J Fluoresc (2012) 22:1627–1632
DOI 10.1007/s10895-012-1106-5

ORIGINAL PAPER

Studies on the Chemical Modification of Goat Liver Cystatin and the Effect on Its Anti-Papain Inhibitory Activity

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Received: 18 March 2012 / Accepted: 27 June 2012 / Published online: 26 July 2012

Abstract Goat liver cystatin was subjected to various chemical modifications in order to ascertain the amino acid residues responsible for its structural and functional integrity. Modification of tryptophan by HNBB led to the complete inactivation of the protein. The inactivation was also accompanied by the complete loss of tryptophan fluorescence at 340 nm. The reaction of liver cystatin with HNBB yielded a characteristic decrease in absorbance at 280 nm. Acetylation of the amino groups of liver cystatin was carried out in the presence of acetic anhydride. The acetylated cystatin showed a decrease in fluorescence intensity at 335 nm which could be attributed to the modification of tyrosine residue due to side reaction.

Keywords Chemical modification · Liver cystatin · Tryptophan modification · Amino group modification HNBB · (2-hydroxy-5 nitro benzyl sulphonium bromide) · Acetic anhydride

Introduction

Cystatins are proteins that tightly bind and regulate the activities of thiol proteinases [1]. These proteins are all related by structure and function to an inhibitor of cysteine proteinases which was first described in egg white and called as chicken egg white cystatins. They have been evolutionary related forming the "Cystatin Superfamily". The members of the protein superfamily were grouped into three families on the basis of their location, size and complexity of polypeptide chains [2, 3]. Members of Family 1, the stefins are found primarily intracellularly, contain about 100 amino acid residues (~11 KDa) and lack disulphide bonds. Members of family 2, the cystatins are found primarily in body fluids and in tissues also. These contain about 120 amino acid residues (~14 KDa) and two intrachain disulphide bonds. Family 3 comprises the plasma kininogens and may therefore also be called the kininogen family with molecular weight of ~70 to 120KD. They contain additional disulphide bonds and are also glycosylated [2]. These inhibitors might protect the cells from unwanted proteolysis which may otherwise cause a number of pathologies [4], like rheumatoid arthritis [5], osteoporosis [6], Alzheimer's disease [7], metastasizing cancer, [8], and microbial invasion [9].

Chemical modification of proteins is a rapidly expanding area in chemical biology [10].The identification of specific amino acid residues within the active sites of proteins is important for understanding the relationship between their structure and biological activity. Chemical modification of proteins serves as a tool to identify the amino acid residues involved in their binding [11]. In this paper, we demonstrated chemical modification of various amino acid side chains of goat liver cystatin (LC) that helped to identify the essential amino acid residues involved in its papain inhibitory activity.

The importance of tryptophan in the inhibitory activity of cystatin was depicted by the use of reagent HNBB or Koshland's reagent (2-hydroxy-5 nitro benzyl sulphonium bromide). It was of interest to determine how tryptophan residues are localized in molecules and whether they are responsible for the manifestation of papain inhibitory activity. Useful information on whether tryptophan residues are

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localized on the surface of a protein molecule or are 'hidden' in the hydrophobic region can be obtained by comparing modification with HNBB in the presence of GdnHCl and in its absence. In the presence of 6 M GdnHCl those tryptophan residues may be exposed that do not undergo oxidation with HNBB under usual conditions. The role of amino groups in the native protein was also determined by acetylation with acetic anhydride.

Materials

Papain, Sephacryl-S100HR, casein, acrylamide, ethylene diamine tetra acetic acid (EDTA), acetone, commassie brilliant blue R-250, Cysteine were from Sigma Chemical company. St.Louis USA. Medium range molecular weight marker were from genei. HNBB (2-hydroxy-5-nitro benzyl sulphonium bromide) was purchased from Sigma Aldrich. Acetic anhydride was also purchased from Sisco research laboratory. All other chemicals were of the reagent grade from commercial sources.

Methods

Purification of Goat Liver Cystatin

Cystatin was isolated and purified from goat liver with a yield of 370 by using a three step procedure [12].

Protein Estimation

The protein content was quantitated by Folins phenol reagent by the method of Lowry et al. [13].

Assay of Papain Inhibitory Activity

The inhibitory activity of cystatins was assessed by its ability to inhibit caesinolytic activity of papain by the method of Kunitz [14].

