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Amyloid-like Fibril Formation by Trypsin in Aqueous Ethanol. Inhibition of Fibrillation by PEG

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Abstract: The formation of amyloid-like fibrils was studied by using the well-known serine protease trypsin as a model protein in the presence of ethanol as organic solvent. Trypsin forms amyloid-like fibrils in aqueous ethanol at pH = 7.0. The dye Congo red (CR) was used to detect the presence of amyloid-like fibrils in the samples. The binding of CR to fibrils led to an increase in absorption intensity and a red shift in the absorption band of CR. Thioflavin T (ThT) and 8-anilino-1-naphthalenesulfonic acid (ANS) binding assays were employed to characterize amyloid-like fibril formation. The ThT binding assay revealed that the protein exhibited maximum aggregation in 60% (v/v) ethanol after incubation for 24 h at 24 ^{o}C. The ANS binding results indicated that the hydrophobic residues were more exposed to the solvent in the aggregated form of the protein. The effects of polyethylene glycol (PEG) on the formation of amyloid-like fibrils was studied *in vitro*. The aggregation of trypsin was followed via the kinetics of aggregation, the far-UV circular dichroism (CD) and transmission electron microscopy (TEM) in the presence and absence of PEG. The CD measurements indicated that the protein aggregates have a cross-beta structure in 60% ethanol. TEM revealed that trypsin forms fibrils with a thread-like structure. The inhibitory effect of PEG on the aggregation of trypsin increased with rising PEG concentration. PEG therefore inhibits the formation of amyloid-like fibrils of trypsin in aqueous ethanol.

Keywords: Amyloid-like fibril, trypsin, organic solvent, polyethylene glycol, circular dichroism, Congo red.

1. INTRODUCTION

Approximately 50 human disorders are currently known to be associated with the misfolding of normally soluble peptides and proteins, and their subsequent conversion into insoluble, highly ordered amyloid fibrils [1]. Amyloid fibrils contribute to the complications in diseases such as the spongiform encephalopathies, Alzheimer's, Parkinson's and Huntington's diseases, type II diabetes mellitus, and primary and secondary systemic amyloidosis [2], which affect millions of people worldwide. It is important to study the behaviour not only of peptides and proteins that are associated with known diseases, but also of those that do not give rise to any pathogenic condition. This reflects a well-defined structural form of the protein that is an alternative to the native state — a form that may in principle be adopted by many, if not all, polypeptide sequences [1]. Most peptides and proteins have the potential to form amyloid fibrils under appropriate conditions**;** amyloid is a generic form of protein conformation, and the ability to form amyloid structures may be a general property of the polypeptide backbone [3-5]. The *in vitro* aggregation of water-soluble proteins can often be observed under mild denaturing conditions, at high temperature, at pH values close to the isoelectric point or in the presence of polar organic solvents [6]. Such conditions promote aggregation because they result in the partial unfolding of structured proteins and they allow the sampling of locally unfolded native-like states, both of which are possibly amyloidogenic states [7]. It has been demonstrated experimentally that the surface charges play an important role in the aggregation of proteins [8, 9].

The stabilization of the native folded structure of a protein through the action of chemical compounds can markedly inhibit its unfolding and increase the energy barrier of fibrillation, and can therefore serve as an effective therapeutic strategy against protein deposition diseases [10-12]. Recently, some authors have reported on inhibition of fibrillation as well as destabilization of preformed fibrils using polyphenols [13, 14]. Polyethylene glycols (PEGs) are nontoxic, biocompatible, water-soluble, hydrophilic non-ionic polymers that can interact with proteins through hydrogen-bonding or hydrophobic interactions [15-17]. They have potential protective effects on various types of protein denaturation [18]. PEGs with high molecular weight stabilize proteins through a preferential hydration mechanism [19, 20]. Depending on the nature of the protein and the experimental

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conditions, PEGs can facilitate or inhibit protein aggregation [21, 22]. PEG treatment after traumatic brain injury reduces the accumulation of β-amyloid precursor protein in degenerating axons [23]. Long-chain PEGs (PEG 20,000 and PEG 35,000) prevent the alkali-salt mediated fibrillation of hen egg white lysozyme at alkaline pH by stabilizing its *native* state, thereby preventing β-sheet formation [24].

