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Autolysis control and structural changes of purified ficin from Iranian fig latex with synthetic inhibitors

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Abstract

The fig's ficin is a cysteine endoproteolytic enzyme, which plays fundamental roles in many plant physiological processes, and has many applications in different industries such as pharmaceutical and food. In this work, we report the inhibition and activation of autolysis and structural changes associated with reaction of ficin with iodoacetamide and tetrathionate using high-performance liquid chromatography (HPLC), ultra filtration membrane, and dynamic light scattering (DLS) methods. The ficin structural changes were also determined using UV-absorption, circular dichroism (CD), fluorescence spectroscopy, and differential scanning calorimetry (DSC) techniques. These techniques demonstrated that iodoacetamide completely inhibited ficin autolysis, which was irreversible. However, tetrathionate partially and reversibly inhibited its autolysis. The ficin structural changes with two synthetic inhibitors were associated with secondary structural changes related to decreased alpha-helix and increased beta sheet and random coil conformations, contributing to its aggregation.

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Keywords

Autolysis; Ficin; Inhibition

1. Introduction

Egypt, Turkey, Iran, Algeria and Morocco are the top five producers of figs worldwide. Iranian fig is produced in rain-fed condition and the lowest aflatoxin contamination is reported in its dried figs than irrigated condition in other countries [1]. Ficin plays fundamental roles in many plant physiological processes, and has different usages in pharmaceutical and food industries [2–11]. Reactive groups of $-\text{SH}$, $-\text{NH}_3^+$ and $-\text{COO}^-$ are necessary for the catalytic action of ficin. Steps of the reaction between ficin and its substrate involves the rapid formation of a ficin-substrate complex, formation of a covalent intermediate as the acyl enzyme that is accompanied by releasing of N-terminal fragment and finally the liberation of C-terminal part by nucleophilic water molecule involvement [12]. Ficin contains at least two sulfhydryl groups, only one of which is directly involved in the catalytic site; the other sulfhydryl group occasionally shows activity. This sulfhydryl group, which is indirectly involved in ficin catalytic site, is buried and can be titrated with *p*-chloromercuribenzoate [13].

Ficin proteolytic and esterolytic activities are irreversibly inhibited by reagents that react with $-\text{SH}$ groups such as iodoacetamide or 1,3-dibromoacetone at almost equal molar ratio [13]. Dibromoacetone is bifunctional and sequentially reacts with the thiol group of cysteine residue and histidine residue at 5 Å distance, respectively, in the active site and intra-active site cross linkage is formed by the inhibitor. The active site is cylindrical with almost 8 Å depth at both ends [14].

Autolysis is generally an intramolecular process. However, several studies have shown that autolysis of the calpain is an intermolecular process [15]. Autolysis of an active enzyme is considered as a fundamental problem in crystallography experiments resulting in formation of non-uniform crystals. Therefore, autolysis enhances the impurities at different stages of the enzyme preparation [16]. Protease autolysis is also a major problem in assessing activity, since the enzyme quickly disables itself. On the contrary, sometimes peptides derived from protease autolysis still continue to have proteolytic activity [17].

Inhibitors affect enzymes activity by blocking their active site or by changing their structure [18]. Spectroscopic studies of papain have revealed that the thiol group in the active site of several inactive papain derivatives were alkylated [19], with minimal changes in the structure [20]. Despite the interesting and informative experiments already performed, ficin has not been well characterized. This may be related partly to its autolysis behavior, which may occur during purification [21]. Still ficin and similar pro-teases purification and identification are important works [22–25] because of their autolysis. Sometimes for get rid of the autolysis, ficin has been successfully purified with special method like pegylation [22]. Thus, ficin autolysis may act as an undesirable barrier for understanding its precise enzyme structure. Although, fortunately the initial studies were done on ficin crystallography and structural transitions in acid denatured ficin were studied in presence of

halogenols and alkanols [23,25]. However, one of the reasons that structural ficin has not been precisely determined are its autolysis. So far, no data on the ficin autolysis control are available, although few experiments have been done on its proteolytic properties inhibition [22,24]. This work was conducted for this purpose. Here, we studied the inhibitory effects of iodoacetamide and tetrathionate on ficin autolysis, structure and activity.