Chemical Modification of Tryptophan

The reaction of goat liver cystatin with HNBB was carried out in 10 % acetone containing sodium phosphate buffer. Modification reactions were initiated by treating the protein solution with the excess of the reagent HNBB. 1 μM protein solution was incubated with increasing concentration of reagent from 0.25 mM to 1 mM. Aliquots of reaction mixtures were removed at a time interval and assayed immediately for the papain inhibitory activity. In each experiment, a control which had undergone the same modification steps without modifying agents was set up. Activity is expressed as the ratio of the activity of the modified protein A, to that of control Ac, multiplied by 100.

The number of tryptophan residues were calculated by the method of Pajot [15] as described in Jin et al. [16].

Modification of Amino Groups

The amino groups were modified by reaction with acetic anhydride. The protein concentration was kept constant as 1 μM and the concentration of acetic anhydride was varied from 0.5 mM to 3 mM. Aliquots of reaction mixture were taken, dialysed against 0.05 M sodium phosphate buffer and assayed for papain inhibitory activity.

Spectroscopic Measurements

The uv absorption measurements of modified cystatin and native cystatin was obtained by measuring the absorption between 200 and 350 nm in a Shimadzu Spectrophotometer using a cuvette of 1.0 cm pathlength.

Fluorescence Measurements

Fluorescence measurements of cystatin treated with HNBB and native cystatin was studied by measuring fluorescence on a Hitachi F-2000 spectrophotometer. The samples were excited at 290 nm for tryptophan fluorescence and emission range was taken at 300–400 nm. Fluorescence measurements was also done with acetic anhydride treated samples and the samples were excited for total protein fluorescence at 280 nm and emission range was taken at 300–400 nm.

Results

Tryptophan Modification

Goat liver cystatin was inactivated by treatment with tryptophan specific reagent HNBB. Goat liver cystatin was found to loose its papain inhibitory activity by incubation with increasing concentration of HNBB. Figure 1 shows that at 1 mM HNBB resulted in the complete loss of papain inhibitory activity.

Fluorescence Measurements

Fluorescence spectroscopy is widely employed and an excellent spectroscopic probe to investigate conformational changes in tertiary structure of proteins and peptides. The aromatic amino acids tryptophan, tyrosine and phenylalanine offer intrinsic fluorescence probes of protein conformation, dynamics and intermolecular interactions. Out of the three, aromatic residues tryptophan is the most popular probe [17].

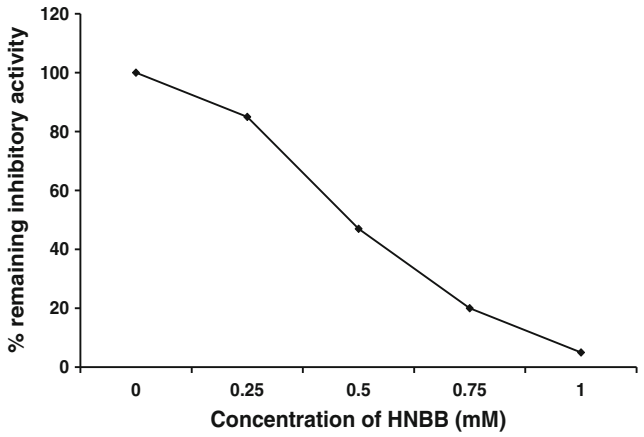


Fig. 1 Percentage loss in activity of goat liver cystatin upon incubation with HNBB. Goat liver cystatin (1 μM) was incubated with increasing concentration of HNBB,0.25 mM,0.5 mM,0.75 mM and 1 mM. The aliquots were withdrawn and assayed immediately for papain inhibitory activity

The native goat liver cystatin upon excitation at 290 nm showed a fluorescence emission at 340 nm, which is typical of tryptophan fluorescence (Fig. 2). However, upon treatment with HNBB a tryptophan specific reagent there was a marked decrease in the fluorescence emission as the tryptophan residues/molecules are sequentially modified. The fluorescence quenching was found to decrease linearly with increasing concentration of HNBB. Upon incubation for 5 min with 1 mM concentration of HNBB fluorescence intensity totally ceased consistent with the modification of tryptophan residues. The emission spectra of modified cystatin showed no wavelength shifts, suggesting that tryptophan residues on the surface of the protein has been modified and denaturation or gross conformational change has not occurred in the modified cystatin.

