H.J., MACHEIDT, W., FRITZ, H., and AUERSWALSD, E.A. (1997) Quail cystatin: Isolation and characterization of a new member of the cystatin family and its hypothetical interaction with cathepsin B. *FEBS Lett* 1997; 412: 551 - 558
GUINEC, N., DALET-FUMERON, V., PAGANO, M. (1993) In vitro study of basement membrane degradation by the cysteine proteinases, cathepsins B, B-like and L. Digestion of collagen IV, laminin, fibronectin, and release of gelatinase activities from basement membrane fibronectin. *Biol. Chem. Hoppe-Seyler* **374**: 1135-1146
KASTELIC, L., TURK, B., KOPITAR-JERALA N., ŠTOLFA, A., RAINER, S., TURK, V., and LAH, T.T. (1994) Stefin B, the major molecular weight inhibitor in ovarian carcinoma. *Cancer Lett*. **82**: 81 - 88
KIJOWSKI, J., LECENIEROWSKI, G., FABISZ-KIJOWSKA, A. (2000) Lysozyme polimer formation and functionality of residuals after lysozyme extraction. In: Egg Nutrition and Biotechnology. Ed.: SIM, J.S., NAKAI, S., GUENTER, W. pp.269-286. CABI Publishing. Canada. ISBN 0-85199-330-3
KYHSE-ANDERSON, J., SCHMITT, C., NORDIN, G., ANDERSSEN, B., NILSSON-EHLE, P., LINDSTEON, V., and GRUBB, A. (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.
LI-CHAN, E. C. Y., POWRIE, W. D., NAKAI, S. (1995) The chemistry of eggs and egg products. In: Egg Science and Technology. Ed: Stableman, W.J., Cotterill, O. J. (pp. 105-175). Food Products Press. N.Y., London. ISBN 1-56022-855-5
MASHITA M., and KONAGAYA, S. (1991) Cysteine proteinase inhibitor in egg of *Chum salmon*. *J Biochem Tokyo* **110**: 762 - 766.
NAKABAYASHI, H., HARA, M., SHIMUZU, K. (2005) Clinicopathologic significance of cystatin C expression in gliomas. *Hum Pathol* **36**:1008-15.
RAWLING, N.D., and BARRETT, A.J. (1990) Evolution of proteins of the cystatin superfamily. BEZIN, J., POPOVIE, T., TUEK, V., BROCHART, U., and MACHLEIDT, W. (1984). Human cystatin, a new protein inhibitor of cysteine proteinases. *Biochem Biophys Res Commun* **118:** 103 - 109.
SALEH, Y., SIEWINSKI, M., SEBZDZ T., JELEN, M., ZIOLKOWSKI, P., GUTOWICZ, J., GRYBOS M., PAWELEC M . (2003) Inhibition of cathepsin B activity in human breast cancer tissue by cysteine peptidase inhibitor isolated from human placenta-immunohistochemical and biochemical studies. *Folia Histochem et Cytobiol* **41:** 161 – 167

SALEH, Y., WNUKIEWICZ, J., ANDRZEJAK R., TRZISZKA T., SIEWINSKI M., ZIOLKOWSKI, P., KOPEC, W. (2006) Cathepsin B and cysteine protease inhibitors in human tongue cancer: Correlation with tumor staging and in vitro inhibition of cathepsin B by chicken cystatin. *J Cancer Mol* **2:** 67-72

SALEH, Y., SEBZDA, T., WARWAS, M., KOPEC, W., ZIOLKOESKI, J., SIEWINSKI, M. (2005) Expression of cystatin C in clinical human colorectal cancer tissues. *J. Exp. Ther. Oncol* **5:** 49 – 53

SALEH, Y., SIEWINSKI, M., KIELAN, W., ZIOLKOWSKI, P. (2003b) Regulation of cathepsin B, L expression in vitro in gastric cancer tissues by egg white cystatin. *J Exp Ther Oncol* 3: 319 - 324.

SALEH, Y., SIEWINSKI, M., SEBZDA, T., GRYBOS, M., PAWELEC, M., JANOCHA, A. (2003a) Effect of combined in vivo treatment of transplantable solid mammary carcinoma in Wistar rats using vitamin E and cysteine peptidase inhibitors from human placenta. *J Exp Ther Oncol* **3**: 95 – 102.

SALEH, Y., ZIOLKOWSKI, P., SIEWINSKI, M., MILACH J., MARSZALIK, P., RYBKA, J. (2001) The combined treatment of transplantable solid mammary carcinoma in wistar rats by use photodynamic therapy and cysteine proteinase inhibitors. *In Vivo* **15:** 351 - 357

SCHWARTA, M.K. (1995) Tissue cathepsin as tumor markers. *Clin Chim Acta* **237**: 67 - 78.

SKALERIC, U., BAKNIK, J., CURIN, V., LAH, T.T., and TURK, V. (1989) Immunochemical quantitation of cysteine proteinase inhibitor cystatin C of inflamed gingival. *Arch Oral Biol* **25**: 589 - 593.

SOKOL, J.P., SCHIEMAN, W.P. (2004) Cystatin C antagonizes transforming growth factor-β signaling in normal and cancer cells. *Mol Cancer Res* **2**:183-195

THOMSSEN, C., SCHMITT, M., GORETZKI, L., OPPELT, P., PACHE, L., DETTMAR, P., JANICKE, F., GRAEFF, H. (1995) Prognostic value of the cysteine proteases cathepsins B and cathepsin L in human breast cancer. *Clin Cancer Res*. **1**:741-746

TRZISZKA, T., SALEH, Y., KOPEC, W., SIEWINSKI, M., WESIERSKA, E. (2004) Effect of Hen's Age on the Level of Cystatin in the Chicken Egg White. *International Journal of Poultry Science* **3:** 471-477,

TURK, V., and BODE, W. (1991) The cystatins: protein inhibitors of cysteine proteinases. *FEBS Lett*. **285**: 213 - 219

WARWAS, M., GBUREK, J., OSADA, J., and GARAB, K. (1995) Purification and characterization of cystatin from duck egg white. *Acta Biochimica Polonica* **42**: 351 - 356.

WESIERSKA, E., SALEH, Y., TRZISZKA, T., KOPEC, W., SIEWINSKI, M. (2005) Antimicrobial activity of chicken egg white cystatin. *World Journal of Biotechnology & Microbiology* **22**: 59-64