2. Materials and methods

2.1. Materials

Iodoacetamide (IAA), potassium tetrathionate (PTT), thioflavin T (ThT), sodium bicarbonate (sodium hydrogen carbonate), bovine serum albumin (BSA), *ortho*-phthalaldehyde (OPA), sodium dodecyl sulfate (SDS), cysteine hydrochloride were obtained from Sigma–Aldrich ChemieGmbH (Munich, Germany). Trichloroacetic acid (TCA), phosphate buffer, peptone, β -mercaptoethanol, sodium tetra borate, ethylene diamine tetra acetic acid (EDTA), phosphoric acid and sodium chloride were obtained from Merck (Germany). Purified ficin (ficin A) from Sabz cultivar of Iranian fig latex and camel casein were prepared with same procedure as previously described for bovine casein [24]. All other chemicals were of analytical grade.

2.2. Assessment of inhibitory effects on ficin autolysis behavior

2.2.1. Ultra-filtration membrane assays—Main and purified isoform of ficin (ficin A) with concentration of 0.1 mg ml^{-1} were studied in 10 mM sodium phosphate buffer at pH 7.0 in the absence or presence of 0.3 mM iodoacetamide, 0.3 mM and 1.0 mM potassium tetrathionate. Neutral pH was selected because the autolysis is a function of the pH [26,27]. Control was immediately separated by 10 kDa ultrafiltration membrane and centrifugation ($630 \times g$, 60 min, 4°C). Each treatment was separately stored at -20°C . Autolysis treatment with inhibitor and a control without inhibitor were performed at 20°C for 10 days. Following incubation, samples were isolated by ultrafiltration membrane. Protein and peptide concentrations were determined by OPA method [28] for all treatments at same room temperature. For comparison, the percentages of peptides with different molecular weights were calculated for each treatment.

2.2.2. Cation exchange column and HPLC assays—The predominant isoform (ficin A) and completely pure ficin with final concentration of 1.5 mg ml^{-1} in the absence or presence of 5 mM iodoacetamide, 3 mM and 10 mM potassium tetrathionate were examined. Samples were filtered using 10 kDa ultrafiltration membrane to ensure the absence of peptide impurities. The first control with exact volume of 1 ml was injected into the HPLC system. The second control and ficin treated with inhibitors after storage at 37°C for 40 h were separately injected into the cation exchange column on HPLC (Shimadzu system with SP-Sepharose column), and changes were separately evaluated. The percentage of autolysis was calculated according to the below formula: $\% \text{autolysis} = [(A_1 - A_2)/A_1] \times 100$, where A_1 and A_2 are the peaks area of the pre- and post-autolysis forms of the enzyme, respectively [24,29].

2.3. Ficin aggregation in the presence of inhibitor

2.3.1. Dynamic light scattering (DLS) studies—The predominate ficin isoform (1 mg ml⁻¹) was dissolved in 100 mM potassium phosphate buffer pH 7.6 containing 12.5 mM EDTA and 12.5 mM of L-cysteine, in the absence or presence of 0.5 mM iodoacetamide, 1.0 mM potassium tetrathionate. DLS (Brookhaven model) was used for the measurement of Ficin diameter in the presence or absence of the inhibitor.

2.3.2. Separation of aggregated ficin with centrifugation—The ficin (1.5 mg ml⁻¹) sediment aggregation in the presence of 5 mM iodoacetamide or 3 and 10 mM tetrathionate was separated by centrifugation (1820 × g, 10 min, and room temperature). The total area under the chromatogram peaks for ficin with or without inhibitor (A₂ and A₁) was calculated, respectively, with the help of HPLC system program. The difference between areas under the chromatogram peaks of A₁ and A₂ is an indication of the amount of aggregation in the presence of inhibitor. The percentages of aggregation were calculated from the following equation:

