

Amyloid fibril formation by pepsin in neutral pH at room temperature†

Cite this: *Soft Matter*, 2013, **9**, 11457

Subhajit Ghosh, Subhrajyoti Dolai and Joykrishna Dey*

Received 12th September 2013

Accepted 29th October 2013

DOI: 10.1039/c3sm52410h

www.rsc.org/softmatter

Herein we report for the first time fibril formation by pepsin at neutral pH and room temperature. Fibril formation was characterized by a number of techniques including fluorescence and circular dichroism spectroscopy, dynamic light scattering, and transmission electron microscopy.

Amyloid fibril formation has gained immense biological and clinical importance in recent years. From a clinical point of view amyloid formation has fatal consequences. It involves several diseases including neurodegenerative disorders such as Alzheimer's, Parkinson's and prion diseases.^{1–5} The mechanism of the formation of amyloids is not only important for the treatment of amyloidosis related diseases but also in the development of novel biomaterials in nanobiotechnology.^{6,7} Proteins are very important functionalizing materials, and amyloid fibrils are a class of protein structure that are very interesting in biology and physical science. There are many reports on amyloid formation by BSA, HSA and lysozyme either in acidic conditions or in the presence of denaturing agents or under heat-induced conditions.^{8,9} Initially, the protein forms unstable intermediates and then aggregation occurs.

Studies pertaining to the formation of amyloid fibrils at neutral pH are extremely important since numerous amyloid-related pathogeneses have been identified under similar conditions. In order to understand the mechanism of fibril formation by proteins, we chose pepsin which is a well-known proteolytic enzyme. Pepsin is secreted from the chief cells in the stomach to degrade food proteins into peptides in the presence of stomach acid. Pepsin attacks most proteins except keratin, nail and other carbohydrate-rich proteins. This is why pepsin is widely used in the detergent industry to remove proteinaceous stains (*e.g.* blood

stains, egg, *etc.*) as well as in the laboratory analysis of different proteins to cleave the bonds between aromatic amino acids. Other applications of pepsin include the preparation of cheese and various protein-containing food-stuffs.

Porcine pepsin is a gastric aspartic protease. It is a single-chain protein with a secondary structure consisting mostly of pleated sheets (see Fig. 1).¹⁰ It is reported that pepsin undergoes a conformational change from the native state to a partially denatured state under a narrow pH range between 6 and 7 (ref. 11 and 12) which is also supported by the results of our steady-state fluorescence anisotropy (*r*) measurements at different pHs (Fig. S1, ESI†). The first part (pH < 5) of the titration curve represents the conformational change of pepsin due to deprotonation of the amino acid residues, while the sigmoid decay of anisotropy at pH > 6 corresponds to the alkaline denaturation of pepsin. The objective of the present study is to investigate if pepsin exhibits amyloid fibril formation at neutral pH. The details of the experimental measurements are described in the ESI.†

One of the most commonly used methods for the detection of amyloid fibril formation is the increase of the fluorescence intensity of thioflavin T (ThT).^{13–17} In fact, the enhancement of ThT fluorescence is considered to be an indication of amyloid fibril formation in many *in vitro* fibrillation studies.^{18–21} The binding of ThT to amyloid fibrils has been reported to be highly

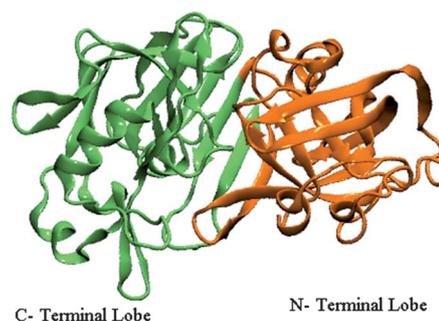


Fig. 1 Structure of porcine pepsin (PDB identification code 4PEP).

Department of Chemistry, Indian Institute of Technology Kharagpur, Kharagpur-721 302, India. E-mail: joydey@chem.iitkgp.ernet.in; Fax: +91-3222-255303; Tel: +91-3222-283308

† Electronic supplementary information (ESI) available: details regarding the materials and experimental techniques employed in this work. See DOI: 10.1039/c3sm52410h

specific as it does not interact with proteins in the native, completely or partially unfolded states, and with amorphous aggregates of proteins. Based on angle-dependent fluorescence polarization measurements, Krebs *et al.* have proposed a model in which ThT binds in monomeric form to the amyloid fibril in parallel to the axis of the fibril.²² This was later confirmed by molecular modeling and near-field scanning optical microscopic measurements.²³ In a recent review,²⁴ the current status of ThT in the diagnosis of amyloid fibrils was discussed. Although there are several explanations,²⁴ including fluorescent dimer²⁵ and excimer^{26,27} formation by ThT molecules upon incorporation into amyloid fibrils, the enhancement of ThT fluorescence is now attributed to the decreased torsional relaxation in the excited molecule.^{28–34}

