Journal of Molecular Structure 1059 (2014) 94–100

Contents lists available at ScienceDirect

Journal of Molecular Structure

journal homepage: www.elsevier.com/locate/molstruc

# Amyloid formation and inhibition of an all-beta protein: A study on fungal polygalacturonase

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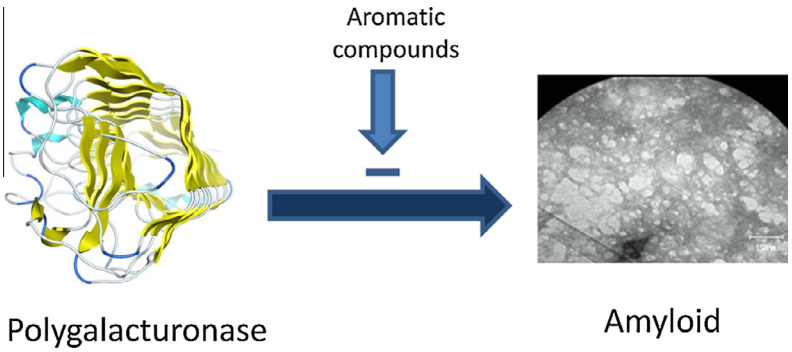
## HIGHLIGHTS

- Amyloid fibril formation of a beta-helix protein.
- General inhibitory effect of aromatic compounds on polygalacturonase amyloid formation.
- Best inhibitory effect seen with Silibinin and chlorpropamide.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Article history:*
Received 18 June 2013
Received in revised form 4 October 2013
Accepted 5 November 2013
Available online 20 November 2013

*Keywords:*
Amyloid
Polygalacturonase
Albumin
Silibinin
Chlorpropamide
Beta-helix

## ABSTRACT

Theoretically, all proteins can adopt the nanofibrillar structures known as amyloid, which contain cross-beta structures. The all-beta folded proteins are particularly interesting in this regard, since they appear to be naturally more predisposed toward this structural arrangement. In this study, methanol has been used to drive the beta-helix protein polygalacturonase (PG), toward amyloid fibril formation. Congo red absorbance, thioflavin T fluorescence, circular dichroism (CD) and transmission electron microscopy have been used to characterize this process. Similar to other all-beta proteins, PG shows a non-cooperative fibrillation mechanism, but the structural changes that are monitored by CD indicate a different pattern. Furthermore, several compounds containing aromatic components were tested as potential inhibitors of amyloid formation. Another protein predominantly composed of alpha-helices (human serum albumin) was also targeted by these ligands, in order to get an insight into their potential anti-aggregation property toward structurally different proteins. Among tested compounds, silibinin and chlorpropamide were able to considerably affect both proteins fibrillation process.



## 1. Introduction

Aggregation of proteins, often characterized by the presence of beta-sheet structures, is associated with pathological consequences in a wide range of diseases, as well as technical problems during *in vitro* studies involving proteins [1]. This process is also problematic in manufacturing steps such as protein purification [2]. One particular type of aggregates, namely amyloids, are highly

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http://dx.doi.org/10.1016/j.molstruc.2013.11.011

ordered nanofibrils which may stem from potentially diverse and heterogeneous precursor structures [3] and show various morphologies [4].

Usually, fibril generation starts from the formation of protofilaments [5]. In these subunits, the protein molecules are arranged in beta strands and positioned perpendicular to the elongation axis. Two to six of these protofilaments may associate or twist around each other, resulting in fibrils of about 10 nm diameter [5]. These common structural properties of fibrils, shared between different proteins, provide the possibility of using common amyloid identifying tools (e.g. Congo red dye and thioflavin T fluorescence probe) [6]. Amyloid structure is now considered to be a generic form [7,8] which could be potentially reached by any protein. However, physicochemical properties of proteins are believed to be determinative in their aggregation rates; these include polar and nonpolar water-accessible surface areas, dipole moment, and aromatic interactions (π-stacking) [9]. The principal requirement of amyloid formation is the occurrence of particular conformational changes, which induce the formation of partially structured intermediates. These intermediates associate further into oligomers and fibrils [10].

An intriguing fact about this process concerns the mechanism by which proteins with diverse secondary structural features achieve a final common cross-beta spine structure. Amyloid formation has been reported in proteins of different folds, from insulin with predominant alpha secondary structures [11,12], and all-alpha proteins such as myoglobin [13–15] to carbonic anhydrase which contains mostly beta strands [16] and alpha-synuclein which is naturally unstructured [17]. Interestingly, about unstructured proteins, some reports mention the formation of alpha-helical intermediates preceding the conversion to the amyloid beta structures [18]. A particular case concerns all-beta proteins, i.e. the structures that would seem to be naturally disposed to form amyloid, but that need also to undergo partial unfolding before making fibrillar structures [19,20]. Proteins possessing beta-barrel fold are even more interesting, since the oligomeric amyoid assemblies of abeta peptides have been proposed to possess beta-barrel structure [21].

