



PRODUCTION OF CYSTATIN C WILD TYPE AND STABILIZED MUTANTS

Joanna Pollak¹, Aneta Szymanska², Veronica Lindstrom³, Anders Grubb³

1. Department of Laboratory Medicine, Nicolaus Copernicus University in Torun, Collegium Medicum in Bydgoszcz, Poland
2. Department of Medicinal Chemistry, Faculty of Chemistry, University of Gdańsk, Gdańsk, Poland
3. Department of Clinical Chemistry and Pharmacology, Lund University, Sweden

Correspondence

Joanna Pollak

Department of Laboratory Medicine, Collegium Medicum UMK

Skłodowskiej-Curie 9, 85-094 Bydgoszcz, Poland

email: kizdiagn@cm.umk.pl

phone: (+48 52) 585-44-90; fax: (+48 52) 585-36-03

Abstract

Cystatin C is produced in all nucleated cells. It has various functions and biological activities. Researchers are focused on its role in kidney diseases as a marker of glomerular filtration but also as a very important link in development of amyloid diseases.

This work describes expression and purification of both wild type (wt) and stabilized form (stab 1 and 2) of wt cystatin C and amyloid-forming L68Q mutant of cystatin C. The recombinant cystatin C can be used in projects requiring pure cystatin C to examine models of dimerization and fibrils formation as well as a standard in clinical tests.

INTRODUCTION

Cystatin C

Human cystatin C (HCC) is a very interesting protein, the biology of which is still not completely known. It is a cysteine-proteinase inhibitor widespread in human biological fluids, secreted by all nucleated cells in the body [1,2]. The serum concentration of HCC is normally about six times lower than in cerebrospinal fluid [3]. HCC is an endogenous inhibitor of cysteine proteases, including cathepsin B (CatB), recently discovered to be an amyloid β -degrading enzyme [4,5]. It is hypothesized that CatB enzymatic activity can be enhanced by reducing levels of cystatin C [5]. Cystatin C can be also be used as a marker in the diagnosis of neurodegenerative diseases [4,6,7].

Cystatin C and amyloid diseases.

Amyloidosis is characterized as a protein conformational disease, where a change in protein conformation results in self-aggregation and tissue deposition [8]. Major components of deposited amyloid are protein fibrils, which have β -sheet configuration [9]. Human cystatin C (HCC) is involved in pathogenesis of two types of amyloid disorders: hereditary cystatin C amyloid angiopathy (HCAA) - where the extremely amyloidogenic variant L68Q HCC is deposited as amyloid, causing brain haemorrhage in early adulthood, and in disorders where the generally occurring normal wild type (wt) cystatin C is deposited together with the amyloid β peptide [10,11], mainly in elderly patients and patients affected with Alzheimer's disease or Down's syndrome [2, 12]. L68Q variant has the substitution of leucine to glutamine in cystatin C caused by a single nucleotide mutation. Observation shows that the L68Q variant forms dimers in body fluids more easily than wt HCC [11].

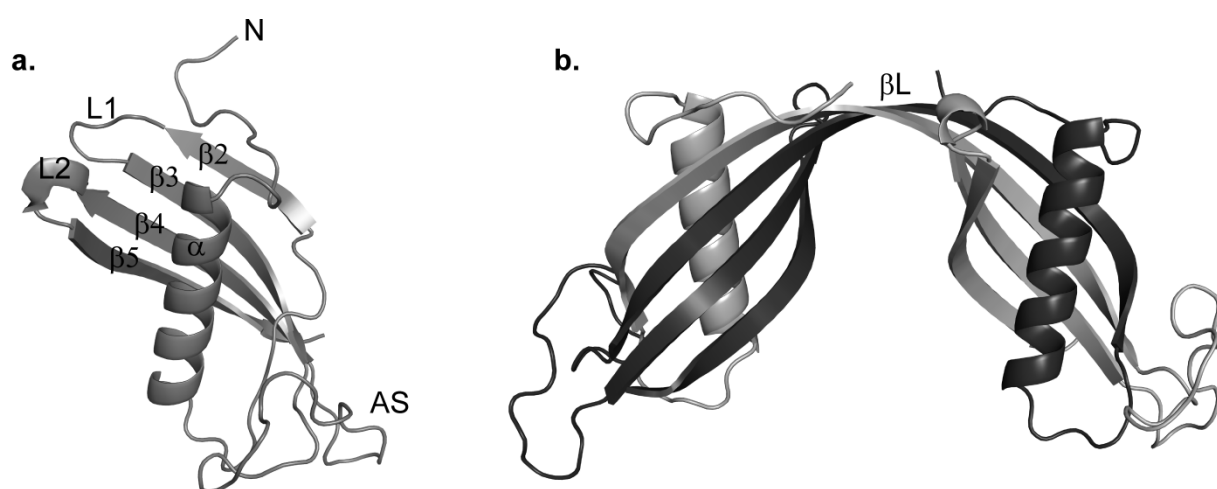
Wild type and stabilized form (stab1, stab2) of cystatin C

HCC is composed of 120 amino acids and contains four cysteine residues forming two characteristic disulfide bonds close to the C-terminal of the protein [11].