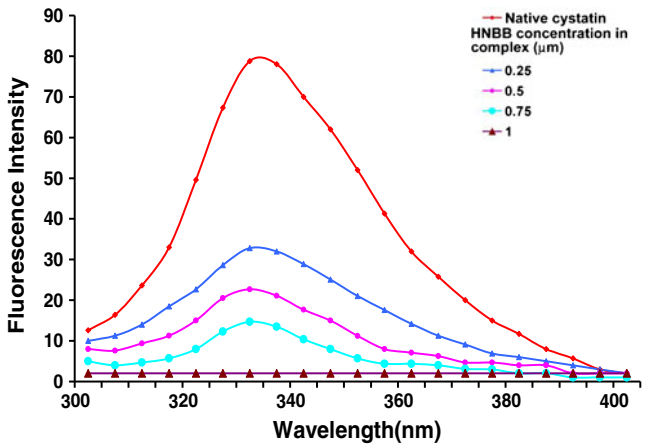


Fig. 2 Fluorescence emission spectra of native and HNBB modified cystatin. Excitation wavelength was 290 nm.The protein concentration in the cuvette was 1 μM

The modification of microenvironment of aromatic residues of liver cystatin in the presence of denaturants has been studied by monitoring the changes in the intensity and wavelength of emission maxima as a function of denaturant concentration. In order to expose the "hidden" tryptophan residues in goat liver cystatin it was treated with 6 M GdnHCl. Fluorescence spectra of the cystatin in the presence of GdnHCl showed unfolding of the inhibitor as manifested by the increase in fluorescence intensity and a red shift (15 nm) of the emission maximum.

Upon incubation of GdnHCl exposed liver cystatin with increasing concentration of HNBB (0.25 mM–0.75 mM) for 5 min the fluorescence intensity was found to decrease with the increasing concentration of HNBB. Figure 3 shows that at 0.75 mM HNBB fluorescence intensity was totally quenched indicating that all the tryptophan residues were modified.

The number of modified tryptophan residues at various concentrations of HNBB were calculated by the method of Pajot [15].

Concentration	Number of tryptophan residues modified
0.25 HNBB	9
0.5 HNBB	14
0.75 HNBB	16

Absorption Measurements

Absorption measurements also clearly revealed the modification of tryptophan residues. Successive addition of HNBB to goat liver cystatin resulted in decrease in the absorbance at 280 nm due to the transformation of tryptophan to oxindole, a much weaker chromophore at this wavelength [18]. As can be seen in Fig. 4 addition of HNBB led to decrease in absorbance at 280 nm. At 1 mM HNBB concentration there was a significant decrease in absorbance at 280 nm. However, addition of higher amounts of HNBB led to the increase in absorbance around 260 nm. The increase in the absorbance at 260 nm could be due to change in the microenvironment of the aromatic residues in the reaction (specifically phenylalanine) or due the reaction products (indolenines) formed upon the interaction of tryptophan with HNBB [19].

Far Uv-CD Analysis

The CD spectra of cystatin upon modification with HNBB showed no substantial changes in the wavelength range of 200–250 nm. (Results not shown). This suggests that loss in activity of the cystatin was mainly

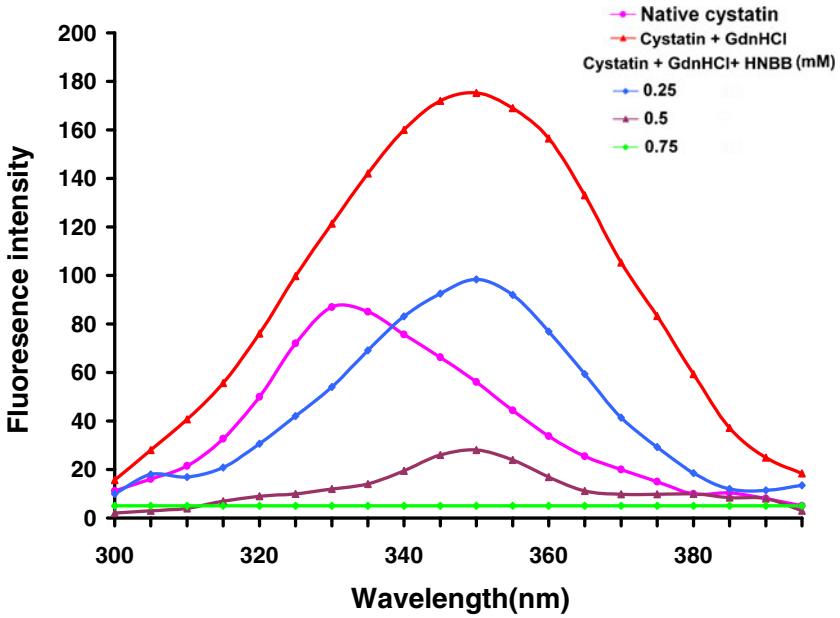


Fig. 3 Fluorescence emission spectra of native, GdnHCL treated liver cystatin and HNBB modified liver cystatin. Excitation wavelength was 290 nm.The protein concentration in the cuvette was 1 μM

dued to modification of the tryptophan involved in complexation with the proteinase [20].

