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The effect of limited proteolysis by different proteases on the formation of whey protein fibrils

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ABSTRACT

Four proteases: trypsin, protease A, pepsin, and protease M were selected to modify whey protein concentrate (WPC) at a low degree of hydrolysis (0.1, 0.2, and 0.3%) before adjusting to pH 2.0 and heating at 90°C to gain insight into the influence of proteolysis on fibril formation. The kinetics of fibril formation were performed on native and modified WPC using the fluorescent dye thioflavin T in conjunction with transmission electron microscopy and far-UV circular dichroism spectroscopy for the morphological and secondary structural analyses. The change in surface hydrophobicity and content of free sulfhydryl groups were also observed during the formation of fibrils for the native and modified WPC. The content of aggregation and thioflavin T kinetic data indicated that the ability of fibril formation was apparently different for WPC modified by the 4 proteases. Whey protein concentrate modified by trypsin aggregated more during heating and the fibril formation rate was faster than that of the native WPC. Whey protein concentrate modified by the other proteases showed slower aggregation with worse amyloid fibril morphology. Compared with the native WPC, the structure of WPC changed differently after being modified by proteases. The state of α-helix structure for modified WPC played the most important role in the formation of fibrils. Under the mild conditions used in this work, the α-helix structure of WPC modified by trypsin caused little destruction and resulted in fibrils with good morphology; the content of α-helices for WPC modified by other proteases decreased to 36.19 to 50.94%; thus, fibril formation was inhibited. In addition, it was beneficial for the modified WPC to form fibrils such that the surface hydrophobicity increased and the content of free sulfhydryl groups slightly decreased during heating.
Key words: whey protein concentrate, fibril, protease hydrolysis, structure change

INTRODUCTION

Amyloid fibrils aggregation has been found in many globular proteins, such as β-LG, soy protein, immunoglobulins, and α-LA at low pH and low ionic strength (Carrotta et al., 2001; Goers et al., 2002; Khurana et al., 2003). Amyloid fibrils share similar morphological features, as they are a few nanometers thick, branched or unbranched, often twisted, and characterized by a stacked-protein β-sheet structure (Dobson, 1999; Morozova-Roche et al., 2000; Rochet and Lansbury, 2000; Couzin, 2002; Khurana et al., 2003). The aqueous solution of a globular protein changes the balance of forces determining the position of the equilibrium between folded and unfolded states. The noncovalent interactions such as hydrophobicity, hydrogen bonding, van der Waals' forces, and the ionic bond play important roles in stabilizing fibrils, whereas the disulfide bonding between protein molecules does not occur to any significant extent because cysteine residues are predominantly protonated for self-assembly into amyloid fibrils (Otte et al., 2000; Alting et al., 2002).

Almost all published studies of heat-induced β-LG self-assembly into amyloid-like fibrils at low pH and low ionic strength have not shown whether hydrolysis is a necessary precursor to the nucleation or growth of fibrils. Some reviews consider that the fibrils consist of small peptides formed by hydrolysis of the globular protein during long-time heating at low pH. The kinetics of hydrolysis and the formation of fibril for β-LG also show that the protein first needs to be hydrolyzed and that subsequently the peptides associate into the fibrils. Adamcik and Mezzenga (2011) worked on the fibril formation of β-LG at pH 2.0 and found that the association of peptides into mature semi-flexible fibrils is quite complex. It involves the formation of proto-fibrils of aligned peptides that associate sidewise into twisted ribbons that subsequently transform into helical structures or even closed nanotubes. However, some papers have reported that not all peptides actually incorporate into fibrils. Lara et al. (2012) found that hydrolysis precedes aggregation into either type of aggregate and suggested that different peptides form different structures, including long semi-flexible fibrils and shorter flexible strands.

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Although the above results suggest that hydrolysis is essential for fibril formation, the hydrolysis in these papers is acid hydrolysis, which occurs during long-time heating in an aqueous medium at low pH. In the current research, whey protein concentrate (**WPC**) was first hydrolyzed by proteases at mild conditions and then the modified WPC was heated to form fibrils at 90°C and pH 2.0 to confirm whether proteolysis is beneficial to fibril formation. To compare the ability of WPC modified by different proteases to form fibrils, 4 proteases that cleave to different peptide bonds were selected. We also analyzed the relationship between the changed secondary structure of modified WPC and the formation of fibrils.