The dimerization process of HCC starts by domain swapping of monomeric cystatin C as seen during crystallization. The domain that swapps is built of the α -helix and it's two flanking β -strands (β 1 and β 2) (Figure 1). It is connected to the C-terminal part of protein (strands β 3- β 5) by a hinge region [11,14].

Figure 1 [13]

Models for cystatin C monomer (a) and dimer (b).



To study the formation of dimers, fibrils and oligomers of cystatin C there were established the mutated variants of cystatin C - stab1 and stab2, where disulfide bonds have been introduced in order to prevent domain swapping [2,15]. Point mutations were introduced by site directed mutagenesis into the vectors encoding wt HCC and L68Q cystatin C (Table 1). In stab1 point mutation L47C/G69C was introduced to stabilize disulfide bond between the strands β 2 and β 3 and in stab2 point mutation F29C/M110C was introduced to stabilize the α -helix and the β 5-strand [10,11,15].

Table 1
HCC variants [2].

Mutations	wt cystatin C	L68Q cystatin C
	wt cystatin C	L68Q cystatin C
L47/G69C	wt cystatin C stab1	L68Q cystatin C stab1
F29C/M110C	wt cystatin C stab2	L68Q cystatin C stab2

METHODS

Construction of expression vectors by Site-directed Mutagenesis

Construction of the mutants was performed by site-directed mutagenesis using plasmid pH313 encoding the gene of human cystatin C as a fusion protein with the *E. coli* outer membrane protein (OmpA) signal peptide, according to previously described protocols [2,15,16]. The QuikChange kit (Stratagene, La Jolla, CA) was used to introduce planned mutations. Different primers were designed to either introduce or delete a restriction site in mutated plasmids (as described in 2). PCR amplification was performed in a PerkinElmer Gene Amp PCR System 2400. The sequencing reactions were performed by 25 incubation cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. Received products were purified (QIAquick nucleotide removal kit, Qiagen, Hilden, Germany) and analyzed (ABI Prism 310 Genetic Analyzer, Applied Biosystems, Foster City, CA). Appropriate and checked vectors were introduced into *E. coli* MC1061 for overproduction of HCC mutants [2, 16].

Expression

The growth of bacteria hosting plasmids for overproduction of wt cystatin C and wt cystatin C stab1 was maintained at 30°C in a shaking incubator at 300 rpm. Induction for both variants was initiated by increasing temperature to 42°C followed by incubation for 3h when the optical density (OD) at 600nm was approximately 5. Induction for wt cystatin C stab2 was performed at 38°C instead of at 42°C. The culture of bacteria producing the L68Q cystatin C stab1 needed to be diluted to an OD of 3, remaining conditions were the same as for wt cystatin C. L68Q cystatin C and L68Q cystatin C stab2 both had initiated induction at 38°C and were incubated for 2 h at an OD ~3. Each bacterial subclone was grown in two 450 ml batches. Periplasmic extract was obtained by cold osmotic shock in a total volume of 20 ml [2].

Purification

Purification of periplasmic extracts was performed in a two-step procedure [2, 15]. Wildtype cystatin C, wt cystatin C stab1, and wt cystatin C stab2 were purified by anion exchange chromatography using Q-Sepharose (Amersham Biosciences, Uppsala, Sweden) in 20 mM ethanolamine, pH 9.0 containing 1mM benzamidine chloride. In the next step the fractions containing cystatin C were concentrated by ultrafiltration. After concentration samples were subjected to gel chromatography using a FPLC system and a Superdex HR 75 column (Amersham Biosciences, Uppsala, Sweden) in 10 mM sodium phosphate buffer, pH 7.4 containing 140 mM sodium chloride, 3 mM potassium chloride and 1 mM benzamidine chloride (PBS). Ultrafiltration was used again to concentrate isolated proteins to ~0.5 mg/ml [2].

L68Q cystatin C, L68Q cystatin C stab1, and L68Q cystatin C stab2 were purified by affinity chromatography with use of the monoclonal mouse antibodies raised against wt cystatin C coupled to CNBr-activated Sepharose (Amersham Biosciences, Uppsala, Sweden). L68Q variants were eluted with 0.1 M ammonium bicarbonate containing 6 M guanidinium chloride. Protein refolding and buffer exchange were performed on a Superdex HR 75 column running in 50 mM ammonium bicarbonate. Finally, L68Q cystatin C and L68Q cystatin C stab1 were concentrated to ~0.2 mg/ml, and L68Q cystatin C stab2 to ~0.05 mg/ml by ultrafiltration [2, 15].