In order to study amyloid formation by pepsin, we monitored the steady-state fluorescence of ThT in buffered aqueous solutions of pepsin. The fluorescence emission spectra of ThT measured at room temperature (30 °C) are depicted in Fig. 2. For comparison purposes, we have also included the ThT fluorescence spectrum in phosphate buffer (pH 7) without any pepsin. In the absence of pepsin, however, no change of ThT fluorescence was observed upon ageing. It can be observed that for a given pepsin concentration, the emission intensity increased with the increase of incubation time (Fig. 2(a)), clearly suggesting amyloid formation. Interestingly, a lag time of *ca.* 36 h was observed to exist in the early periods of incubation, suggesting the existence of a nucleation step. The presence of a lag phase is related to the changes in the nature and strength of the intermolecular interactions upon incubation. During this time a fast formation of small oligomers occurs, which need to reach a critical concentration and more time to grow to form larger aggregates. This is also supported by the results of dynamic light scattering (DLS) measurements. It is interesting to note that the emission spectrum after incubation for 7 days exhibits a small blue shift relative to the spectrum obtained after 5 days incubation. This might be an

indication of ThT binding to another less polar site which has no β -structure typical of amyloid fibrils. In fact, binding of ThT to the P-site of acetylcholinesterase with high selectivity has been reported recently.³⁵

For a fixed incubation time (7 days), the ThT fluorescence intensity increased almost eight-fold (Fig. 2(b)) as the pepsin concentration increased from 0.025 to 0.2% (w/v). Thus we can conclude that amyloid formation is facilitated when pepsin concentration is increased. Furthermore, the fibril formation by pepsin was monitored by a ThT fluorescence assay at different pHs. The spectra measured at different pH after 5 days of sample preparation are presented in Fig. 2(c). From the figure it can be seen that the enhancement of ThT fluorescence is highest at pH 7. Both above and below this pH, ThT fluorescence enhancement was observed to be less which means fibril formation is optimal only at pH 7. This is indicated by the plot (Fig. 2(d)) of the fluorescence intensity *versus* pH. Since the N-terminal lobe of pepsin is denatured at pH 7, the fibril formation can be attributed to the conformational change in the N-terminal lobe which is more mobile. At pH >7, the pepsin is in an alkaline-denatured state (I_p) as a result of the ionization of some buried aspartic acid (Asp¹¹ and Asp¹⁵⁹) residues.¹¹ Since the alkaline denaturation is irreversible in nature, fibril formation is inhibited. The decrease in ThT fluorescence at alkaline pH further confirms its specificity to amyloid fibrils.

In order to study the conformational change, we measured circular dichroism (CD) spectra (Fig. 3) of pepsin in phosphate buffer (pH 7) at different incubation periods. The CD spectrum obtained immediately after the solution preparation clearly shows that pepsin is predominantly a β -sheet rich protein as indicated by a minimum at *ca.* 215 nm. After 12 h, the 215 nm band exhibits a shift to 208 nm which might be an indication of oligomer formation. However, the depth of the minimum decreased by a large extent when incubated for 7 days. The spectrum is characterized by the appearance of two new minima at *ca.* 225 nm and 208 nm, which suggests the formation of a non-native helical structure at the expense of β -sheet conformation. This non-native α -helix structure is more prone to aggregation³⁶ as indicated by the increase in aggregate size as per the discussion below.

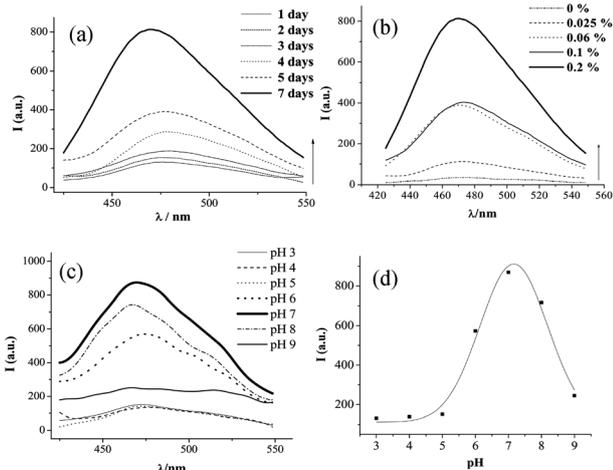


Fig. 2 Fluorescence spectra (uncorrected) of ThT (a) in 0.2% pepsin at different time intervals, (b) at different pepsin concentrations, and (c) in 0.2% pepsin at different pHs; (d) the variation of fluorescence intensity of ThT in 0.2% pepsin with pH.