Trypsin, a well-known serine protease, with a polypeptide chain of 223 amino acid residues and contains six disulfide bridges. Its catalytic triad is composed of serine, histidine and aspartic acid residues. Trypsin is a globular protein with a predominance of antiparallel β-sheet and α-helix in its secondary structure. Trypsin is composed of two domains, in both of which *6* strands form an *anti-parallel beta-barrel* in the native *structure of trypsin* [25-27]. Trypsin forms different secondary structures in organic solvents, depending on the nature and concentration of the solvent, circular dichroism (CD) measurements indicating that the extent of β-sheet structure formation varies in the range 31–61% at pH 3.0 [28, 29].

We report here the *in vitro* formation of amyloid-like fibrils by trypsin in aqueous ethanol at pH = 7.0 in the presence or absence of different polyols. However, unfortunately at pH = 7.0 autolysis proceeds. The autolysis can alter the formation of amyloid fibrils, thus it is necessary to modify the serine residue at the active site of the enzyme with PMSF. The PMS-trypsin is inactive. PMSF binds specifically to a serine residue in the active site of serine protease [30]. The amyloid-like formation by trypsin was studied by measuring the kinetics of aggregation, through thioflavin T (ThT), 8-anilino-1-naphthalenesulfonic acid (ANS) and Congo red (CR) binding assays and CD measurements, and by means of transmission electron microscopy (TEM).

2. MATERIALS AND METHODS

2.1. Materials

Trypsin (EC 3.4.21.4; from the bovine pancreas), ANS, ThT (4-(3,6-dimethylbenzothiazol-2-yl)-N,N-dimethylaniline), and *N*-benzoyl-L-arginine ethyl ester (BAEE) were purchased from Sigma-Aldrich. PEG (average molecular weight 2000) was from Fluka. All other reagents and buffer components used were of analytical grade.

2.2. Enzymatic activity assay

The measurement of trypsin activity was based on spectral differences between BAEE and the carboxylate form of *N*-benzoyl-L-arginine. Enzymatic activity was determined according to Schwert and Takenaka [31] by measuring the increase in absorbance at 253 nm in a reaction mixture at pH 8.0. Reactions were initiated by the addition of 20 µl 0.15 mg/ml enzyme solution from the different samples.

2.3. Modification of trypsin with phenylmethylsulfonyl fluoride (PMSF)

The reaction of trypsin with PMSF modifies the serine residue at the active site of the enzyme, inactivating it. This is necessary because the autolysis of trypsin at neutral pH can alter its formation of amyloid fibrils. Solutions of 8.4 µl PMSF (100 mM) in 2-propanol and 20 µl trypsin (50 mg/ml in 0.001 N HCl) were added at 24 ^{o}C to 2 ml 0.05 M potassium phosphate buffer (pH = 7.0) with stirring. Such aliquots were added to the buffer on four occasions. The reaction was allowed to proceed for 30 min. To remove the excess reagent, the solution was then filtered on Sephadex G-25, followed by elution with 0.05 M potassium phosphate buffer (pH = 7.0). The enzymatic activity assays revealed that 95% of the trypsin was modified.

2.4. ANS binding

ANS binding assays were performed on a Hitachi F-2500 FL fluorescence spectrophotometer, using 50 µM ANS solution with 30 µg/ml protein, in the absence or presence of 60% ethanol. The fluorescence spectra were recorded 30 min after the addition of ANS to the protein aliquots. The excitation wavelength was 365 nm and the emission was collected between 400 nm and 600 nm. The emission and excitation monochromator slit widths were 10 and 5 nm, respectively.

2.5. ThT binding assay

Trypsin fibril formation was monitored in ThT binding assays. Samples containing 300 µg/ml trypsin were incubated at 24 ^{o}C for 24 h in various concentration of ethanol. 100 µl of protein sample was then diluted into buffer (10 mM phosphate, 150 mM NaCl, pH 7.0) containing 65 µM ThT. 5 min after the addition of the protein aliquot, the fluorescence intensity was recorded. The emission was measured at 485 nm and the excitation wavelength was 440 nm. The emission and excitation monochromator slit widths were 10 and 5 nm, respectively.

