The Formation of Amyloid-Like Fibrils of α-Chymotrypsin in Different Aqueous Organic Solvents

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Abstract: The formation of amyloid-like fibrils of α-chymotrypsin was studied in aqueous ethanol, methanol, *tert*-butanol, dimethylformamide and acetonitrile. Thioflavin T (ThT), Congo red (CR) and 1-anilino-8-naphthalenesulfonic acid (ANS) binding, turbidity, intrinsic fluorescence and far-UV circular dichroism measurements were employed to characterize the amyloid fibril formation. The greatest extent of fibril formation after incubation for 24 h at pH 7.0 and at 24 °C was in ethanol at 55%, in methanol and dimethylformamide (DMF) at 60-70% and in *tert*-butanol at 60-80%. The ANS binding and intrinsic fluorescence results showed that the hydrophobic residues are more solvent-exposed in the aggregated form of α-chymotrypsin. The ThT, CR binding and far-UV CD measurements indicated that the formation of the cross-β structure of α-chymotrypsin depends on the polarity of the organic solvent. To determine the role of surface charges in the aggregation, chemically modified forms of α-chymotrypsin were prepared. The citraconylated and succinylated enzymes exhibited a higher and the enzyme forms modified with aliphatic aldehydes a lower propensity for aggregation. These results suggest the important role of surface charges in the aggregation of α-chymotrypsin.

Keywords: Amyloid-like fibril, chemical modification, chymotrypsin, cross β-structure, organic solvent.

INTRODUCTION

The amyloidoses are a group of protein misfolding disorders characterized by the accumulation of insoluble fibrillar protein. More than 40 human diseases are associated with the deposition of normally soluble and functional proteins as insoluble aggregates. The proteins known to form amyloid fibrils *in vivo* display no obvious sequence or structural similarities. The mechanism by which the amyloidogenic proteins undergo conversion from a soluble globular form to the cross-β conformation manifested by the disease-associated fibrils has not yet been elucidated [1,2]. A number of observations suggest that amyloid is a generic form of polypeptide conformation and most proteins have the potential to form amyloid-like structures under appropriate conditions [3-5]. Amyloid formation appears to start from partially structured forms of proteins. The similar features displayed by all amyloid fibrils, regardless of their source, indicate that at least some elements of this process may be common to all amyloidogenic proteins. It has recently been demonstrated that α-chymotrypsin is driven towards amyloid aggregation by the addition of 2,2,2-trifluoroethanol (TFE) at intermediate concentrations and at high temperature [5-7]. TFE is a solvent known to stabilize partially folded proteins and promote amyloid formation in several models [5,8]. The TFE-induced conformational transitions of proteins have been found to be similar to those induced by the lipid environment of biological membranes [9].

We report here the *in vitro* formation of amyloid fibrils by α-chymotrypsin in different aqueous organic solvents (ethanol, methanol, *tert*-butanol, dimethylformamide and acetonitrile). Measurements of the binding thioflavin T (ThT), Congo red (CR) and 1-anilino-8-naphthalenesulfonic acid (ANS), intrinsic fluorescence, turbidity and far-UV circular dichroism were employed to characterize amyloid fibril formation. The fibrils were visualized by transmission electron microscopy (TEM). The amino groups of the enzyme were modified with organic acid anhydrides (succinic, citraconic) to study the role of surface charges on the aggregation of α-chymotrypsin. The role of the hydrophobicity of the enzyme in organic solvent-induced aggregation was studied through the use of α-chymotrypsin modified with aliphatic aldehydes (propanal, decanal or tridecanal).

MATERIALS AND METHODS

Materials

α-Chymotrypsin (EC 3.4.21.1; type II from bovine pancreas), thioflavin-T, N-acetyl-L-tyrosine ethyl ester (ATEE) and the organic solvents were purchased from Sigma. All other chemicals used were reagent grade products of Sigma. Enzyme solutions were made in 5 mM sodium phosphate buffer (pH 7.0), unless otherwise stated. Incubations were carried out at 25 °C.

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Fluorescence Spectroscopy

Fluorescence experiments were performed on a Hitachi F-2500 FL fluorescence spectrophotometer. Intrinsic fluorescence measurements at a protein concentration of 15 μg/ml were made by using an excitation wavelength of 280 nm, and emission was collected between 300 nm and 450 nm with a slit width of 5 and 10 nm for excitation and emission, respectively.

ANS binding was studied at 50 μM with 15 μg/ml protein, in the absence or presence of various concentrations of organic solvents. ANS was excited at 365 nm and the emission was collected between 400 nm and 600 nm, with a slit width of 5 and 10 nm, respectively.

CD Measurements

CD spectra were recorded in the far-UV range from 190 to 250 nm, in a 0.02 cm pathlength optical cell on a Jasco J-815 CD spectrometer at 25 ^{o}C. Four spectra were accumulated and averaged for each sample. The enzymes (0.3 mg/ml) were dissolved in phosphate buffer (final concentration 5 mM) (pH 7.0) containing the appropriate amount of organic solvents and were preincubated for different times before measurements. The ellipticity, (Θ), was expressed in mdeg.