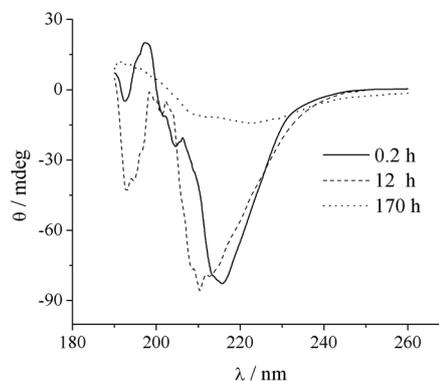


Fig. 3 CD spectra of pepsin (0.2%) at different periods of incubation in neutral buffer at room temperature.

Because amyloid fibrils have an aspect ratio much larger than that of native globular proteins, fibril formation was also tested by DLS measurements. Fig. 4 depicts the hydrodynamic size distributions in the protein solutions after different periods of incubation. It is observed that there is a substantial increase in the hydrodynamic diameter (d_h) of pepsin when incubated above 8 h. This is consistent with the steady-state fluorescence and CD spectral data described above. This must be associated with the aggregation of partially unfolded pepsin molecules forming first oligomeric structures which slowly grow to form long fibrils.

In order to visualize the amyloid fibrils we have taken transmission electron microscopic (TEM) images of 0.2% pepsin solution. The TEM image shown in Fig. 5(a) clearly reveals the presence of long unbranched microfibers of high aspect ratio. The micrograph in Fig. 5(b) shows that the globular aggregates fuse to form large rod-like aggregates (oligomers) which grow to produce long flexible fibrils. The thicker fibrils are probably formed by the lateral assembly of two or more individual fibrils. This supports the results obtained from the fluorescence, CD and DLS measurements.

It is clear from the fluorescence probe, CD, and DLS studies that the fibrillation kinetics are very slow. This is illustrated by only a forty-fold increase of ThT fluorescence in 0.2% pepsin solution (Fig. 2) with respect to the pH 7 buffer even after 7 days. Recalling that the ThT intensity is at a maximum for the fully amyloid fibril structure, the increment of the fluorescence intensity is expected to be less in the intermediate step, such as oligomer formation. The fibrillation kinetics of pepsin were measured at pH 7 and room temperature. Fig. 6 shows the variation of the ThT fluorescence intensity with time. The fibrillation rate constant of pepsin was observed to be $ca. 38 \times 10^{-2}$ per day, but in the presence of 100 mM NaCl the rate constant decreased to $ca. 28 \times 10^{-2}$ per day. This suggests that the salt has a role in the fibrillation phenomena. In other words, electrostatic interaction has a significant effect in the fibrillation process. As reflected in the rate constant values, NaCl retards the fibrillation process, which may be attributed to a structural change of the pepsin at a moderate salt concentration. Unlike BSA, HSA, lysozyme *etc.* where normal salt effects enhance the fibrillation by shielding the intermolecular

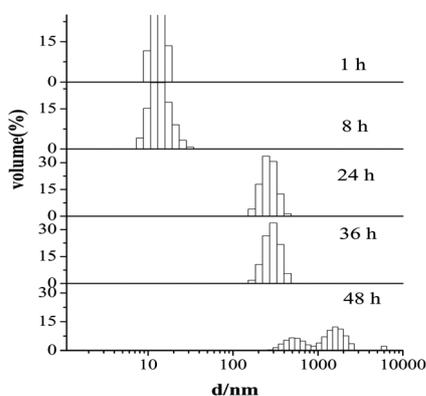


Fig. 4 The hydrodynamic size distributions of pepsin (0.2%, w/v) solution (pH 7) at different periods of incubation at room temperature.