2.6. CR binding

Amyloid-specific CR binding assays were carried out in 5 mM phosphate buffer (pH 7.0) containing 150 mM NaCl. The final concentrations of CR and protein were 11 µM and 26 µg/ml, respectivelly. Aliquots (200 µl) taken from 1-day-aged trypsin samples containing 60% ethanol at pH = 7.0 were combined with 0.8 ml CR solution, and incubated for 15 min at room temperature before the measurements. The absorption spectra of CR were recorded in the wavelength region 400–600 nm in a 10 mm pathlength cell. The presence of trypsin amyloid-like fibrils was indicated by characteristic spectral changes: a red shift of the absorption band and an increase in absorption intensity. The spectra of CR alone and the protein alone were subtracted from spectrum of trypsin + CR, yielding the difference spectrum. The shape of the difference spectra clearly revealed the spectral change in the presence of amyloid fibrils.

2.7. Turbidity measurements

Turbidity at 350 nm was followed at 24 ^{o}C in a 10 mm pathlength cell in the presence or absence of different polyols in 60% ethanol. The PMS-trypsin concentration in the turbidity experiments was 0.15 mg/ml in 10 mM phosphate buffer, pH 7.0. The samples were incubated for 24 h *before measurements*.

2.8. Aggregation kinetics

Aggregation kinetic assays were performed in buffered 60% ethanol on a Hitachi U-2000 spectrophotometer by monitoring the absorption at 350 nm during 30 min at 24 °C in the presence or absence of PEG 2000 at pH 7.0. The enzyme concentration was 0.13 mg/ml in 60% ethanol.

2.9. CD measurements

CD measurements were used to determine the changes in protein secondary structure in the peresence of 60% ethanol. CD spectra were recorded in the far UV range from 185 to 260 nm, in a 0.01 cm pathlength quartz cell on a Jasco J-815 CD spectrometer at 24 °C. The PMS-trypsin was dissolved in potassium phosphate buffer (final concentration 10 mM, pH 7.0). The CD spectra were recorded in the presence of 50 mg/ml PEG 2000 in buffered 60% ethanol, in buffered 60% ethanol and in buffer. The enzyme concentration was 0.15 mg/ml. The ellipticity, (Θ), was expressed in mdeg.

2.10. 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, USA) and stained with 2% (w/v) uranyl acetate. Images were taken on a Philips CM 10 transmission electron microscope (FEI Company, Hillsboro, OR, 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 analysed with an Analy-Sis® 3.2 software package (Soft Imaging System GmbH, Münster, Germany).

3. RESULTS AND DISCUSSION

A variety of experimental methods, including ThT, ANS, CR binding assays, aggregation kinetics, turbidity and CD measurements and TEM were employed to follow the aggregation of the enzyme. Amyloid-like fibril formation was observed in aqueous ethanol at pH 7.0. The aggregation of trypsin was studied in ethanol at different concentrations. The ThT fluorescence intensity at 485 nm increases markedly upon binding to the surface of the crossed β-sheet structured amyloid fibrils [32]. The investigations of ThT binding demonstrated that maximum fibril formation was observed in 60% ethanol after incubation for 24 h at 24 °C (Fig. **1**).

Hydrophobic interactions of ANS with proteins is one of the methods commonly used in studies of protein folding and amyloid fibril formation for the detection and characterization of partially folded and molten globule states of proteins. ANS is a non-covalent extrinsic fluorescent probe; its quantum yield is increased up to 100 times upon binding to hydrophobic regions of a protein, together with a blue shift of the emission maximum [33, 34]. We succeeded in determining the loosening of the protein structure by ANS binding to the hydrophobic region of the partially unfolding protein in 60% ethanol. The ANS fluorescence proved to be increased and its binding was accompanied by a blue shift of 22 nm (from 512 nm to 490 nm) upon binding with the protein (Fig. **2**). This clearly indicates the conformational changes of trypsin in the presence of ethanol.