Thioflavin-T Binding

Samples containing 150 μg/ml of α-chymotrypsin were incubated at 24 ^{o}C for different times in various concentrations of different organic solvents. Then, 100 μl aliquots were drawn from the protein samples and were diluted into buffer (10 mM sodium phosphate, pH 7.0, 150 mM NaCl) containing 65 μM ThT. Fluorescence emission spectra were recorded 5 min after the addition of protein aliquots. The excitation wavelength was 440 nm and the emission was measured at 485 nm. The excitation and emission monochromator slit widths were 5 and 10 nm. The difference spectra were calculated by subtracting the background emission spectrum from that obtained after protein addition.

CR Binding Assay

CR assays were carried out in 5 mM phosphate buffer (pH 7.0) containing 150 mM NaCl to which CR and amyloid fibrils had been added to yield final concentrations of 11 and 8 μM. The mixtures were then incubated for 15 min. The absorption spectra of the samples were recorded between 400 and 600 nm and were corrected for buffer and protein effects. The spectra of CR alone and of CR with protein were compared. A red shift of the absorption band and an increase in absorption intensity were together taken as indicative of the presence of the formation of amyloid fibrils.

Enzyme Activity Assay

For the measurement of α-chymotrypsin activity, ATEE was used: the decrease in absorbance at 237 nm was followed in a reaction mixture (3 ml) containing 45 mM Tris/HCl (pH 7.0) and 1 mM ATEE [10]. The reactions were initiated by the addition of 50 μl enzyme solution from the different samples.

Modification of Lysine Residues

Chemical modifications were carried out according to Mozhaev *et al.* [11] with some modifications. A solution (0.5 ml) of the anhydride (10-800 mM) in dimethyl sulfoxide was added dropwise at 0 ^{o}C to 4 ml of 40 μM α-chymotrypsin in 0.1 M sodium phosphate buffer (pH 8.0) with stirring. The pH of the reaction mixture was kept constant by the addition of 1 M KOH solution. The reaction was allowed to proceed for 1 h. The solution was then fractionated by filtration on Sephadex G-25 to remove the excess reagent. The degree of modification was calculated from the number of amino groups in the modified enzyme form which reacted with trinitrobenzenesulfonic acid as compared with the unmodified enzyme [12]. The modifications with different aldehydes were performed in 0.1 M phosphate buffer (pH 8.4) containing 4 mg α-chymotrypsin dissolved in 10 mM HCl. A solution (0.4 ml) of 5-27 mM of the aldehyde in dimethyl sulfoxide was added dropwise at 0 ^{o}C with stirring for 30 min. To protect the active site of the enzyme during modification, 0.4 ml 0.55 mM N-acetyl-L-Tyr was used. For reduction of the Schiff base formed, 0.2 ml 40 μM sodium borohydride was used. The solution was gel-filtered on Sephadex G 25 to remove the excess reagents.

Turbidity Measurements

Turbidity measurements in 55% ethanol were carried out at 350 nm on a Hitachi U-2000 spectrophotometer at 24 ^{o}C [6]. The pathlength of the sample cell used was 10 mm. The concentration of the protein used in turbidity experiments was 150 μg/ml in 5 mM sodium phosphate buffer, pH 7.0.

Transmission Electron Microscopy (TEM)

10 μl aliquots of the protein solutions were placed on carbon-coated 400-mesh copper grids (Electron Microscopy Sciences, Washington, PA) and stained with 2% (w/v) uranyl acetate. Images were taken on a Philips CM 10 transmission electron microscope (FEI Company, Hillsboro, Oregon, USA) operating at 100 kV. Images were captured with a Megaview II Soft Imaging System, routinely at magnifications of ×46,000 and ×64,000, and analyzed with an Analy-Sis® 3.2 software package (Soft Imaging System GmbH, Münster, Germany).

RESULTS AND DISCUSSION

The structure-activity relationship of enzymes in organic solvents is not well understood. α-Chymotrypsin forms different secondary structures in water/alcohol mixtures, depending on the nature of the alcohol and the water/alcohol composition [13,14]. The aggregation of α-chymotrypsin was studied in ethanol, methanol, *tert*-butanol, dimethylformamide and acetonitrile at different concentrations. The aggregation was followed by turbidity measurements and ThT and CR binding. Amyloid-like fibril formation was observed in all of the studied organic solvents except for acetonitrile. The optimum solvent concentration necessary for amyloid-like filament formation differed in the different organic solvents. The highest extent of fibril formation after incubation for 24 h at pH 7.0 and at 24 ^{o}C was in ethanol at 55%, in methanol and in dimethylformamide at 60-70% and in *tert*-

butanol at 60-80% Fig. (**1A**,**B**). The time dependence of the aggregation of α-chymotrypsin followed by ThT binding was greatest after incubation in 55% ethanol for 5 h at 24 °C at pH 7.0 Fig. (**2A**). The aggregation of the enzyme was followed by the loss of catalytic activity Fig. (**2B**). Molecular dynamic studies of α-chymotrypsin in the presence of 2,2,2-trifluoroethanol revealed distortion of the active site of the enzyme, which may result in an inactive enzyme [15].