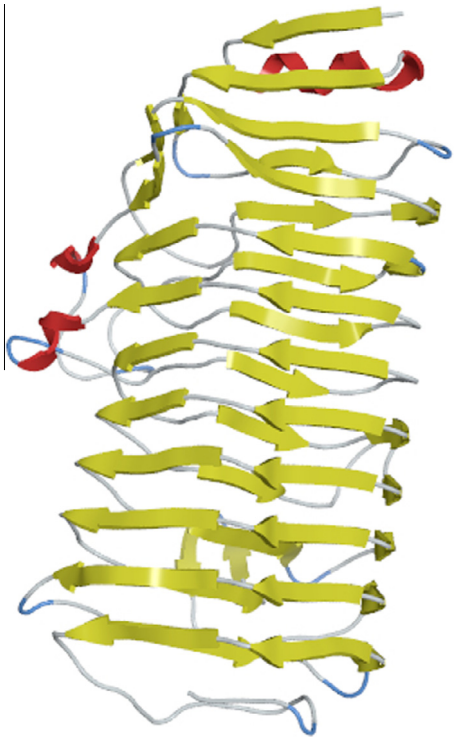

**Fig. 1.** Representation of polygalacturonase three-dimensional structure based on the 1CZF.pdb file.

With regard to these specific features, the present study was aimed to investigate the amyloid formation of polygalacturonase, an all-beta protein which contains a beta-helix fold. The beta-helix structure has been suggested to be present in the amyloid fibrils from various proteins [22,23], but to our knowledge, amyloid formation from a protein with native beta-helix fold has not been yet reported. Polygalacturonases have been assigned to family 28 of glycosyl hydrolases [24]. These enzymes hydrolyze the α(1, 4)-glycosidic bonds between galacturonic acid units of plant cell wall pectin [25,26]. The *Aspergillus niger* endopolygalacturonase II (PG) has a right-handed parallel β-helix fold with 10 complete turns, and is composed of four parallel β-sheets (Fig. 1). One small α-helix is positioned near its N-terminus, and acts as a "cap", shielding the enzyme hydrophobic core from exposure to environment [27]. The enzyme is active at low pH (around pH = 4) [28].

Amyloid formation process of PG has been studied with the use of various detection methods. The effect of some aromatic compounds (as potential anti-amyloidogenics) has also been investigated on the process. In parallel, the same experiments have been done on a protein with predominant alpha helix structure to test the potential of these compounds in acting toward amyloid formation of different proteins.

## 2. Materials and methods

### 2.1. Materials

Polygalacturonase (EC 3.2.1.15) (PG), Human serum albumin (HSA), Thioflavin T (ThT), Congo red (CR), silibinin as well as other aromatic ligands were purchased from Sigma (St. Louis, MO, USA). All organic solvents were obtained from Merck (Darmstadt, Germany). Protein concentration was determined spectrophotometrically by Bradford assay method. Stock solution of tested ligands were prepared using 50 mM glycine buffer (pH = 1.5) as solvent. The final concentrations of the ligands were 50, 100, 150, 200 and 250 μM in the preliminary experiment. Average data points regarding best concentrations of ligands have been presented with error bars representing standard deviation. In order to improve readability and visualization of some results (such as CD), typical data has been represented, where all experiments have been performed at least twice to confirm reproducibility.

### 2.2. Preparation of HSA and PG solutions

HSA and PG were dissolved at a concentration of 3 mg/mL in 50 mM glycine buffer (pH 1.5), mixed with ethanol 60% and methanol 10% respectively, and incubated at 57 °C for 24 h while being stirred gently by Teflon magnetic bars. Samples were taken out at mentioned intervals and stored on ice before addition of CR or ThT.

### 2.3. CR assay

CR was dissolved at 7 mg/mL in a buffer consisting of 0.15 m NaCl and 5 mm potassium phosphate (pH = 7.4). The prepared solution was first filtered through a 0.2 μm filter. Subsequently, 5 μL of well-mixed incubation sample were added to 300 μL of the CR solution and incubated for 30 min. Absorbance spectra were recorded at 400–600 nm using a Shimadzu UV–visible spectrophotometer (Kyoto, Japan). Spectra in the presence of the dye were compared with those of the buffer containing CR in the absence of protein [6]. As a mean to monitor the kinetics of fibril formation, absorbance intensity at 400–600 nm was followed over time, and the absorbance at 520 nm was taken as representative of amyloid