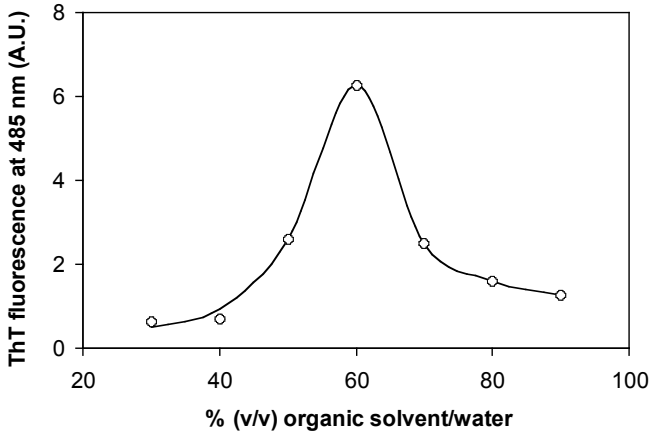


Figure 1. Dependence of ThT binding on the ethanol concentration after incubation for 24 h at 24 °C (○); enzyme concentration: 30 µg/ml.

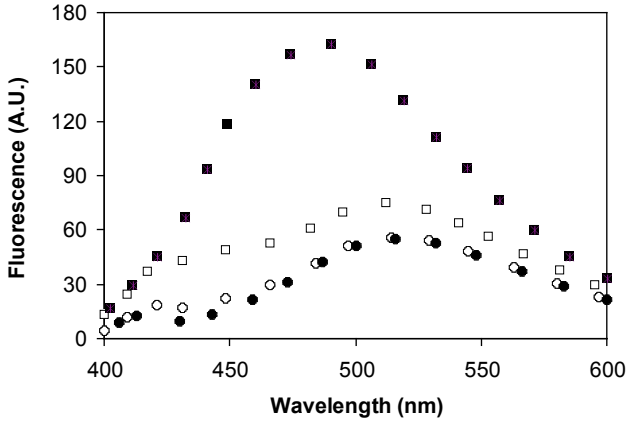


Figure 2. Fluorescence spectra of trypsin-ANS in 5 mM sodium phosphate buffer (pH = 7.0) (●), in 60% ethanol (■) after incubation at 24 °C for 24 h, and of ANS alone in buffer (○) and in 60% ethanol (□); enzyme concentration: 30 µg/ml.

The turbidity measurements were performed to detect the effects of different polyols on the aggregation of the protein. These experiments revealed that the greatest effect was exerted by PEG 2000 in 60% ethanol. In the presence of 50 mg/ml PEG 2000, the absorption at 350 mn had decreased to 41.9% after incubation for 24 h relative to the sample not containing PEG 2000 (Fig. **3**). Our results have shown, that the number of the hydroxyl groups of the sugars not only are responsible for their inhibitory potency, but also the configuration of hydroxyl groups are important. The D-ribose and the L-arabinose contains the same number of hydroxyl groups, however their inhibitory effect are different.

The aggregation kinetic assays were performed to establish the effects of PEG 2000 on the protein aggregation. The kinetics of aggregation of PMS-trypsin in 60% ethanol was investigated by monitoring the time-dependent changes in the absorption at 350 nm in the presence or the absence of PEG 2000 at pH = 7.0. The absorption at 350 nm was monitored for 30 min, with readings every minute. It has been demonstrated that the secondary structure-retaining effect of