Modification of Amino Groups

Acetylation is the process of introducing an acetyl group into a compound for an active hydrogen atom. Acetic anhydride is commonly used as an acetylating agent. Goat liver cystatin upon incubation with acetic anhydride was found to loose its papain inhibitory activity. Figure 5 shows that incubation with 3 mM acetic anhydride resulted in complete loss of papain inhibitory activity.

Fluorescence Measurements

The changes in the native protein as a result of modification of amino groups were also analysed for total protein fluorescence at 280 nm (Fig. 6).The acetylated goat liver cystatin showed a blue shift of 5 nm. The fluorescence intensity was also found to decrease with increasing concentration of acetic anhydride and at 3 mM concentration of acetic anhydride the fluorescence intensity at 335 nm was totally quenched. Upon acetylation all the amino groups including the amino group of lysine residues were modified. These results clearly indicate that acetic anhydride is not specific for

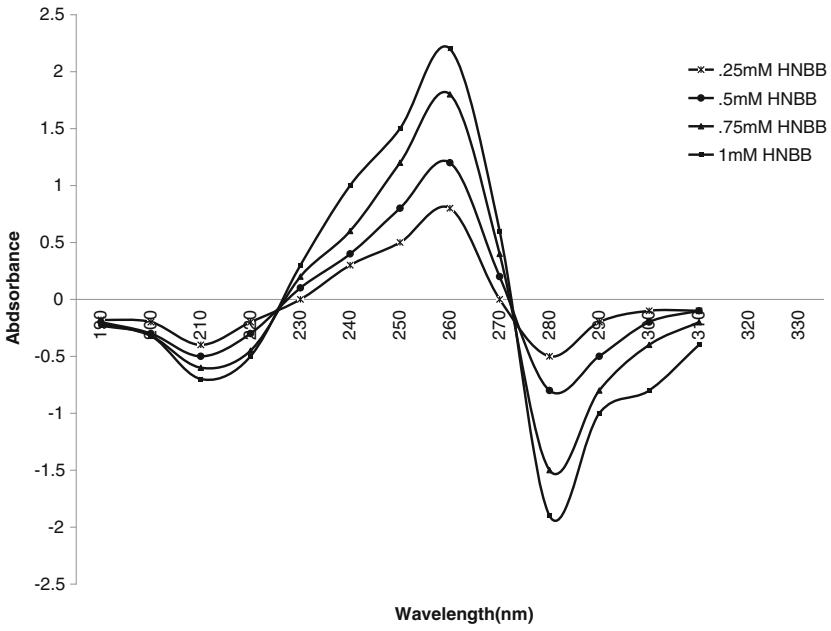


Fig. 4 Difference spectra of HNBB modified liver cystatin measured against native liver cystatin

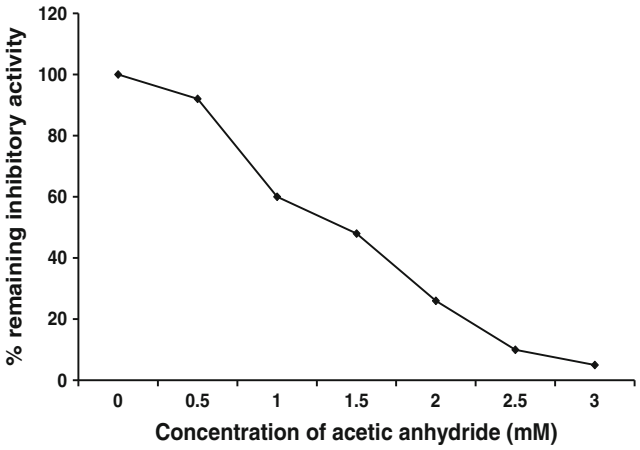


Fig. 5 Percentage loss in activity of goat liver cystatin upon incubation with acetic anhydride. Goat liver cystatin (1 μM) was incubated with increasing concentration of acetic anhydride 0.5 mM, 1 mM,1.5 mM, 2 mM, 2.5 mM and 3 mM. The aliquots were withdrawn and assayed immediately for papain inhibitory activity