The purity of proteins was tested using native electrophoresis on 1% agarose gel in 75 mM barbital buffer containing 2 mM EDTA, pH 8.6 and by SDS PAGE (sodium dodecyl sulfate-poly-acrylamid) using precast NuPage gradient gels (4-12%) from Invitrogen. The gels were run according to the manufactures protocols. Gels were stained by Comassie Blue R-250. An immunofixation of the agarose gel electrophoresis using polyclonal anti-cystatin C was performed to verify the identity of the purified protein as cystatin C.

RESULTS AND DISCUSSION

Human cystatin C wild type is a stable protein that can be effectively produced using heterologous bacterial system. The observed yield of the overexpression was the highest for the wild type protein but the differences observed for

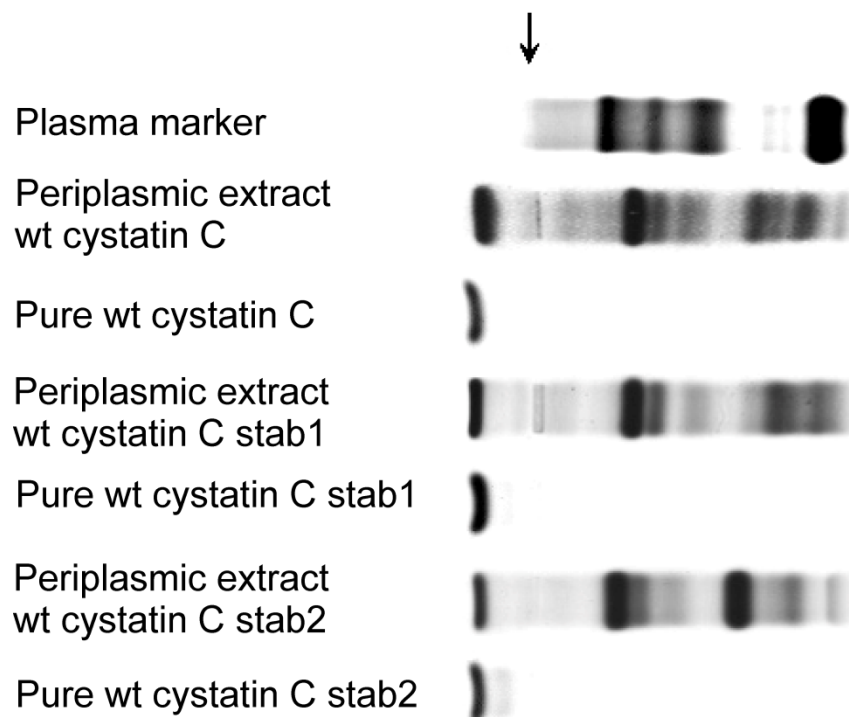
stab1 and stab2 were not striking (Figure 2). Therefore it can be stated that the established system is well suited for obtaining desired mutants of cystatin C and, very likely, also other proteins. In the applied protocol proteins are targeted to the periplasmic space. This approach has two advantages. First, in this cellular compartment oxidation of the native or engineered cysteine residues can directly take place leading to the formation of two native disulphide bridges in the wild type protein as well as the additional bond in the stab1 and stab2 variants. Additionally, the cold osmotic shock procedure, commonly used for isolation of protein from the periplasma can be treated as an additional, preliminary purification step due to lower content of bacterial proteins and cellular organelles in the periplasma comparing to the cytosol.

The purification procedures were chosen to fit the stability profile of the particular protein. For wt cystatin C (17) and its stabilized forms (stab 1 and 2) two step protocol involving ion exchange chromatography on Q-Sepharose followed by gel filtration turned out to be sufficient (see Figure 2). Pure and homogenous protein at the concentration of ~0.5 mg/ml was obtained using this approach.

For highly amyloidogenic L68Q cystatin C, L68Q cystatin C stab1, and L68Q cystatin C stab2 a different system of purification had to be used due to low expression levels of these proteins. L68Q cystatin C has very high tendency to form aggregates (Wahlbom et. al (15) showed that oligomers are present after 3 days of incubation of human L68Q cystatin C even at reduced protein concentration) and needs therefore to be purified rapidly. L68Q and its stabilized variants were purified in two steps. The first step was affinity chromatography with immobilized monoclonal mouse antibodies raised against HCC. Because cystatin C had to be eluted from the column using chaotropic agent (guanidinium hydrochloride), final purification in combination with protein refolding was performed on a gel filtration column.

Figure 2

Native electrophoresis on 1% agarose gel. Periplasmic extracts obtained from expression and pure proteins: cystatin C wt and wt cystatin C stab1 and stab2. The position of protein application was marked with an arrow.



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