$$\% \text{Ficin aggregation} = \left[\frac{A_1 - A_2}{A_1} \right] \times 100$$

2.3.3. Aggregation into amyloid fibrils test—Thioflavin T (ThT) is assessed for its suitability as probe of the aggregated state [30]. When is added to suspensions of aggregated protein; fluorescence emission is enhanced at 482 nm for amyloid fibrils, one of the aggregation forms. The ThT assay was carried out for protein samples before and after incubation. Aliquots from each solution were added to a fresh solution containing 0.025 mM ThT in 25 mM sodium phosphate buffer, pH 6.4, 25 °C. Fluorescence of solutions was monitored on a PerkinElmer LS 55 spectrofluorimeter with excitation at 440 nm, emission at 465 to 535 nm, and slits of 10 nm.

2.4. The effects of synthetic inhibitors on ficin structural changes

2.4.1. Spectroscopic studies—Evaluations were performed using UV-absorption, fluorescence and circular dichroism spectroscopy, as described earlier in ficin experiments [22,25,31,32] in 10 mM sodium phosphate buffers pH 7.0.

2.4.1.1. Absorption spectroscopy: Absorption measurements were carried out in room temperature with Shimadzu model 3100 using a quartz cuvette of 1 cm path length. Spectra were recorded in the absence and in the presence of 0.1 mM iodoacetamide or 0.7 mM potassium tetrathionate in the range 240–320 nm at a protein concentration of 0.25 mg ml⁻¹. Blank solutions containing 0.1 mM iodoacetamide or 0.7 mM potassium tetrathionate were prepared using the same buffer, and their spectra were recorded in the same range.

2.4.1.2. Fluorescence spectroscopy: Fluorescence measurements were made on a Cary eclipse fluorescence spectrophotometer equipped with a data recorder. The excitation and emission slits were set at 10 nm each and the path length of the sample cuvette was 1 cm. Intrinsic fluorescence spectra were recorded in the range 300–500 nm after exciting the

sample at 280 nm, at a protein concentration of 0.005 mg ml⁻¹. Spectra were recorded in the absence and in the presence of 0.03 mM iodoacetamide or 0.2 mM potassium tetrathionate.

2.4.1.3. Circular dichroism spectroscopy: Far-UV CD spectra were performed in the absence and in the presence of 0.17 mM iodoacetamide or 0.3 mM potassium tetrathionate in the range 205–250 nm at a protein concentration of 0.3 mg ml⁻¹. Circular dichroism measurements were obtained using a J-810CD spectropolarimeter with 0.1 cm path length at 25 °C. All CD spectra were converted to $[\theta]_{\lambda}$, the mean residue ellipticity (degcm² mol⁻¹) at wavelength λ (nm) using the Eq. (1) shown below:

$$[\theta]_{\lambda} = \frac{\theta_{\lambda} M_0}{10 \times c \times l} \quad (1)$$

where θ_{λ} is ellipticity (millidegrees) at λ , M_0 is the mean residue weight, c is protein concentration (mg ml⁻¹), and l is path length (cm). The percentage of secondary structure was obtained using the CDNN CD Spectra Deconvolution Software (Version 2.1).

2.4.2. Differential scanning calorimeter (DSC) studies—DSC curves were recorded by Nano-DSC II instrument with a heating rate of 2 °C per min in the temperature range of 25–95 °C. The thermal profile (heat capacity versus temperature) was plotted for ficin (0.8 mg ml⁻¹) in the absence and in the presence of 0.3 mM iodoacetamide or 1.0 mM potassium tetrathionate.

2.5. Estimation of the reversibility of ficin activity by dialysis

Ficin treatments in the presence or absence of 0.5 mM iodoacetamide, 1.0 mM potassium tetrathionate were separately dialyzed against 100 mM sodium phosphate buffer pH 7.6 at 4 °C. The dialysis buffer was changed for each 2 h. Samples were collected every hour for up to 45 h. The ficin activity was measured at 37 °C using camel casein as the substrate in 100 mM sodium phosphate buffer at pH 7.6 containing 250 mM EDTA and 250 mM cysteine. Reversibility of ficin activity was estimated by comparing the dialyzed samples at different periods.