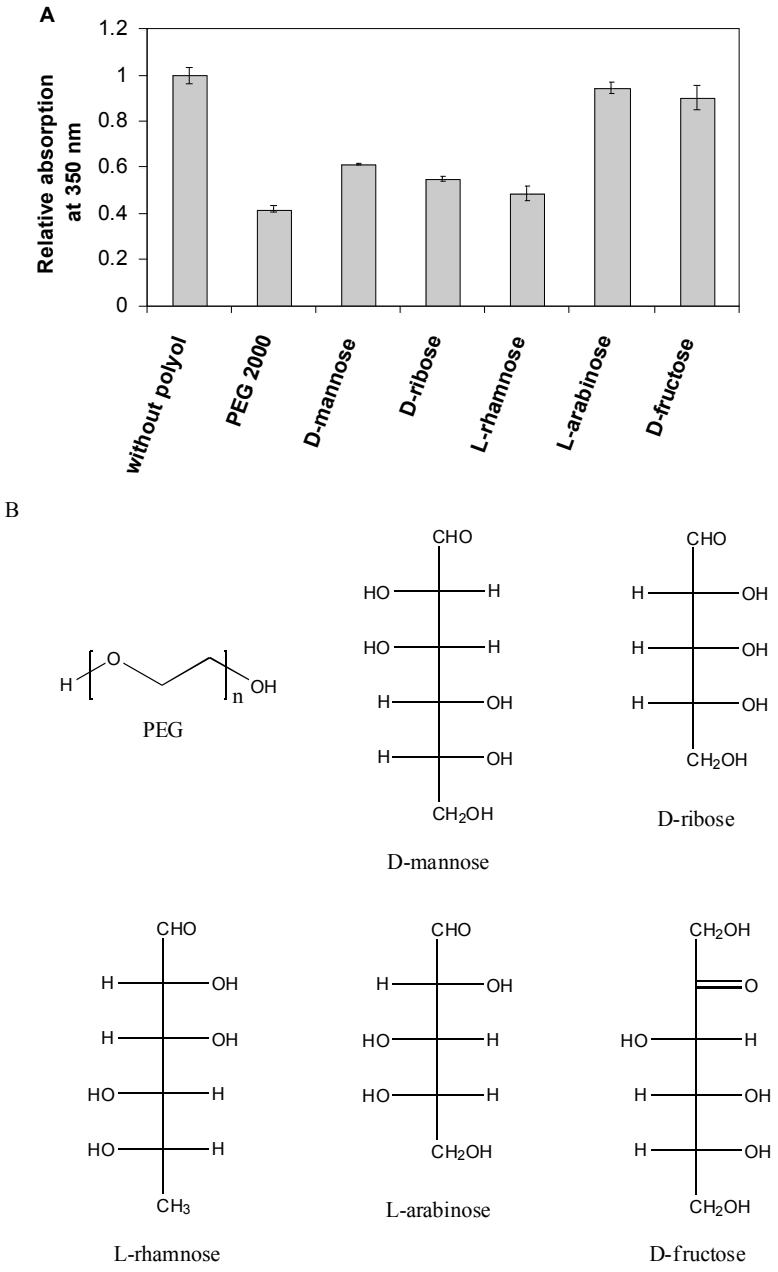


Figure 3 (**A**). Turbidity changes of PMS-trypsin at 24 °C at pH 7.0 in 60% ethanol, monitored via the absorption at 350 nm after incubation for 24 h in the presence of various polyols or without polyol. Polyol concentration: 50 mg/ml, PMS-trypsin concentration: 0.15 mg/ml. (**B**) The chemical structures of the studied polyols.

polyols increases with increasing concentration and number of hydroxy groups [35]. PEG 2000 moderated the formation of amyloid-like fibrils to an extent depending on its concentration in 60% ethanol (Fig. **4**), indicating that PEG 2000 has a stabilizing effect on the protein structure. The inhibitory effect of PEG 2000 on the aggregation increased with rising concentration. These findings led to the conclusion that the stability of the native structure of trypsin can be increased by the presence of PEG, and aggregation can therefore be inhibited through the application of PEG.

The results of CD measurements support the changes in the secondary structure of protein in 60% ethanol. The CD spectra of PMS-trypsin were measured in the presence of PEG 2000 in buffered 60% ethanol and without PEG 2000 as compared with the CD spectrum of the protein in 10 mM phosphate buffer at pH 7.0 (Fig. **5**). The CD spectrum of PMS-trypsin in 10 mM phosphate buffer exhibited a positive maximum at 186 nm with an intense broad minimum between 211 and 217 nm, thus the protein consists of a mixture of alpha-helices and beta-sheets. This spectrum indicates the dominance of the structure of the β-sheet, with the presence of the α-helix. The maximum at 186 nm is primarily indicative of the presence of the α-helix. The maximum of the protein with β-sheet structure is observed at around 195-200

