International Journal of Biological Macromolecules 80 (2015) 208–216

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Amyloid fibril formation from a 9 amino acid peptide, 55th–63rd residues of human lysozyme

Yuhei Tokunaga[a,*], Mitsuharu Matsumoto[b], Yasushi Sugimoto[a,*]

[a] The United Graduate School of Agricultural Sciences, Kagoshima University, 1-21-24, Korimoto, Kagoshima 890-0065, Japan
[b] Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24, Korimoto, Kagoshima 890-0065, Japan

ARTICLE INFO

Article history:
Received 21 February 2015
Received in revised form 9 June 2015
Accepted 11 June 2015
Available online 16 June 2015

Keywords:
Human lysozyme
Hen egg white lysozyme
Amyloid fibril
Core peptide
Acid hydrolysis
β-Structure

ABSTRACT

Hen egg white lysozyme (HEWL) readily forms amyloid fibrils *in vitro*. We have previously identified a core structure, termed HEWL K-peptide, involved in fibril formation. Two major peptides, peptide #3 (50th–102nd residues of human lysozyme (hLZ)) and peptide #5 (54th–102nd residues), were isolated from the hLZ amyloid fibrils that had been exposed to pH 2.0 and 58 °C, and precipitated by ultracentrifugation. These peptides cover most of the beta-domain and C-helix of hLZ including a 9 residues sequence corresponds to a human counterpart of HEWL K-peptide, GIFQINSRY (55th–63rd residues of hLZ, thus named "human K-peptide"). Chemically synthesized human K-peptide readily formed amyloid fibrils, as in HEWL K-peptide. It was demonstrated that both at least 9 residue length and Phe residue at 3rd position of the human K-peptide were crucial for amyloidogenesis *in vitro*. Short peptides covering COOH-terminal region of peptides #3 and #5 did not form amyloid fibrils. These data suggested that human K-peptide region with high propensity of amyloidogenesis plays a key role as a fibril-forming core sequence of hLZ. Interestingly, human K-peptide region is flanked by two predicted semi-disordered regions (39th–52nd and 67th–75th residues). We discuss the possible role of these regions.

1. Introduction

Human amyloidoses are associated with alteration in protein structure from a normal soluble form to an insoluble amyloid fibril and cause severe diseases such as Alzheimer's, Parkinson's, Creutzfeldt-Jakob diseases and Type II diabetes [1,2]. Amyloid fibrils exhibit common structural characteristic of filamentous aggregates of proteins, or their fragments, with extensive cross-beta secondary structure [3,4]. In addition to originally found disease-related proteins, it is now becoming clear that many proteins unrelated to amyloid diseases can form amyloid fibrils and that amyloid-fold is one of the generic feature of the polypeptide chain folding [5,6].

Hen egg white lysozyme (HEWL) has been extensively studied as a model of human lysozyme (hLZ) with regard to amyloid fibril formation [7–10]. We have observed that the addition of preheated (at 72 °C) ovalbumin (OVA) to the native HEWL resulted in formation of fibrous precipitates, which were positive for thioflavin T (Tht) fluorescent emission, under the conditions in which each protein alone formed no fibrils [11]. We isolated a nona-peptide (GILQINSRW, 54th–62nd region) of HEWL, named K-peptide, by chymotryptic/tryptic digestion of HEWL, which exhibited binding to immobilized pre-heated OVA. This has suggested a possibility that pre-heated OVA binds to the K-peptide in HEWL and causes HEWL to undergo structure changes and fibril formation [11]. Interestingly, chemically synthesized K-peptide was found to readily form amyloid fibrils either in neutral buffer solution at 37 °C or at acidic pH, which was biochemically and morphologically indistinguishable from the amyloid fibrils formed from HEWL at acidic and high temperature conditions. Thus, we proposed in the previous papers that the K-peptide region located in the hydrophobic cluster 3 might work as a core part for the amyloidogenesis of HEWL [11,12]. This region is within a longer peptide of 49th–101st residues, which has been generated by chemical degradation of HEWL and has formed amyloid fibrils under acidic pH and high temperature [8,9]. In addition, the 62nd Trp has been found to be crucial for amyloidogenesis of HEWL by mutation analysis [13].

Mutant forms of hLZ isolated from patients, i.e., I56T, D67H and others, have been shown to associate with the deposition of amyloid fibrils responsible for non-neuropathic systematic amyloidosis [14–20]. These mutations cause structural instability of hLZ and facilitated partial unfolding, leading to the ultimate formation of hLZ amyloid fibrils. Funahashi et al. reported that a

* Corresponding authors. Tel.: +81 99 285 8781; fax: +81 99 285 8781.
E-mail addresses: k0085039@kadai.jp (Y. Tokunaga), yasushi@chem.agri.kagoshima-u.ac.jp (Y. Sugimoto).

http://dx.doi.org/10.1016/j.ijbiomac.2015.06.015
0141-8130/

I56T mutant hLZ exhibited the same crystal structure as that of wild-type hLZ, but showed remarkably decreased equilibrium and kinetic stabilities [21]. Dumoulin et al. further reported that both I56T and D67H mutations caused a cooperative destabilization of the beta-domain and the adjacent C-helix region of hLZ [15]. They also described population of partially unstructured intermediate, in which the beta-domain and the C-helix of the mutants are substantially unfolded, whereas their remaining alpha-domain still have native-like structure [15] Wild-type hLZ can also form amyloid fibrils under such a condition as low pH, high temperature and addition of solvents or denaturants [22–24].

In the present paper, we attempt to elucidate the properties and functions of hLZ K-peptide (GIFQINSRY, 55th–63rd residues of hLZ) homologous to HEWL K-peptide, which may have high propensity of fibril formation and may play a crucial role as a fibril-forming core region of hLZ.