3. Results and discussions

3.1. Ficin autolysis assessment

3.1.1. Peptides isolation (size exclusion method)—A few concentration change of ficin with filtration was observed in the presence of iodoacetamide (0.3 mM) up to 10 days of storage at 20 °C. Meanwhile, it showed 27.5% decrease in ficin concentration in the absence of inhibitor. Tetrathionate potassium (0.3 mM concentration) decreased severely ficin concentration (above 10 kDa weight) after 10 days of storage at 20 °C. While, Tetrathionate potassium with 1 mM concentration changed a little ficin concentration after 10 days of storage at 20 °C (Fig. 1). Unchanged ficin concentration suggests that they control its autolysis. It is obvious that activity inhibition could bring out the prevention of protein degradation upon ficin autolysis by iodoacetamide and high concentration of tetrathionate. But low concentration of tetrathionate activated ficin autolysis as observed in

Fig. 1 that peptides percentages with below 10 kDa size were increased. The increase in enzymatic activity following tetrathionate usage was already reported for dihydrofolate reductase from chicken liver that is activated approximately six-fold accompanied by stoichiometric binding of a sulfur-containing moiety of tetrathionate [33].

3.1.2. Charge separation method (cation exchange column)—Cation exchange column was used for peptide fragments separation from intact ficin.

3.1.2.1. Storage effects: The chromatogram for each treatment showed that stored control without inhibitor had several peptide peaks before the main peak; however the fresh control had only the main peak (Fig. 2a). Peptide peak areas were considered as an indication of ficin autolysis. Previous works also showed that ion exchange column is suitable for separation of autolysis products from intact proteins [24,34]. Produced fragments from autolysis were emerged faster than intact protein during the chromatography as reported earlier [24,35]. The peak areas sum related to stored control ficin decreased compared to fresh control ficin. Reduction in peaks areas referred to ficin aggregation (Fig. 2a).

3.1.2.2. Iodoacetamide effects: Ficin main peak intensity in the presence of iodoacetamide decreased after 40 h of storage at 37 °C, while the initial peaks remained unchanged (Fig. 2b). The initial peaks were related to formation peptides. Thus, it can be stated that ficin autolysis was inhibited in the presence of iodoacetamide. Decrease in the main peak intensity or intact ficin peak showed that ficin aggregation did occur. Ficin aggregation was easily visible, and was removed with centrifugation ($1820 \times g$, 10 min, 4 °C). The supernatant solution was injected into a cation exchange HPLC column with a flow rate of 3 ml per minute. Iodoacetamide was successful in inhibition of ficin autolysis from Sabz cultivar latex of fig and at the same time it induced aggregation. For calculating the aggregated amounts of ficin in the presence of iodoacetamide, the peak area related to fresh ficin in the presence of iodoacetamide (A_1) and the peak area related to stored ficin in the presence of iodoacetamide (A_2) were measured that were 252.7 and 217.1 mm² respectively. Difference between these peak areas ($A_1 - A_2$) indicated aggregated ficin in the presence of iodoacetamide. The aggregated ficin percentage was calculated and its amount was 14.1%. Thus, an appropriate method for estimating the amount of protein aggregation using centrifugation and a HPLC cation exchange column was provided.

3.1.2.3. Tetrathionate effects: The total area of the chromatogram peak related to before and after ficin maintenance unchanged (Fig. 2c). This indicates that ficin aggregation is not formed, while the area under the first peaks increased (Fig. 2c). Therefore, ficin autolysis in the presence of 3 mM potassium tetrathionate reduced more than control (without inhibitor), but it was not completely inhibited. Ficin autolysis was also studied in the presence of 10 mM potassium tetrathionate before and after storage at 37 °C for 40 h. The total area of the chromatogram peaks related to before and after of maintenance (Fig. 2d). This indicates that ficin aggregation is formed. While the initial peak unchanged and showed that ficin autolysis in the presence 10 mM potassium tetrathionate was inhibited.