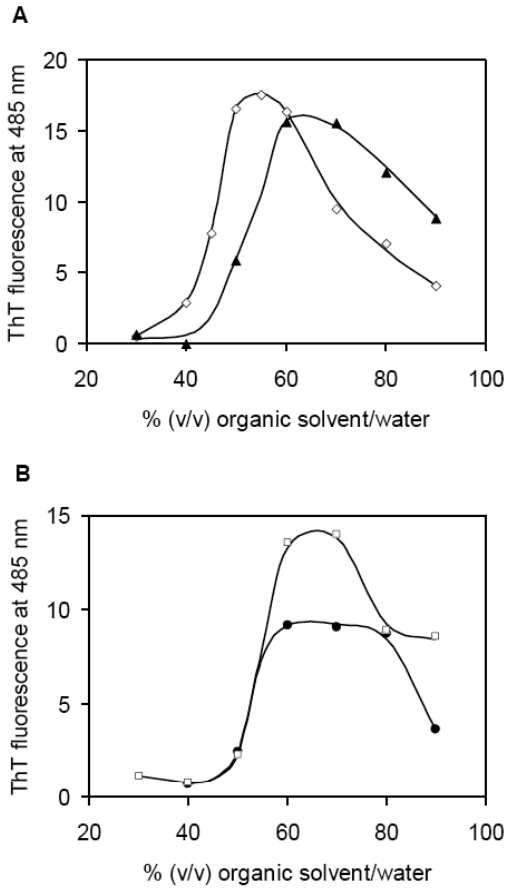


Figure 1. Dependence of ThT binding on the organic solvent concentration after incubation for 24 h at 24 °C. Enzyme concentration: 15 μg/ml. A: methanol (▲), ethanol (◊). B: *tert*-butanol (•), DMF (□)

The structural changes in the enzymes during incubation with the different organic solvents were followed through the intrinsic fluorescence, ANS binding and far-UV CD measurements. α-Chymotrypsin is an all-beta protein, folding in two antiparallel β-barrel domains, each containing six β-strands with the same topology and one short α-helix. α-Chymotrypsin contains eight Trp residues [16]. Both the solvent polarity and the protein conformation in the vicinity of the Trp residues contribute to the changes in the characteristic fluorescence parameters of Trp, the maximum intensity (I_{max}) and the maximum emission wavelength (λ_{max}) [17]. Fig. (**3**) illustrates the dependence of the intrinsic fluorescence of α-chymotrypsin on the duration of incubation in 55% ethanol. The fluorescence intensity of α-chymotrypsin increased after the addition of ethanol (t=0 h), but was then

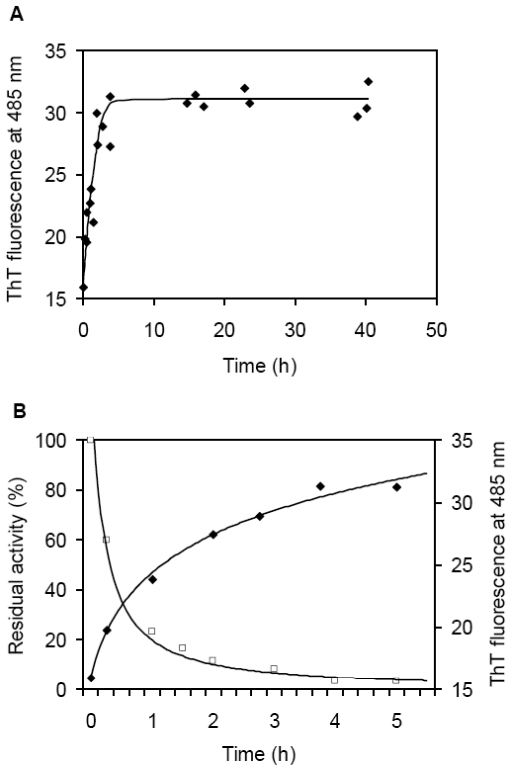


Figure 2. Time dependence of the aggregation of α-chymotrypsin in 55% ethanol, followed via ThT binding (◆) (A,B) and enzyme activity measurements (□) (B). Enzyme concentration: 15 μg/ml.