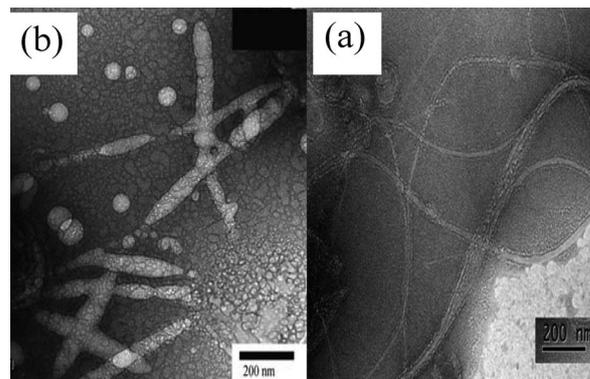


Fig. 5 Transmission electron micrographs of pepsin (0.2%) after 7 days of incubation at pH 7 and room temperature.

electrostatic repulsions,⁸ pepsin may go to a relatively stable state at moderate salt concentration and hence retard fibrillation. Also it should be noted that the rate of amyloid formation by pepsin is much slower compared to BSA⁹ or HSA.⁸

In summary, we have shown that pepsin forms amyloid fibrils in neutral pH (pH 7) at room temperature. Less amyloid formation occurred at pH values higher and lower than pH 7. The pH dependence of fibril formation suggests that first conformational changes occur to the pepsin at pH 7, which then self-assemble to form oligomers and finally to fibrils of high aspect ratio. Since the partial unfolding of pepsin exposes the hydrophobic parts of the protein, the hydrophobic interaction between the proteins contributes to the oligomer and hence fibril formation. The fully alkaline-denatured state (I_p) of the pepsin, however, is not a precursor of amyloid fibrils. Mainly the N-terminal lobe of pepsin which is relatively mobile in pH 7 facilitates the protein aggregation process forming fibrils. The rate of amyloid formation was observed to be much slower than that of BSA or HSA which undergo amyloid formation at low pH (3.0) and high temperature (65 °C). In contrast to BSA or HSA, sodium chloride (NaCl) was observed to play an important role in the retardation of the fibrillation process. In the case of pepsin, NaCl imparts an extra stability to the protein structure. A detailed investigation on the effect of additives, temperature and concentration on the kinetics of amyloid formation is currently under way in this laboratory.

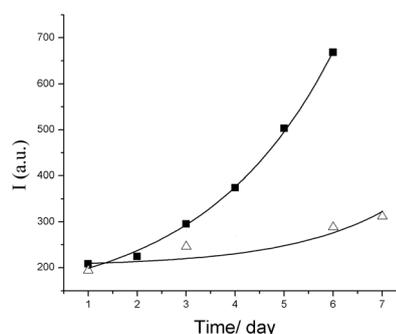


Fig. 6 Fibrillation kinetics in 0.2% pepsin solution at pH 7 and room temperature in the absence (■) and presence (Δ) of 100 mM NaCl.

Acknowledgements

We thank the Indian Institute of Technology Kharagpur for partial support of this work. SG thanks UGC, New Delhi for a research fellowship. We thank Dr. N. Sarkar for the DLS measurements.