3.2. Molecular dimensions

Ficin diameter did not increase in the absence of inhibitor (Fig. 3a), but its diameter increased in the presence of iodoacetamide (Fig. 3b). The analytical sedimentation velocity experiments of papain incubated with iodoacetamide showed that papain had only slight or no aggregation at pH 4.0. However, it reversibly formed dimers or larger aggregate at pH 7.0 [36]. Ficin diameter enhancement in the presence of potassium tetrathionate increased slightly compared to the without inhibitor sample (Fig. 3c). However, this increase is less than the diameter of two ficin molecules or in other word less than the diameter of the accumulation of ficin dimers. Therefore, potassium tetrathionate did not play any role in ficin aggregation.

No clear change in the emission intensity for aggregated ficin was observed after incubation of protein suggested that under these conditions the protein does not form amyloid fibrils (Fig. 4).

The fluorescence enhancement of ThT by aggregated amyloid proteins is quite specific. Previous work showed that ovalbumin protein induced a slight (2.3 fold) fluorescence enhancement of the dye by comparison with high (until 22-fold) enhancement for β -sheet structure in aggregated amyloid proteins [30].

3.3. Structural changes of ficin in the presence of the inhibitors

3.3.1. UV-absorption spectra—Structural changes in proteins due to conformational perturbations or ligand binding can be monitored using absorption spectroscopy by studying changes in chromophore and consequently in spectroscopic characters of protein [37–39]. These changes in chromophore can arise either due to change in the chromophore environment as a consequence of conformational changes or can come from direct chemical interactions of binding molecule with chromophore. These differences can be quantitatively studied by measuring the change in absorbance. UV absorption spectra of ficin (black line) are shown in Fig. 5. For native ficin the spectrum was in the range 240–320 nm with a maximum around 280 nm. With iodoacetamide and with potassium tetrathionate there was a hyperchromism around in this wavelength. Absorption in 280 nm wavelength is related to the tryptophan residues aromatic rings. The change in the absorption spectra of native ficin upon addition of iodoacetamide or of potassium tetrathionate can suggest that its structure has changed.

3.3.2. Intrinsic fluorescence spectra—Ficin fluorescence intensity decreased in the presence of iodoacetamide and potassium tetrathionate (Fig. 6). This result was an indication of ficin structural changes. Ficin structural changes raised tryptophan or other natural fluorophores to the surface of ficin structure and in a more polar environment, which caused a decrease in the fluorescence quantum yield. The quantum yield value is dependent on some environmental factors like temperature, solvent and presence of other chemical species that might contribute to increase or decrease of fluorescence intensity [40–42]. Therefore, fluorescence emission declined due to the enzyme structural changes with ligand (inhibitors) binding. An earlier work showed that ficin fluorescence intensity increased with two

denaturant usages [32]. The decrease in the fluorescence intensity suggested that these inhibitors play quencher role on Trp residues and other fluorophores in ficin structure.

3.3.3. Far-UV CD spectra—The main centers of asymmetry present in proteins are the peptide bond formed by interaction between amino acids. Further contribution to the optical activity of proteins comes from their ability to form well defined super asymmetric secondary structural elements like alpha-helices, beta-sheets and beta-turns. These secondary structural elements have distinctive CD spectra which in turn contribute to properties CD spectra of individual proteins, depending on the relative contribution of structural elements towards overall structure of the protein. Consequently CD spectroscopy has been used as a method to study structural changes of proteins. CD spectroscopy has found all the more important role in studying changes in protein structure and conformation in response to ligand binding or in response to presence of structural perturbants like temperature and denaturing agents. Secondary structure composition of proteins by CD spectroscopy has been studied exploiting the asymmetry generated by amide bond. This is accomplished by utilizing the data recorded in CD spectra of proteins between 200 and 250 nm (far UV CD) for estimating fraction or percentage of different secondary structural elements in proteins [43–46]. Far-UV CD spectra of a polypeptide chain depends on its alpha-helix, beta sheet, beta-turns and random coil contents. Ficin secondary structure changed in the presence of inhibitors (Fig. 7), showing a decrease in alpha-helix and an increase in the beta sheets and random coil contents (Table 1). Also, ficin structural transition was studied with different alcohols. It found that two short chain alkanols (methanol and ethanol) were not effective in inducing significant α -helical structure in acid denatured ficin (ADF). Halogenols had higher influence in inducing the α -helical structure in ADF compared to alkanols [25].