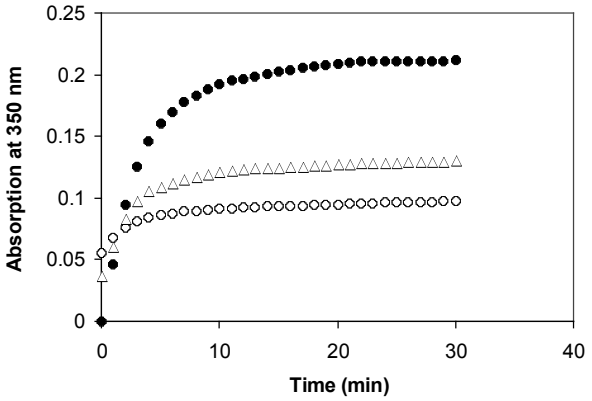


Figure 4. Kinetics of aggregation of PMS-trypsin in 60% ethanol without PEG 2000 (•), or in the presence of 50 mg/ml PEG 2000 (○) of 25 mg/ml PEG 2000 (Δ), monitored via the time-dependent increase in absorption at 350 nm at pH 7.0. Enzyme concentration: 0.13 mg/ml.

nm. In the case of the sample containing 60% ethanol, the maximum absorption characteristic of the α-helix cannot be seen. The positive band at 195 nm and the negative band at 220 nm point to the β-sheet structure of the polypeptide chains, which is characteristic of amyloid fibrils. In the presence of 60% ethanol and 50 mg/ml PEG 2000, the maximum at 200 nm an intense negative band at 217-220 nm indicate the β-sheet structure. However, the discrete rise at 186 nm, the shoulder at 210 nm on the broad minimum, and the stronger intensity as compared with that of the sample in 60% ethanol may refer to the partial presence of the α-helix. The partial presence of the α-helix shows that PEG stabilizes the native structure of the protein.

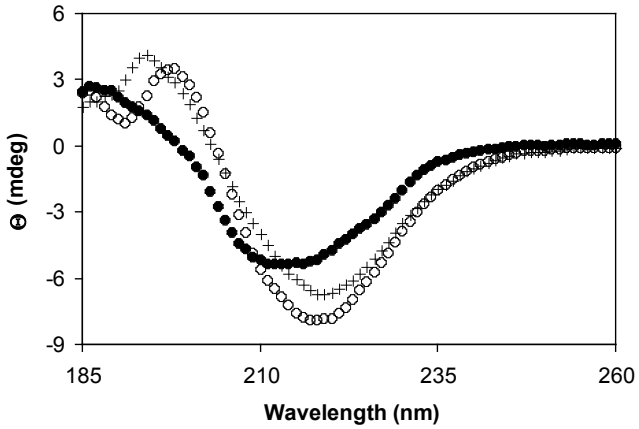


Figure 5. CD spectra of PMS-trypsin in 10 mM phosphate buffer (pH 7.0) (•), in 10 mM phosphate-buffered 60% ethanol without PEG 2000 (+) and in the presence of 50 mg/ml PEG 2000 (○); enzyme concentration: 0.15 mg/ml.

One of the methods most commonly used for the detection of amyloid fibril formation is the CR binding assay. CR exhibits maximum absorption at 490 nm. Its binding to β-sheet-rich amyloid fibrils induces a characteristic increase in absorption intensity and a red shift in the absorption band of CR from 490 to 540 nm [36]. Binding of the dye was detected both by an increase in the absorption intensity and by a red shift in the absorption maximum. The changes were better quantified by the difference spectrum between the dye-free situation and that when CR was incubated with the amyloid fibrils. The maximum spectral difference in ethanol in the absence of PEG 2000 was observed at 550 nm (Fig. **6**). CR binding experiments suggested that the aggregates have amyloid-like properties. PEG 2000 is capable of inhibiting trypsin amyloid-like fibril formation *in vitro* in ethanol.