Notes and references

- 1 A. T. Alexandrescu, *Protein Sci.*, 2005, **14**, 1–12.
- 2 K. Lundmark, G. T. Westermark, A. Olsen and P. Westermark, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 6098–6102.
- 3 M. B. Pepys, *Annu. Rev. Med.*, 2006, **57**, 223–241.
- 4 G. Jones and R. Mezzenga, *Soft Matter*, 2012, **8**, 876–895.
- 5 H. Jang, L. Connelly, F. T. Arce, S. Ramachandran, R. Lal, B. L. Kagan and R. Nussinov, *Phys. Chem. Chem. Phys.*, 2013, **15**, 8868–8877.
- 6 C. Li, J. Adamcik and R. Mezzenga, *Nat. Nanotechnol.*, 2012, **7**, 421–427.
- 7 T. P. Knowles, A. W. Fitzpatrick, S. Meehan, H. R. Mott, M. Vendruscolo, C. M. Dobson and M. E. Welland, *Science*, 2007, **318**, 1900–1903.
- 8 J. Juárez, S. G. López, A. Cambón, P. Taboada and V. Mosquera, *J. Phys. Chem. B*, 2009, **113**, 10521–10529.
- 9 M. Bhattacharya, N. Jain and S. Mukhopadhyay, *J. Phys. Chem. B*, 2011, **115**, 4195–4205.
- 10 D. R. Davies, *Annu. Rev. Biophys. Biophys. Chem.*, 1990, **19**, 189–215.
- 11 X. Lin, J. A. Loy, F. Sussman and J. Tang, *Protein Sci.*, 1993, **2**, 1383–1390.
- 12 T. Tanaka and R. Y. Yada, *Protein Eng., Des. Sel.*, 2001, **14**, 669–674.
- 13 H. Naiki, K. Higuchi, M. Hosokawa and T. Takeda, *Anal. Biochem.*, 1989, **177**, 244–249.
- 14 H. LeVine, III, *Protein Sci.*, 1993, **2**, 404–410.
- 15 H. LeVine, III, *Arch. Biochem. Biophys.*, 1997, **342**, 306–316.
- 16 Y. Yoshiike, D. H. Chui, T. Akagi, N. Tanaka and A. Takashima, *J. Biol. Chem.*, 2003, **278**, 23648–23655.
- 17 D. Allsop, L. Swanson, S. Moore, Y. Davies, A. York, O. M. El-Agnaf and I. Soutar, *Biochem. Biophys. Res. Commun.*, 2001, **285**, 58–63.
- 18 J. Goers, S. E. Permyakov, E. A. Permyakov, V. N. Uversky and A. L. Fink, *Biochemistry*, 2002, **41**, 12546–12551.
- 19 T. Ban, D. Hamada, K. Hasegawa, H. Naiki and Y. Goto, *J. Biol. Chem.*, 2003, **278**, 16462–16465.
- 20 J. R. Kumita, C. J. Weston, L. P. Choo-Smith, G. A. Woolly and O. S. Smart, *Biochemistry*, 2003, **42**, 4492–4498.
- 21 L. Zhu, X. J. Zhang, L. Y. Wang, J. M. Zhou and S. Perrett, *J. Mol. Biol.*, 2003, **328**, 235–254.
- 22 M. R. H. Krebs, E. H. C. Bromley and A. M. Donald, *J. Struct. Biol.*, 2005, **149**, 30–37.
- 23 C. C. Kitts and D. A. V. Bout, *J. Phys. Chem. B*, 2009, **113**, 12090–12095.
- 24 M. Groenning, *J. Chem. Biol.*, 2010, **3**, 1–18.
- 25 M. Groenning, L. Olsen, M. van de Weert, J. M. Flink, S. Frokjaer and F. S. Jorgensen, *J. Struct. Biol.*, 2007, **158**, 358–369.
- 26 C. R. Raj and R. Ramaraj, *Chem. Phys. Lett.*, 1997, **273**, 285–290.
- 27 R. Sabate, L. Rodriguez-Santiago, M. Sodupe, S. J. Saupé and S. Ventura, *Chem. Commun.*, 2013, **49**, 5745.
- 28 E. S. Voropai, M. P. Samtsov, K. N. Kaplevsky, A. A. Maskevich, V. I. Stepuro, O. I. Povarova, I. M. Kuznetsova, K. K. Turoverov, A. L. Fink and V. N. Uverskiid, *J. Appl. Spectrosc.*, 2003, **70**, 868–874.
- 29 N. Amdursky, Y. Erez and D. Huppert, *Acc. Chem. Res.*, 2012, **45**, 1548–1557.
- 30 V. I. Stsiapura, A. A. Maskevich, V. A. Kuzmitsky, K. K. Turoverov and I. M. Kuznetsova, *J. Phys. Chem. A*, 2007, **111**, 4829–4835.
- 31 A. A. Maskevich, V. I. Stsiapura, V. A. Kuzmitsky, I. M. Kuznetsova, O. I. Pavarova, V. N. Uversky and K. K. Turoverov, *J. Proteome Res.*, 2007, **6**, 1392–1401.
- 32 V. I. Stsiapura, A. A. Maskevich, V. A. Kuzmitsky, V. N. Uversky, I. M. Kuznetsova and K. K. Turoverov, *J. Phys. Chem. B*, 2008, **112**, 15893–15902.
- 33 P. K. Singh, M. Kumbhakar, H. Pal and S. Nath, *J. Phys. Chem. B*, 2009, **113**, 8532–8538.
- 34 A. I. Sulatskaya, A. A. Maskevich, I. M. Kuznetsova, V. N. Uversky and K. K. Turoverov, *PLoS One*, 2010, **5**, e15385.
- 35 M. Harel, L. K. Sonoda, I. Silman, J. L. Sussman and T. L. Rosenberry, *J. Am. Chem. Soc.*, 2008, **130**, 7856–7861.
- 36 N. A. P. Ulrih, G. Anderluh, P. Macek and T. V. Chalikian, *Biochemistry*, 2004, **43**, 9536–9545.