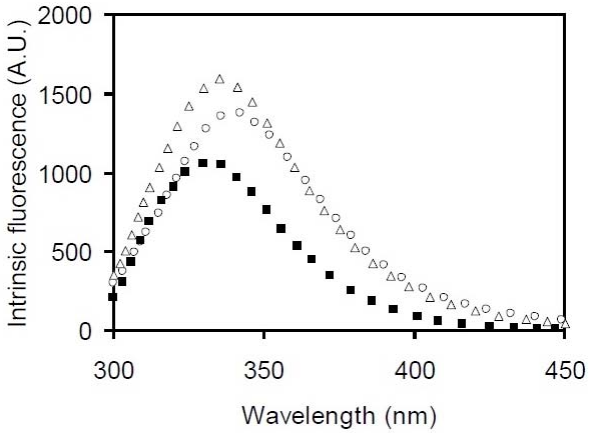


Figure 3. Intrinsic fluorescence of α-chymotrypsin in 5 mM sodium phosphate buffer (pH 7) (■), in 55% ethanol at 0 h (Δ) and at 24 h (○). Excitation wavelength: 280 nm. Enzyme concentration: 15 μg/ml.

decreased at t=24 h, accompanied by a red shift in λ_{max}. The λ_{max} value of the enzyme in water was 330 nm, in 55% ethanol it was 334 nm (t=0 h) and after incubation for 24 h it was 339 nm. The increase in I_{max} and the red shift in λ_{max} suggest structural changes in α-chymotrypsin during incubation in 55% ethanol. Similar changes were measured with chemically modified α-chymotrypsin forms too Table **1**.

ANS is widely used to detect the formation of intermediates in the folding pathway [18]. ANS binds to the hydrophobic surface or clusters in the unfolding enzyme, resulting

Table 1. Comparison of intrinsic fluorescence and dye binding of different α-chymotrypsin forms. Excitation wavelength: 280 nm. Turbidity at 350 nm: in 55% ethanol, after 10 min. ThT binding: ThT fluorescence intensity at 485 nm in 55% ethanol after 23 h

α-Chymotrypsin forms	Intrinsic fluorescence		Turbidity at 350 nm (OD)	ThT binding (A.U.)
	λ_{max} (nm) in buffer	λ_{max} (nm) in 55% ethanol		
Unmodified e – NH_2	330	339	0.53	33.6
Citraconylated e – NH – C(=O) – C(CH_3)=CH – C(=O) – O^-	330	338	0.71	46.2
Tridecyl e – NH – $(CH_2)_{12}$ – CH_3	331	340	0.32	29.3

in an increase in its fluorescence emission, accompanied by a blue shift in the emission maximum (ANS does not bind to the hydrophilic surface of the native enzyme). Fig. (**4**) presents the fluorescence of ANS bound to α-chymotrypsin in 5 mM sodium phosphate buffer (pH 7), 60% methanol and 60% DMF. After incubation for 24 h at 24 °C in these aqueous organic solvents, the binding of ANS to the enzyme resulted in an ~14 nm blue shift in λ_{max} and an increase in fluorescence intensity relative to that of the native enzyme with ANS in buffer. This indicates the exposure of some of the hydrophobic residues and conformational changes in the vicinity of the aromatic side-chains of α-chymotrypsin in these aqueous organic solvents. The increase in ANS fluorescence intensity accompanied by a blue shift in λ_{max} after binding to α-chymotrypsin, and the red shift in the intrinsic fluorescence of the enzyme after incubation for 24 h, show

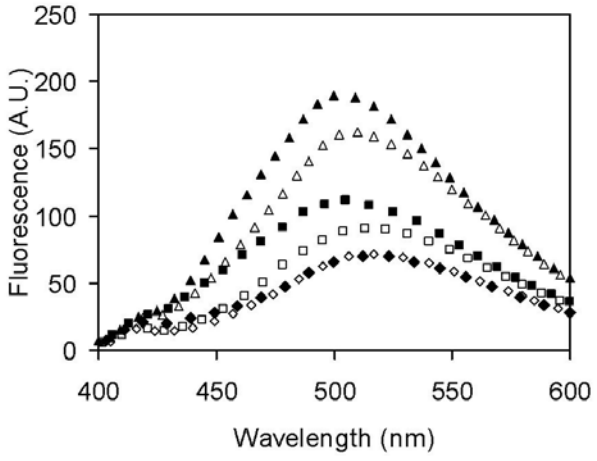


Figure 4. Fluorescence spectra of α-chymotrypsin-ANS in 5 mM sodium phosphate buffer (pH 7) (◆), in 60% methanol (■), in 60% DMF (▲) after incubation at 24 °C for 24 h, together with the spectra of ANS alone in buffer (◊), in 60% methanol (□), in 60% DMF (Δ). Enzyme concentration: 15 μg/ml. Excitation wavelength: 365 nm.

that the hydrophobic residues (including the aromatic side-chains) are more solvent-exposed in the aggregated form of α-chymotrypsin. Studies with α-chymotrypsin at high temperature and at pH 2.5 also demonstrated the higher solvent exposure of the hydrophobic and aromatic residues as compared with the native enzyme [19]. In the early stages of the trifluoroethanol-induced fibrillogenesis of α-chymotrypsin, the hydrophobicity was a major determinant [20].