MATERIALS AND METHODS

Materials

Whey protein concentrate was purchased from Hilmar Cheese Co. (Hilmar, CA). The composition of the WPC was 76.94 weight percent protein (measured from Kjeldahl analysis: N × 6.38) with the major proteins. Trypsin (EC 3.4.21.4) and pepsin (EC 3.4.23.1) were purchased from Sigma-Aldrich (St. Louis, MO); protease A "Amano" 2G and protease M "Amano" G were purchased from Amano Enzyme Inc., Japan. Thioflavin T (**Th T**) powder and 1-anilino-8-naphthalene sulfonate (**ANS**) powder were purchased from Sigma-Aldrich. All other reagents and chemicals were of analytical grade and purchased from the local market.

Limited Proteolysis of Whey Protein Concentrate

Whey protein concentrate was added to Milli-Q water (Millipore Corp., Billerica, MA) to make a 7.9% (wt/vol) dispersion. Hydrolysis was performed for 30 min at 30°C (pH 6.7), except pepsin, for which it was performed at pH 2.0. The degree of hydrolysis (**DH**) with different amounts of proteases added was controlled and determined by using the pH-state method (Adler-Nissen, 1986), which determined the DH percentage on the basis of the number of free titratable amino groups produced by hydrolysis of peptide bonds. The DH was calculated using the following equation:

$$\mathrm{DH} = \frac{V_{NaOH} \times N_{NaOH}}{\alpha \times MP \times h_{tot}} \times 100\%,$$

where V_{NaOH} was the titrant volume of NaOH, N_{NaOH} was the concentration of NaOH (0.1 mol/L), α was the degree of dissociation of α-amino groups, MP was the mass of protein (g), and h_{tot} was the number of peptide bonds in the substrate (mEq/g of protein). The α value was 0.44 and h_{tot} was 7.8 (Adler-Nissen, 1986). When the DH reached 0.1 to 0.3% , the WPC hydrolysate was set to pH 2.0 and centrifuged at 19,000 × g for 30 min at 4°C as soon as possible, as hydrolysis proceeded very slowly at 4°C.

Modified WPC Aggregation

Whey protein concentrate aggregation was determined by the method of Kurouski et al. (2012) with some modification. The native and hydrolyzed WPC solution was set to pH 2.0 by adding a 6 M HCl solution and centrifuged 19,000 × g for 30 min at 4°C to remove undissolved protein. The supernatant was diluted to 3 weight percent with Milli-Q water, the protein concentration was determined by Kjeldahl analysis (N × 6.38), and then the solutions were adjusted to pH 2.0 with 6 M HCl. To induce fibril formation, the protein solution was heated for 10 h at 90°C in a water bath. Following the requisite heating time, the tube was cooled in ice water for 5 to 10 min. Aliquots were 2 mL for the Th T assay, 20 mL for the content of aggregation, and 4 mL for the other assays.

Fractionation of Protein Aggregation

The 3 weight percent (protein basis) solutions of native and modified WPC by different proteases were added to tubes and heat treated at 90°C (pH 2.0) in a water bath. The beginning of the holding time was, therefore, defined as the moment when the tubes were placed in the water bath. The concentration in the untreated sample was called C_0. After each hour passed, the samples were immediately cooled with cold water. The denatured and aggregated WPC were separated by a selective precipitation method (centrifuged at 15,000 × g for 20 min at 4°C; Veerman et al., 2002). The supernatant was defined by the non-aggregation concentration at time t (C_t), which was determined by means of the Kjeldahl method (N × 6.38).

The kinetic parameters (reaction rate constant k and activation energy E_a) of disappearance of native whey protein were determined using nonlinear regression after integration of the general rate equation (Kessler and Beyer, 1991):

$$-\mathrm{d}_c/\mathrm{d}_t = k_n \times C^n,$$

where k_n was the apparent rate constant and C^n was the exponential function.

After a heating time t (s), the initial concentration C_0 decreased to C_t (g/L) and the ratio of C_t/C_0 could be obtained by integration:

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