Preparation and Characterization of Purified Amyloid Fibrils

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Amyloid fibrils are highly organized proteinaceous aggregates associated with pathogenic conditions such as Alzheimer's disease and the spongiform encephalopathies.[1] They are also being explored as novel nanostructures with a wide variety of potential applications.[2] Despite the considerable advances in the characterization of such fibrils[3,4] there are still a number of important aspects of their structure that remain to be clarified. Among these are the detailed definition of the conformational state of the protein chains within an amyloid fibril and the manner in which the constituent protofilaments are assembled. Studies to investigate such issues are hindered by the intrinsic heterogeneity of fibril samples prepared in vitro,[4] as these invariably contain soluble precursors and nonfibrillar aggregates.[5] The development of strategies to isolate fibrils is therefore of great importance. Here we report a method to achieve this objective and use it to characterize the fibrils formed at low pH by the SH3 domain of the α-subunit of bovine phosphatidylinositol-3′-kinase.

Separation of fibrils from other species, particularly large aggregates, is difficult to achieve by common biochemical procedures. The method developed here was based on the knowledge that amyloid fibrils deposited in tissue as a result of disease can be highly protease resistant.[6] Samples of two types of SH3 aggregates, the first containing predominantly amorphous aggregates and the second predominantly amyloid fibrils, were prepared at low pH as described elsewhere.[5] These samples were then exposed to pepsin, a protease with high activity under such conditions, for different periods of time and the extent of digestion was monitored by FTIR (Figure 1). In amorphous aggregates spectral features associated with β-sheet structure (1612 and 1684 cm^{-1} respectively) disappear rapidly leaving a band at ca. 1649 cm^{-1} characteristic of unstructured species. Samples containing amyloid fibrils, by contrast, experience much smaller changes in appearance even after 3 h of incubation.[7] Interestingly, among the changes occurring in the latter samples again we see the loss of the band at ca. 1684 cm^{-1} whereas the rest of the aggregation components remain largely intact and only a small shift of the main peak, centered at ca. 1618 cm^{-1}, to higher wavenumbers is detected. These changes suggest that the amorphous species present in the sample are digested to leave just the protease-resistant fibrils. The increase in intensity at ca. 1640−1660 cm^{-1} after digestion is again attributable to a higher population of disordered species, such as peptide fragments.

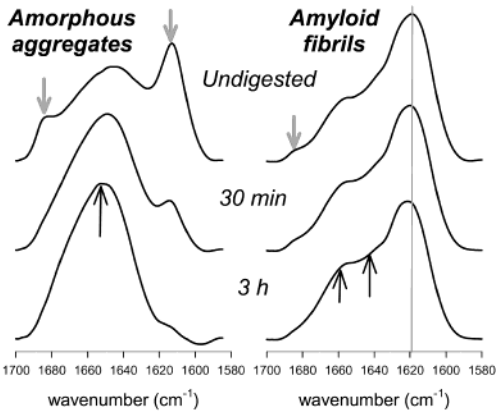

Figure 1. FTIR spectra of samples of SH3 initially containing predominantly either amorphous aggregates (left) or amyloid fibrils (right) following digestion by pepsin for different lengths of time.[11] Arrows point to components altered by digestion as described in the text.