CR binding to amyloid fibrils is fibril-specific. CR alone exhibits an absorption spectrum with a maximum at 490 nm. When fibrils are present, the intensity increases and the absorption maximum shifts to 510 nm. Fig. (**5**) illustrates the spectral change on adding fibrils to CR solution. The difference spectrum in the different aqueous organic solvents exhibited a characteristic shift in the absorption maximum to 540 nm respectively Fig. (**5B**,**C**).

The aggregation of the various forms of α-chymotrypsin in different aqueous organic solvents was followed by TEM. A TEM image taken after incubation for 24 h at 24 °C is presented in Fig. (**6**). Filamentous aggregates were observed in each sample, although the quality of the images was strongly influenced by the staining properties of the aggregates. In many cases, positive staining (dark object - light background) was observed instead of the common negative one (light object – dark background), possibly due to the unique composition of the surface solvate layer in the different solvent mixtures, which may influence the ability of the aggregates to adsorb. Positive staining unfortunately caused a considerable loss of structural detail, the aggregates often appearing uniformly black without any indication of the fine structure. The aggregates formed extensive three-dimensional assemblies resembling loose tangles of string in almost every case; in DMF, short, curly fibrils were seen which were intertwined or adhered to each other Fig. (**6**).

The results of far-UV CD measurements supported the structural rearrangement and amyloid-like fibril formation of α-chymotrypsin. To compare the effects of the polarity of

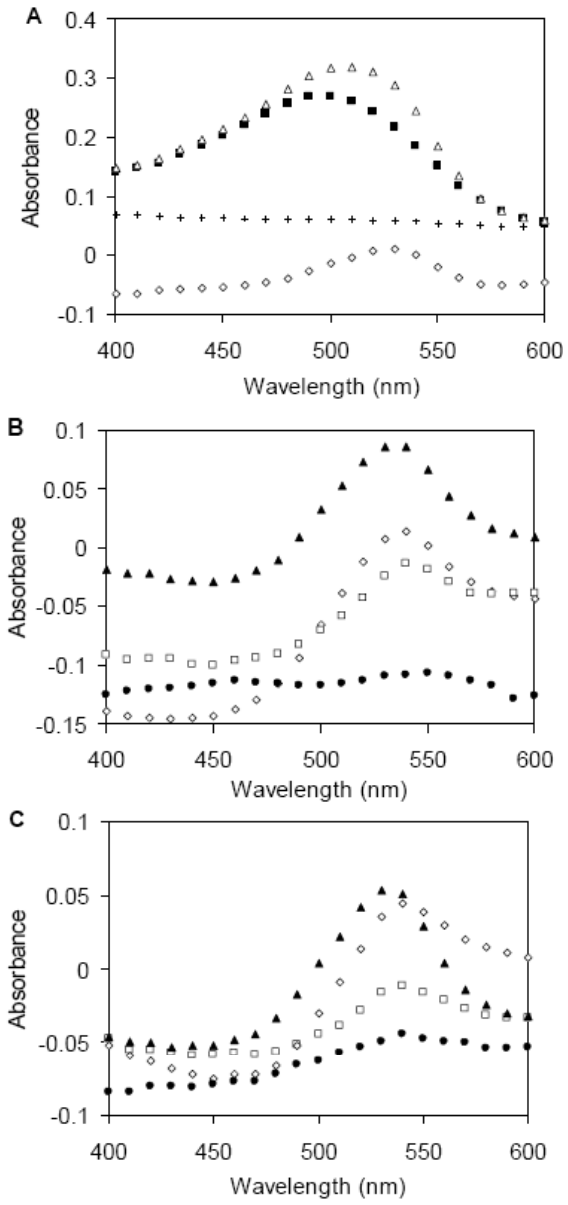
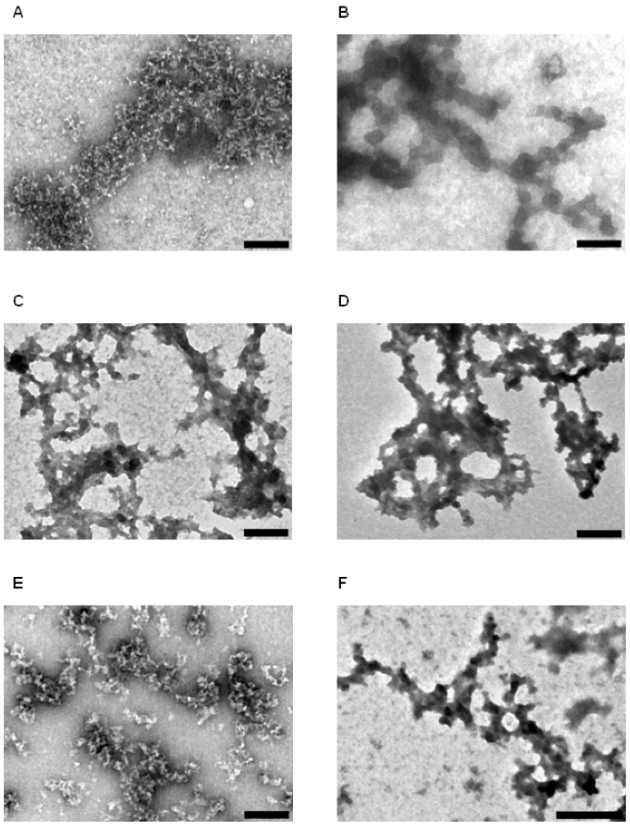
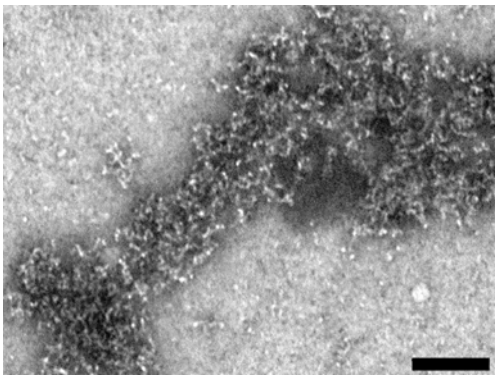