3.3.4. Differential scanning calorimeter (DSC)—Differential scanning calorimetry profile of ficin in the absence of iodoacetamide showed a single peak with melting temperature of 78 °C, whereas the iodoacetamide results yield two melting temperatures of 75 °C and 82 °C for ficin. Two melting temperatures show that two transitions are produced in ficin structure. One transition has higher thermal stability than other. Ficin in the presence of this inhibitor revealed three peaks, which indicated ficin dissociation. The first peak manifested between 50 and 60 °C, second and third peaks at higher temperatures (Fig. 8). A previous study reported that the increasing amounts of iodoacetamide in the reaction mixture caused an extra band formation on the basic side of the large subunit of ribulose biphosphate carboxylase [47].

Differential scanning calorimetry peak of ficin in the presence of potassium tetrathionate was given as a single peak, so the energy content of ficin structure remained unchanged in the presence of potassium tetrathionate (Fig. 9). DSC profile of ficin in the presence of potassium tetrathionate showed a single peak with melting temperature of 73 °C that its thermal stability decreased (Fig. 9).

3.4. Reversibility or irreversibility of inhibited ficin

The specific activity of inhibited ficin with iodoacetamide and potassium tetrathionate was measured after dialysis. Ficin activity after inhibition by iodoacetamide and potassium tetrathionate was irreversible and reversible respectively (Fig. 10).

Ficin reversibility in the presence of potassium tetrathionate was evaluated by differential scanning calorimetry after heating to two degree above melting point temperature and then was re-evaluated after re-cooling. Differential scanning calorimetry peak of ficin in the presence of potassium tetrathionate showed that ficin reversibility value of 13.5% (Fig. 9), while DSC reversibility peak of ficin in the presence of iodoacetamide was 0% (Data not shown).

Irreversible inhibitors always bind to enzymes covalently [48]. When an inhibitor has a chemical group that reacts with the active site of the enzyme, which cannot be separated again by either gel filtration or dialysis, it is called an irreversible inhibitor [48]. Iodoacetamide reacts with cysteine residue of the active site, thus, the enzyme is irreversibly alkylated [48]. There are two possible pathways: (a) the thiolate anion can react with the carbon of the halomethyl group in a nucleophilic substitution; (b) the sulfur attacks the carbonyl carbon and the product is indirectly formed via a tetrahedral intermediate and a sulfonium ion [48]. The inhibition by iodoacetamide was also irreversible for Cathepsin C, HMG-CoA reductase, and carboxydismutase [49–51]. It was shown that irreversible inhibition of Guinea pig liver transglutaminase by iodoacetamide occurs only in the presence of the essential cation, calcium [52]. However, iodoacetamide was found to be a reversible inhibitor of human erythrocyte carbonic anhydrase B activity [53,54].

3.5. Evaluation of inhibitory effects on ficin

Iodoacetamide completely controlled ficin autolysis, while it also promoted the aggregation of ficin. Iodoacetamide plays a positive effect (autolysis control) and a negative effect (aggregation) on ficin. Its effect on autolysis prevention and aggregation enhancement is comparable. In this study by measuring the area under the chromatogram peaks we found that autolysis damage in the absence of iodoacetamide was more than aggregation damage in its presence (Table 2). Ficin absorption increased in the presence of the inhibitor at 280 nm and beta sheets formation was enhanced in ficin secondary structure. However, when beta sheets are formed in a protein structure, the protein tends to aggregate more quickly. Thus, ficin structural changes and beta sheet formation caused its aggregation. Other studies have shown that iodoacetamide caused rapid aggregation of cellular proteins [55].