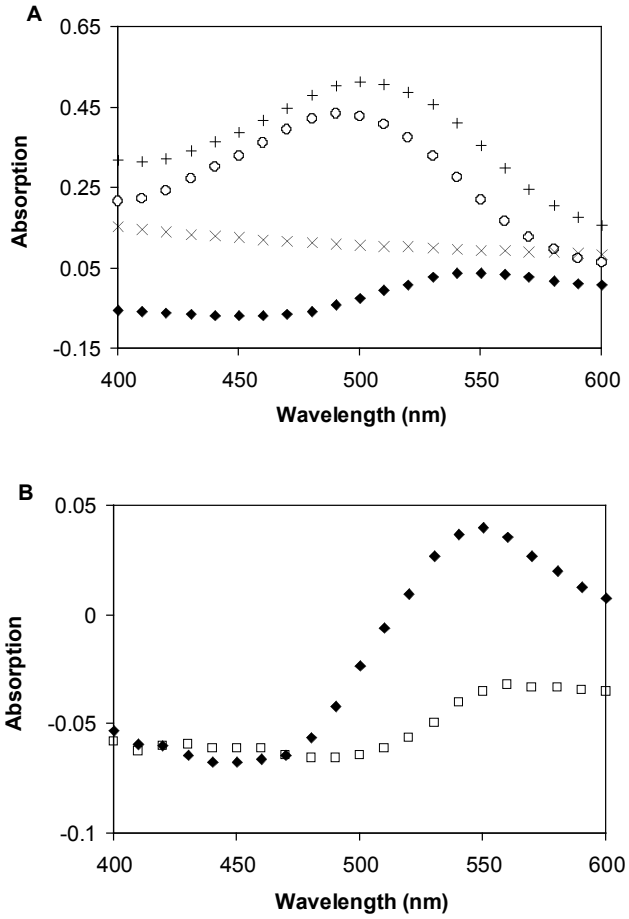
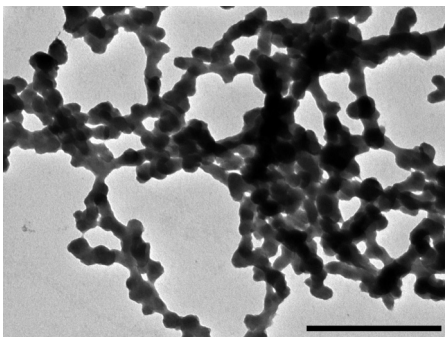


Figure 6. CR absorption and difference spectra of PMS-trypsin (**A**). PMS-trypsin + CR (+), CR alone (○), PMS-trypsin alone (x), difference spectrum in 60% ethanol (♦). Difference spectra of PMS-trypsin in 60% ethanol (**B**) in the presence of 50 mg/ml PEG 2000 (□) and without PEG 2000 (♦). Final protein concentration: 26 μg/ml.

TEM images recorded after incubation for 24 h at 24 °C in the presence or the absence of PEG 2000 revealed that the trypsin aggregates have a fibrillar morphology in 60% ethanol (Fig. **7**). The inhibitory effect of PEG 2000 on trypsin fibrillation was confirmed by TEM. In the presence of PEG we can observe the formation of non-fibrillar aggregates instead of the formation of amyloid fibrils.

A



B

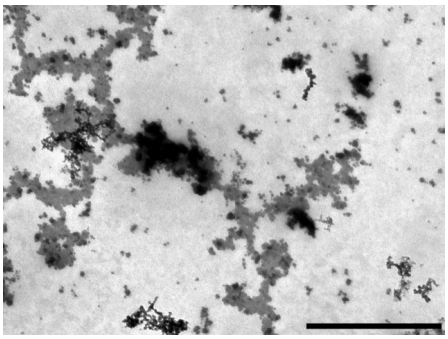


Figure 7. TEM micrographs of trypsin samples after incubation at 24 °C for 24 h. PMS-trypsin in 60% ethanol (**A**), PMS-trypsin in 60% ethanol in the presence of 50 mg/ml PEG 2000 (**B**). The scale bar indicates 1 μm. Enzyme concentration: 0.13 mg/ml in ethanol.

In summary, we have demonstrated that trypsin forms amyloid-like fibrils at pH 7.0 at 24 °C in aqueous ethanol. The greatest extent of formation of amyloid-like fibrils occurred in 60% ethanol. PEG 2000 retarded trypsin aggregation because it stabilizes the native structure of trypsin against deleterious conformational changes, preventing protein–protein aggregation interactions. PEG 2000 inhibits the amyloid-like fibril formation of trypsin in ethanol.

LIST OF ABBREVIATIONS

ANS	=	8-anilino-1-naphthalenesulfonic acid
BAEE	=	N-benzoyl-L-arginine ethyl ester
CD	=	circular dichroism
CR	=	Congo red
PEG	=	polyethylene glycol
PMSF	=	phenylmethylsulfonyl fluoride
TEM	=	transmission electron microscopy
ThT	=	thioflavin T

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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