Figure 5. Congo red absorbance and difference spectra of citraconylated α-chymotrypsin (A). α-Chymotrypsin + Congo red (Δ), Congo red alone (■), α-chymotrypsin alone (+), difference spectrum in 55% ethanol (◊). (Difference spectrum: spectra of Congo red alone and α-chymotrypsin alone were subtracted from spectrum of α-chymotrypsin + Congo red). Difference spectra of unmodified α-chymotrypsin (B) and α-chymotrypsin modified with tridecanal (C) in 55% ethanol (◊), in 60% methanol (▲), in 60% *tert*-butanol (·) and in 60% DMF (□). Enzyme concentration: 8 μM, Congo red concentration: 11 μM.

different alcohols on the aggregation of α-chymotrypsin, the CD spectrum was measured in the presence of buffered 60% methanol, 55% ethanol and 60% *tert*-butanol at t=0 h and after incubation for 24 h Figs. (**7** and **8**). The relative polarities (*rp*) of these solvents are as follows: 0.762 for methanol, 0.654 for ethanol and 0.389 for *tert*-butanol (water: 1.000) [21]. α-Chymotrypsin is an all-β protein characterized by a CD spectrum which resembles that of a random-coil confor-





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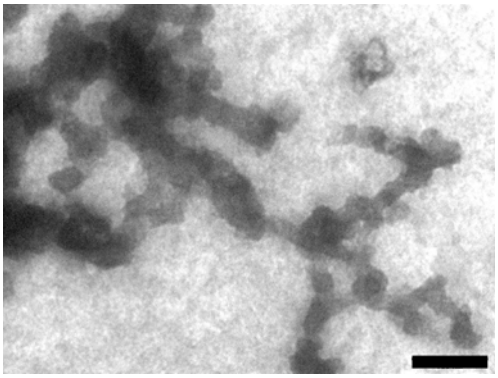

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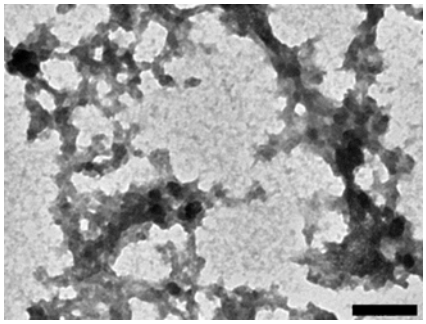

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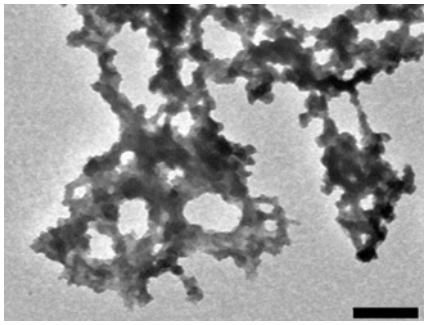

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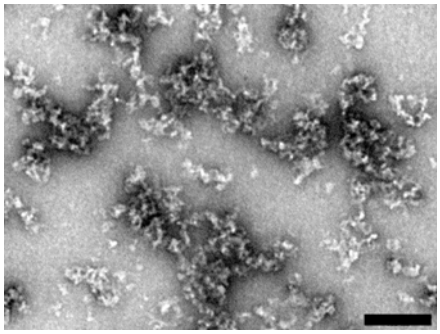

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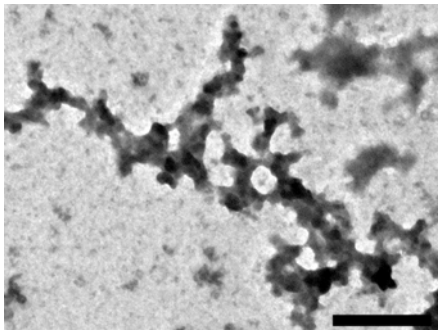