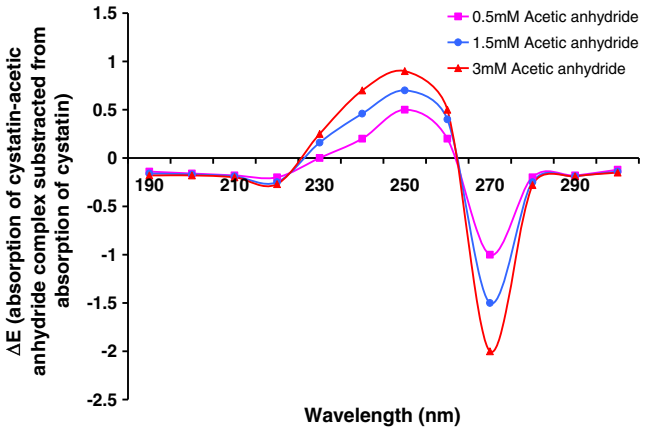


Fig. 7 Difference spectra of acetylated liver cystatin measured against native liver cystatin

amino groups, but may also modify the aromatic residues such as tyrosine. This decrease in fluorescence intensity around tyrosine may be because of side reaction due to acetylation.

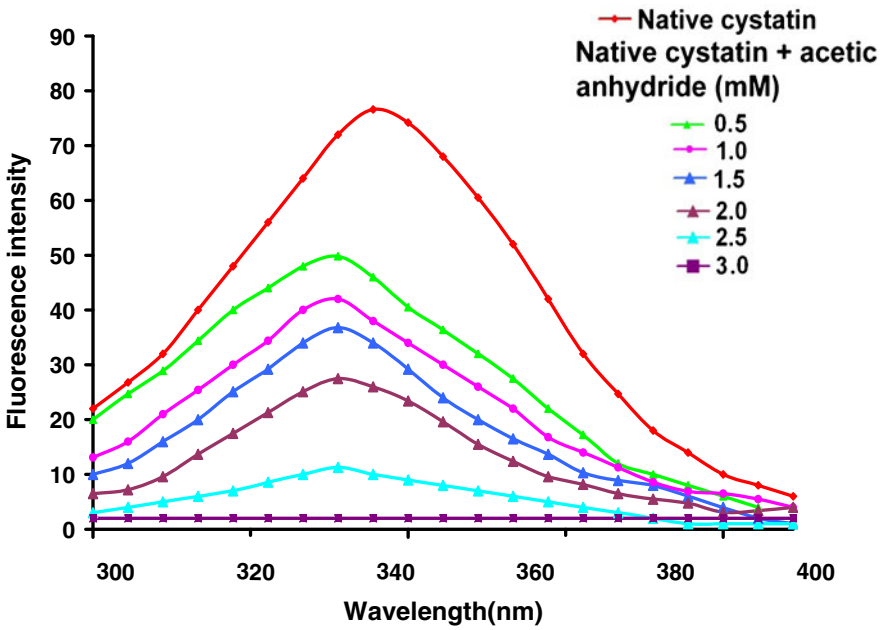
Absorption Measurements

The difference absorption spectrum (the system containing acetylated cystatin measured against cystatin alone) is shown in Fig. 7. As can be seen the absorption of acetylated goat liver cystatin decreased around 270 nm. The decrease in absorption around 270 nm might be due to modification of tyrosine. It has been reported that acetic anhydride modifies amino groups as well as phenolic hydroxyl groups such as tyrosine residues [21].

Conclusion

Thus, based on the above studies it can be concluded that chemical alterations of liver cystatin led to the loss of anti-papain activity of liver cystatin. These results further indicate that tryptophan might be involved in papain inhibition process of liver cystatin. This shows similarity of liver cystatin to other cystatin superfamily members with respect to its involvement of tryptophan inhibition as is already established, (Trp-104 for chicken cystatin [22]. Loss in the inhibitory activity incurred by

Fig. 6 Fluorescence emission spectra of native and acetylated cystatin. Excitation wavelength was 280 nm.The protein concentration in the cuvette was 1 μM



amino group modification points to the importance of charged interactions (through lysine) in maintaining the active conformation of protein.

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