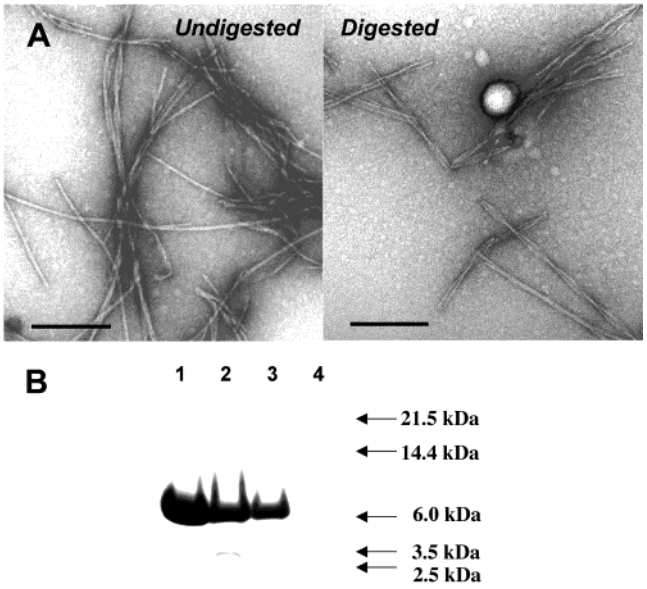

Figure 2. Analysis of fibril preparations after pepsin digestion. (A) Electron micrographs of fibril preparations before and after digestion. Bar = 200 nm. (B) SDS-PAGE of solubilized fibrils before and after treatment with pepsin. Lane 1, native protein; lane 2, fibrils before exposure to pepsin; lane 3, pellet obtained after treatment of the sample in lane 2 with pepsin; lane 4, supernatant obtained after treatment of the sample in lane 2 with pepsin (see text for details).

Morphological changes in the amyloid fibrils during pepsin digestion were analyzed by electron microscopy. The data show that abundant intact fibrils remain after pepsin digestion with the overall appearance of the fibrils in the original preparations (Figure 2a). The integrity of the protein molecules within the fibrils was confirmed in a variety of ways. 1D-NMR spectra were recorded after the fibrils had been disrupted in 6 M GndHCl and the protein refolded; the spectra were indistinguishable from those of the native protein prior to aggregation.[9] SDS-PAGE analysis of samples containing fibrils before and after treatment with pepsin and subsequent ultra-centrifugation shows in both cases a band migrating at the same position as the SH3 monomer, again indicating that the fibrils contain intact full-length protein (Figure 2b). The supernatant obtained after ultra-centrifugation of pepsin-

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(7) The morphology of the samples was assessed by EM before proteinase digestion. Digestion was performed at 37 °C at a ratio of pepsin:PI3-SH3 1:200 in weight. Aliquots taken at different times were analyzed by FTIR.

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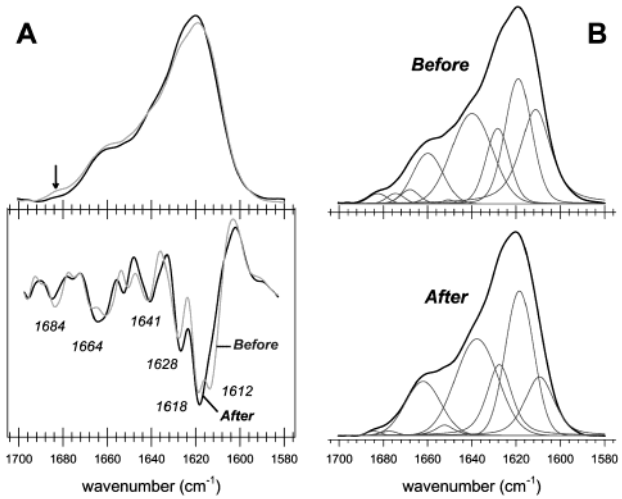

Figure 3. (A) FTIR spectra and second-derivative analysis of untreated (grey trace) and purified (black trace) SH3 amyloid fibrils. (B) Curve fitting of FTIR spectra corresponding to amyloid fibrils before and after the purification procedure.

digested fibrils shows no detectable peptide fragments (Figure 2b), showing that any soluble protein present in the original sample was completely degraded. Finally, mass spectrometric (MS) analysis of samples of solubilized amyloid fibrils after pepsin treatment yielded mass/charge peaks corresponding only to the full length SH3 domain.[10]

FTIR analysis of the original and purified fibrillar samples shows an overall similarity in their spectra (Figure 3),[11] indicating that the majority of the contributions to the amide I band of the untreated sample can be attributed to the fibrils present in the solution. A detailed examination of the spectra, however, reveals some significant differences, particularly when the second derivatives of both spectra are compared. The component at 1684 cm^{-1} is essentially absent after digestion, and one of the three components of the main aggregation band, that at 1612 cm^{-1}, is not detectable (Figure 3). This result strongly supports the conclusion that the bands at 1684 and 1612 cm^{-1}, the main components in samples containing amorphous aggregates (see Figure 1),[5] are characteristic of nonfibrillar material present in the sample before the purification.