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Figure 6. TEM micrographs of modified and unmodified α-chymotrypsin samples after incubation at 24 ^{o}C for 24 h. **A:** Citraconylated α-chymotrypsin in 55% ethanol; **B:** α-chymotrypsin modified with tridecanal in 55% ethanol; **C:** α-chymotrypsin in 55% ethanol; **D:** α-chymotrypsin in 60% methanol; **E:** α-chymotrypsin in 60% DMF; **F:** α-chymotrypsin in 60% *tert*-butanol. The scale bar represents 100 nm. Enzyme concentration: 0.3 mg/ml.

mation [22,23]. The crystal structure data demonstrated that this kind of protein consists of antiparallel pleated sheets which are either highly distorted or form very short irregular strands [23]. This may cause the negative CD band to shift from the ideal β-sheet position (~216 nm) towards the lower wavelength region. Fig. (**7**) shows that the CD spectrum in 5 mM phosphate buffer at t=0 h has a negative band at ~203 nm and a low-intensity positive band below 193 nm. Incubation for 24 h resulted in decreases in intensity of both the positive and the negative band, but the position of the negative maximum did not change. In buffered 55% ethanol at t=0 h, the spectrum displays a more typical β-sheet character (a negative band at ~216 nm and an increased positive band intensity; Fig. (**8**)). On increase of the incubation time (0.5, 4 and 24 h), a gradual red shift (216 → 220 nm) and a decrease in the intensity of the negative band were observed Fig. (**8**). The intensity of the positive band was decreased significantly after incubation for 4 and 24 h. The decreases in intensity of both the positive and the negative band and the red shift in the negative band indicate twisted β-sheet enrichment in aggregates of increasing size. Similar red-shifted β-sheet spectra have been observed for a number of other peptides and the shifts have been attributed to the appearance of distorted (helically twisted) β-strands [24-26]. In methanol, the negative maximum at ~220 nm indicates the presence of twisted β-sheet already at t=0 h, together with a normal β-sheet structure (a shoulder at ~213 nm). After incubation for 24 h, the intensity of the negative maximum at 213 nm was more decreased indicating a relative enrichment of the twisted β-sheet over the normal β-sheet Fig. (**7**).

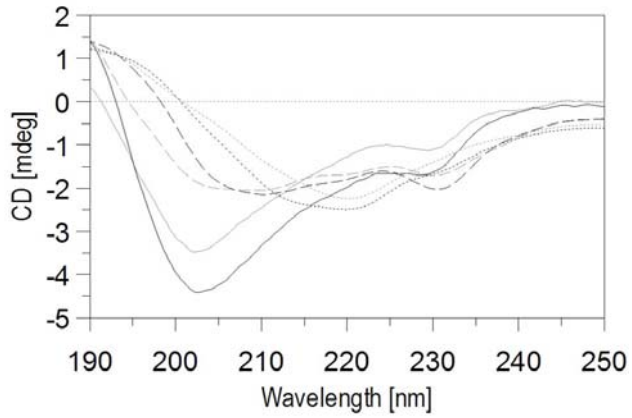

Figure 7. CD spectra of α-chymotrypsin in 5 mM phosphate buffer (pH 7.0) (——), in 5 mM phosphate-buffered 60% methanol (– –) and 60% *tert*-butanol (……). Enzyme concentration: 0.3 mg/ml. t= 0 h black lines, t=24 h grey lines.

It may be seen in Fig. (**7**) that the most apolar of these solvents *tert*-butanol, has the lowest effect on the CD spectrum of α-chymotrypsin: the spectrum underwent a red shift of only a few nm, indicating the presence of a considerable amount of irregular β-sheet besides the normal β-sheet at t=0 h. Incubation for 24 h resulted in broadening of the negative band towards the low-wavelength region of the spectrum, which is possibly due to the further formation of irregular β-strands. The CD measurements are in accordance with ThT and CR binding studies. It is generally accepted that the surfaces of cross-β structures form a ThT-binding site [*e.g*. 27].

It can be seen in Fig. (**1A**, **B**) that the increase in ThT fluorescence after incubation for 24 h was less in *tert*-butanol than in ethanol or methanol, indicating that a smaller fraction of enzyme forms the cross-β structure in *tert*-butanol.

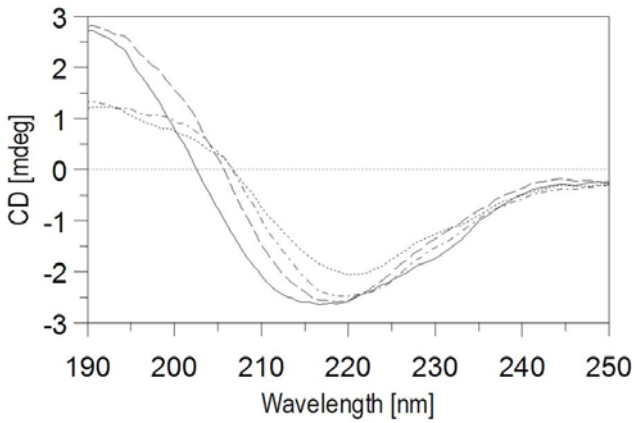

Figure 8. CD spectrum of α-chymotrypsin in buffered 55% ethanol at 0 h (——) and after incubation for 0.5 h (— —), 4 h (— · —) and 24 h (**…..**). Enzyme concentration: 0.3 mg/ml.