4. Conclusion

Ficin is threatened by two main factors, autolysis and aggregation. In this paper, these two losses of ficin in the presence and in the absence of synthetic inhibitors were resolved. Autolysis of ficin in the absence of inhibitor is very large but in the presence of iodoacetamide was completely inhibited; however ficin aggregation was greatly induced. This is because of beta-sheets increasing in the second structure, structure opening, ficin molecules adhesion to each other and molecules sedimentation. Iodoacetamide inhibits ficin

activity irreversibility and aggregates large amount of protein. Therefore, iodoacetamide was not recommended for autolysis inhibition because of large aggregation. Tetrathionate has both activator and inhibitor effect on ficin autolysis at very low concentrations and higher concentrations respectively. Tetrathionate had a few side effects on structure opening, thermal stability decreasing and aromatic residues exiting. A tetrathionate inhibitory effect was reversible on ficin by dialyzing.

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Fig. 1.

Inhibitory effect of 0.3 mM iodoacetamide and 1 mM potassium tetrathionate were compared with control and 0.3 mM potassium tetrathionate; Ficin concentration without inhibitor during 1 (■) and 10 (□) days, and with iodoacetamide (■), 0.3 mM potassium tetrathionate (▒) and 1.0 mM potassium tetrathionate (▣) during 10 days at 20 °C storage. Separation of produced peptide fragments from ficin autolysis was performed using 10 kD ultrafiltration membranes.

Fig. 2. Ficin chromatogram changes before (—) and after (.....) storage at 37 °C for 40 h, after removal of the aggregates. Ficin in the absence of inhibitor (a) or presence of 5.0 mM iodoacetamide (b), 3.0 mM potassium tetrathionate (c) and 10 mM potassium tetrathionate (d).

Fig. 3. Ficin diameter: (a) in the absence or presence of; (b) iodoacetamide, (c) potassium tetrathionate.

Fig. 4. ThT fluorescence spectra for buffer (.....) and for ficin before (—) and after 40 h incubation in 37 °C in the presence of iodoacetamide (—).

Fig. 5.
Ficin UV-absorption spectra; in the absence (—) or presence of iodoacetamide (.....) or potassium tetrathionate (—).

Fig. 6.
Ficin intrinsic fluorescence spectra: in the absence (—) or presence of iodoacetamide (.....)
or potassium tetrathionate (—).

Fig. 7.
Ficin Far-UV CD spectra: in the absence (—) or presence of iodoacetamide (.....) or potassium tetrathionate (—).

Fig. 8. Differential scanning calorimeter of ficin: in the absence (—) or presence of iodoacetamide (.....).

Fig. 9.
Differential scanning calorimeter of ficin in the presence of potassium tetrathionate (—) and its reversibility (.....).

Fig. 10. Evaluation of ficin activity changes during dialysis in the absence of inhibitor (—) and reversibility of ficin activity that was inhibited with iodoacetamide (.....) or potassium tetrathionate (—).

Table 1

Inhibitors effects on ficin secondary structure contents.

%Content	Without inhibitor	With iodoacetamide	With tetrathionate
Alpha-helix	26.2 ± 0.5	20.4 ± 0.2	23.5 ± 0.3
Antiparallel beta sheet	9.8 ± 0.2	11.4 ± 0.1	10.5 ± 0.1
Parallel beta sheets	10.0 ± 0.1	11.4 ± 0.2	10.6 ± 0.1
Beta turn	17.6 ± 0.1	17.9 ± 0.2	17.8 ± 0.1
Random coil	36.4 ± 0.4	38.9 ± 0.6	37.6 ± 0.6

Table 2

The effects of inhibitors on aggregation, autolysis and total loss of ficin by comparing the areas under HPLC chromatogram peaks.

Treatment	Autolysis (%)	Aggregation (%)	Total losses (%)
Ficin without inhibitor	14.0	5.1	19.1
Ficin with iodoacetamide	0.0	14.1	14.1
Ficin with 3.0 mM tetrathionate	4.1	0.0	4.1
Ficin with 10.0 mM tetrathionate	0.0	5.4	5.4