2. Materials and methods

2.1. Recombinant human lysozyme and chemically synthetic peptides

Recombinant hLZ was purchased from Wako (#189-02064). The K-peptide and its derivatives were chemically synthesized by Greiner Bio-one GmbH (Germany), SCRUM Inc. (Tokyo, Japan) and GeneNet (Fukuoka, Japan).

2.2. In vitro experimental conditions for amyloid fibril formation

The recombinant hLZ protein was dissolved at 3.5–15 mg/ml in 50 mM glycine-HCl buffer, pH2.0 and incubated at 58 °C, following the protocol used to generate amyloid fibrils for HEWL [12]. Synthetic peptides were first dissolved in dimethyl sulfoxide (DMSO) at 10 mg/0.1 ml, and then incubated in 50 mM of various buffer solution at 37 °C. The concentration of peptides was 2 mg/ml in the final buffer solutions.

2.3. Measurement of thioflavin T fluorescence intensity

Samples (10 μl) were mixed with 250 μl of 10 μM thioflavin T (Tht) dissolved in 50 mM Na-phosphate buffer, pH 6.7. Fluorescence intensity arising from Tht bound to amyloid fibrils was measured at 485 nm with excitation at 435 nm using a fluorescence microplate reader (Corona, Hitachi). The fluorescence spectra with excitation at 440 nm were also measured.

2.4. Transmission electron microscope (TEM) and atomic force microscope (AFM) observations

Amyloid preparations were examined by TEM and AFM as described previously [12]. Dilution of samples 10-fold for TEM and 50- to 100-fold for AFM observations were carried out with water. For TEM, diluted samples were placed on copper grid covered with carbon coated collodion film (Nisshin excel support film #649), treated with 1% phosphotungstic acid, and observed by an electron microscope (Hitachi 7000) at 80 kV. For AFM, diluted samples were placed on freshly cleaved mica, dried and observed by a JEOL JSPM-5200 (Nihon Denshi) AFM. Cantilevers with etched silicon tips (Olympus) having a resonance frequency of 300 kHz were used with a tapping mode.

2.5. CD measurement

Far UV CD spectra were measured on a Jasco J-820 spectropolarimeter at room temperature. The samples were diluted with 20 mM buffer solution to 0.1 mg/ml for the measurement. Four scans were accumulated at a response time of 4 s and at a scan rate of 10 nm/min. The spectrum was converted to the mean residue ellipticity using the path-length of the cell, 0.1 cm, and the protein concentration, 0.1 mg/ml.

2.6. Peptide analysis by reverse-phase HPLC column chromatography, amino-terminal amino acid sequencing, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (TOF-MS)

Recombinant hLZ was dissolved at 15 mg/ml in 50 mM glycine-HCl buffer, pH2.0 and incubated at 58 °C for one month to form amyloid fibrils. The incubated sample (1 ml) was ultracentrifuged at 150,000 × *g* for 5 h. The resultant precipitates were dissolved in 0.5 ml of 8 M guanidine-HCl in 50 mM glycine-HCl buffer, pH 2.0, containing 150 mM beta-mercaptoethanol. This dissolved sample was applied to Octyl-80Ts reverse-phase HPLC column (TOSOH 0017344, 0.46 × 15 cm) equilibrated with 10% acetonitrile/0.1% trifluoroacetic acid (TFA) in H_2O. The bound peptides were eluted from the column with 10–59.5% acetonitrile gradient at a flow rate of 0.8 ml/min. Peptide fractions separated by Octyl-80Ts column chromatography were dried and applied to Tricin-SDS-PAGE [25]. Peptide bands were electro-blotted to ProBrott membrane (Applied Biosystems), cut out, and subjected to sequence analysis. Molecular mass of purified peptide was determined by TOF-MS (Bruker Daltonics, Autoflex Speed TOF/TOF-KG) analysis: peptides were dissolved in 55% acetonitrile/0.1% TFA/H_2O solution and spotted onto target plate (MTP 384 target plate polished steel TF) after mixing with the same volume of 67% acetonitrile/0.033% TFA/H_2O saturated with alpha-cyano-4-hydroxycinnamic acid. Molecular mass calibration was carried out with Bruker Bacterial Test Standard.

2.7. Others

Tris-Glycine SDS-PAGE was carried out using the Laemmli system [26]. Tricine-SDS-PAGE was done according to Schagger and Jagow [25]. Protein or peptide amount was measured by bicinchoninic acid method following Smith et al. [27]. The absorption coefficient of purified protein was calculated according to Schmid [28].

3. Results

3.1. Time course of amyloid fibril formation from recombinant hLZ and HEWL at acidic pH and high temperature

HEWL and recombinant hLZ (3.5 mg lysozyme/ml 50 mM Gly-HCl buffer, pH 2.0) were incubated at 58 °C for 7 days and subjected to thioflavin T (Tht) fluorescence analysis. As shown in Fig. 1, fluorescence intensity of both samples slowly increased with time during the initial 3 days incubation followed by a more prominent increase, indicating formation of amyloid fibrils. The Tht fluorescence of HEWL leveled off at 5 days incubation. On the contrary, the fluorescence intensity of hLZ showed a steady increase even after 5 days. The fluorescence intensity was ~2.4-fold higher for HEWL than for hLZ at 7 days, indicating that the apparent efficiency of amyloid fibril formation is stronger for HEWL than for hLZ under the conditions used.

3.2. Precipitation of amyloid fibril by centrifugation and analysis of its peptides

The core structure of amyloid fibrils of hLZ has been analyzed by limited proteolysis with pepsin [23]. A long peptide, corresponding to 32nd–108th residues of hLZ covering nearly 60% of total mass, was identified as a protease-resistant core structure [23]. Here, we