To determine the roles of surface charges and hydrophobicity in the aggregation of α-chymotrypsin, chemically modified forms of α-chymotrypsin (citraconylated, succinylated and modified with propanal, decanal and tridecanal) were prepared. Some measured data of citraconylated α-chymotrypsin and enzyme form modified with tridecanal are shown in Table **1**. α-Chymotrypsin contains 14 lysyl ε-amino and 3 N-terminal α-amino groups [16]. 6-9 amino groups of the enzyme were modified with both organic acid anhydrides and aliphatic aldehydes. Data on the aggregation of the citraconylated form and that modified with tridecanal are shown in Fig. (**9**), followed via turbidity measurements at 350 nm. The results of ThT binding of modified α-chymotrypsin forms are presented in Fig. (**10**). The citraconylated and succinylated forms, (data not shown) exhibited higher propensity for aggregation than that of the unmodified enzyme whereas the forms modified with aliphatic aldehydes

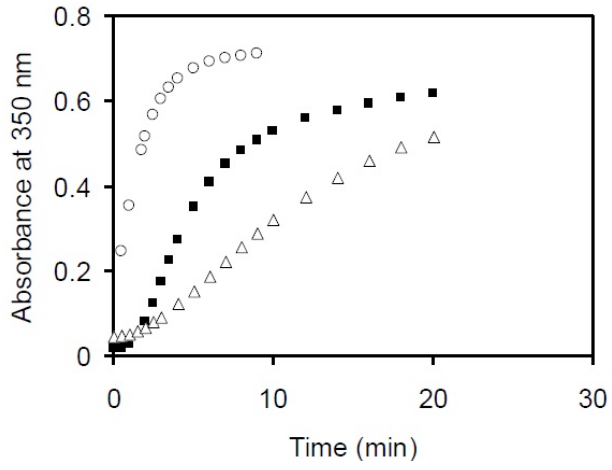

Figure 9. Changes in turbidity of different enzyme forms during aggregation in 55% ethanol. Control (■), citraconylated enzyme (○), and enzyme modified with tridecanal (Δ). Enzyme concentration: 150 μg/ml.

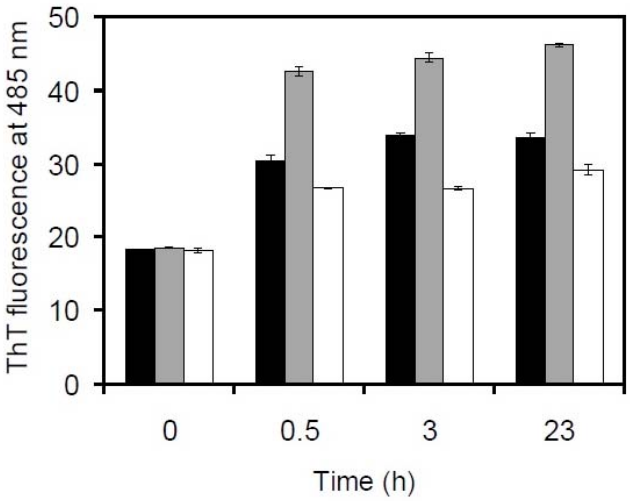

Figure 10. Time dependence of ThT binding of unmodified α-chymotrypsin (■), citraconylated enzyme (■), and enzyme modified with tridecanal (□). Enzyme concentration: 15 μg/ml.

exhibited a lower propensity. Citraconic and succinic acids each contain 2 carboxylic groups; accordingly, the positive charges of the amino groups on the enzyme are converted into negative ones, while the modifications with aldehydes result in decreases in the positive charges. The lower propensity of α-chymotrypsin modified with propanal, decanal and tridecanal for aggregation, followed either via ThT binding or via turbidity measurements at 350 nm, suggests that the surface charges play an important role in the aggregation of α-chymotrypsin.

The critical role of electrostatic interactions was demonstrated in the aggregation of α-chymotrypsin induced by 32% 2,2,2-trifluoroethanol at pH 3 [7].

CONCLUSIONS

Our results reveal that the aqueous organic solvents ethanol, methanol, *tert*-butanol and dimethylformamide may induce the amyloid-like aggregation of α-chymotrypsin. Intrinsic fluorescence and ANS binding measurements indicate that the process of aggregation is accompanied by structural rearrangement. ThT and CR binding and far-UV CD measurements indicated that the formation of a cross-β structure of α-chymotrypsin depends on the polarity of the different alcohols. Chemical modifications of α-chymotrypsin demonstrate the significance of surface charges in the aggregation of the enzyme.

ACKNOWLEDGEMENTS

This work was supported by the European Union and co-financed by the European Regional Fund, project TÁMOP 4.2.2-08/1-2008-0005.

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Received: January 31, 2012 Revised: November 29, 2011 Accepted: August 30, 2011