We can assume on this basis, therefore, that the characteristic bands of the β-structure present in amyloid fibrils occur principally at 1618 and 1628 cm^{-1}. Other FTIR components at 1641 and 1664 cm^{-1} are, however, still present in the fibrils after digestion; these can be attributed to disordered structures and β-turns, respectively, suggesting that some regions of the protein form flexible loops and turns within the amyloid fibrils. This latter conclusion is consistent with the model of the SH3 fibrils based on cryo-EM analysis[4] but is remarkable given the lack of susceptibility of the protein in the fibrils to proteolysis. It must reflect the fact that even those regions of the polypeptide chains not in well-defined β-sheet structures are tightly held within the fibrillar assemblies.

As well as providing evidence for different types of secondary structure, FTIR analysis is reported to distinguish between parallel and antiparallel configurations in β-sheets.[12] It is possible, however, that the specific geometry of the β-strands in the fibrils could perturb the IR frequencies from those classical of β-sheet structures. Nevertheless, the almost complete absence of spectral components at ca. 1684 cm^{-1} in the FTIR spectra of purified fibrils suggests a negligible contribution of antiparallel relative to parallel interactions.[12] FTIR analysis of highly fibrillar samples of insulin has also suggested a mainly parallel β-sheet structure,[13] as have several FTIR[14] and solid-state NMR[15] studies of amyloid fibrils formed from a range of peptides. Other studies on fibrillar samples by solid-state NMR and FTIR, however, have reported the presence of antiparallel β-sheet structure.[16] Further studies of highly purified materials are therefore needed to establish conclusively the orientation of the β-strands in amyloid fibrils. The ability to study fibrillar material, generated by approaches such as that we report here, should be of particular importance in these studies.

In conclusion, amyloid fibrils free of amorphous aggregates and soluble precursors can be prepared by using a combination of proteolytic digestion and ultra-centrifugation. This approach has enabled us to demonstrate that the full-length protein is preserved within the SH3 fibrils studied here. FTIR analysis shows that the protein within the fibrils has FTIR bands typical of a β-sheet structure that is mainly parallel in character. Other regions of the polypeptide chain appear to form turns and disordered structures that are likely to link the β-strands, although these seem to be tightly held within the fibril structure. The approach described here should be applicable to a wide range of other amyloid systems, and to be important not only to investigate disease-related aggregates but also as a means of preparing and characterizing novel materials assembled from protein fibrils.

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(8) Amorphous aggregates were prepared by incubating a 0.5 mM solution of SH3 in ^2H_2O at pH 1.5 for 5 days at 35 °C. Amyloid fibrils were prepared by incubating a 0.5 mM solution of SH3 in ^2H_2O at pH 2.0 for 30 days at 35 °C.

(9) Refolding of the protein was carried out by dialysis in 20 mM sodium phosphate buffer at pH 7.2 after incubation in 6 M GndHCl.

(10) SDS-PAGE loading buffers containing 1% SDS or LDS completely solubilized the fibrils. Before MS analysis, fibrils were dissolved by incubating samples in 10% ammonium hydroxide.

(11) Samples containing amyloid fibrils were prepared as detailed above and digested with pepsin at a ratio of pepsin:SH3 1:100 in weight for 3 h at 37 °C, after which the samples were ultra-centrifuged for 1 h at 300000*g*. The pellet containing the fibrils was then resuspended in ^2H_2O at pH 2.0, ultra-centrifuged for a second time, resuspended again in the same buffer, and analyzed by FTIR.

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