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Peptide–peptide and protein–peptide interactions in mixtures of whey protein isolate and whey protein isolate hydrolysates

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Abstract

The extent of aggregation in whey protein isolate (WPI) hydrolysates induced by *Bacillus licheniformis* protease was quantified as a function of degree of hydrolysis (DH), temperature and ionic strength. The capacity of the hydrolysates to aggregate added intact protein was also studied. The amount of aggregated material and the size of the aggregated peptides were measured by nitrogen content and size exclusion chromatography, respectively. Aggregation increased with DH up to the practical end point of hydrolysis (DH 6.8%). The aggregates formed under the various conditions studied consisted of peptides with masses ranging from 1.4 to 7.5 kDa. The hydrolysates were also able to aggregate added WPI. The additional amount of aggregated material increased with increasing DH. Peptides involved in peptide–peptide interactions were also involved in protein–peptide interactions. It is hypothesized that hydrophobic interactions dominated peptide–peptide interactions, while protein–peptide interactions depended on the balance between hydrophobic attraction and electrostatic repulsion.

Keywords: *Bacillus licheniformis* protease; Aggregation; Whey proteins; Peptides

1. Introduction

Whey proteins are widely used to improve the texture of food products. The ability to form gels is one of the important functional properties of whey proteins. The general mechanism of protein gelation involves an initial unfolding of protein molecules that subsequently leads to aggregation via various interaction forces (disulphide bridges, electrostatic attractions, hydrogen bonds, hydrophobic and/or Van der Waals interactions). Aggregation then proceeds with further association among protein particles thus creating a three-dimensional network.

The gelling properties of proteins can be modified in various ways: chemically, physically, and also via enzymatic hydrolysis. Enzymatic hydrolysis of proteins causes a decrease in molecular weight, an increase in the number of ionizable groups, and an increased exposure of hydrophobic groups (Panyam & Kilara, 1996). In most cases, the propensity of the hydrolysed protein to form a gel is less than that of the intact protein (Kester & Richardson, 1984).

Some cases of improved enzyme-induced gelation of whey proteins have been reported in the literature. Doucet, Gauthier, and Foegeding (2001) showed that enzyme-induced aggregation and gelation occurs during extensive hydrolysis of whey protein isolate (WPI) with Alcalase 2.4L® (an enzyme preparation derived from *Bacillus licheniformis*). Hydrophobic interactions among peptides, with a molecular mass less than 2 kDa, were observed to be involved in the formation of aggregates that associated further to form a gel (Doucet, Otter, Gauthier, & Foegeding, 2003). Also, it has been shown that partial hydrolysis of WPI with a serine protease from *Bacillus licheniformis* (BLP) may lead to the formation of enzyme-induced aggregates that eventually form a gel (Otte, Ju,

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Faergemand, Lomholt, & Qvist, 1996). A similar type of aggregate is formed when β-lactoglobulin (β-lg), the most abundant protein in bovine whey, is hydrolysed with BLP (Otte et al., 1997), suggesting that β-lg is mainly responsible for the effects observed with WPI. Aggregates formed during hydrolysis of β-lg with BLP consist of peptides of intermediate size (2–6 kDa) held together by mainly hydrophobic interactions (Otte et al., 1997). The aggregates are made up of six to seven major peptides, of which four have been identified (Otte, Lomholt, Halkier, & Qvist, 2000). Hydrolysis of α-lactalbumin (α-la), the second major protein in bovine whey, with BLP also leads to the formation of aggregates and, under certain conditions, to so-called nanotubules (Graveland-Bikker, Ipsen, Otte, & de Kruif, 2004; Otte, Ipsen, Ladefogrd, & Sorensen, 2004).

The conditions (degree of hydrolysis—DH—, pH, ionic strength, temperature) that favour peptide–peptide interactions, or even more interestingly, the interactions among peptides and intact proteins, have not been studied in detail. This information could help understanding of the gelation mechanism in protein hydrolysates. In addition, previous studies have shown the ability of β-lg to interact with peptides (Barbeau, Gauthier, & Pouliot, 1996; Noiseux, Gauthier, & Turgeon, 2002).

In the present work, the extent of aggregation in hydrolysates of WPI made with BLP was quantified as a function of DH, temperature and ionic strength. The aggregates formed were analysed with respect to peptide composition. Furthermore, the capacity of the hydrolysates to aggregate added intact protein was studied using titration experiments with intact WPI. The composition of the resulting aggregates (peptides and intact protein) was also investigated.

2. Materials and methods

2.1. Materials

A commercial WPI powder (trade name Bipro, Davisco Foods International Inc., Le Sueur, MN, USA) was used for the experiments. According to the manufacturer, it consisted of (by weight) 74.0% β-lg, 12.5% α-la, 5.5% bovine serum albumin (BSA), and 5.5% immunoglobulins. The protein content of the powder was 93.4% (w/w) and it contained 0.12% (w/w) calcium. The enzyme used was a serine proteinase from *Bacillus licheniformis* specific for Glu-X bonds and to a lesser extent for Asp-X bonds (Breddam & Meldal, 1992) and was kindly provided by Novozymes (BLP, product name NS-46007, batch PPA 6219; E.C. 3.4.21.19; Novozymes A/S, Bagsvaerd, Denmark).

All reagents were of analytical grade and purchased from Sigma (Sigma Chemical CO, St Louis, USA), Merck (Darmstadt, Germany) or Roche (Roche Diagnostics, Almere, The Netherlands).

2.2. Hydrolysis of WPI

WPI was dispersed in Millipore water at a concentration of 120 mg g^{-1} and stirred overnight at 4 °C. The suspension was centrifuged for 30 min (19,000 *g*, 20 °C) and the supernatant was subsequently filtered (Rezist 30GF92 and PVDF 0.45 μm, Schleicher and Schuell GmbH, Dassel, Germany). The supernatant was diluted to 50 mg g^{-1}, preheated at 40 °C for 15 min and the pH was adjusted to pH 8, 5 min before addition of the enzyme solution. The enzyme/substrate ratios (E/S) used were (v/v) 1/1250, 1/500, 1/192 and 1/100 to reach DH values of 1.8%, 3.2%, 5.3% and 6.8%, respectively. During hydrolysis, the reaction mixture, incubated at 40 °C, was maintained at pH 8 by addition of a 0.4 M NaOH solution in a pH-STAT (719 S Titrino, Metrohm Ion Analysis, Metrohm Ltd., Herisau, Switzerland). Incubation times were between 50 and 180 min. To permanently inactivate the enzymatic reaction of BLP (as checked with size exclusion chromatography), the pH was adjusted to pH 2 with a 6 M HCl solution. A solution of WPI incubated under the same conditions as the longest hydrolysis incubation and containing heat-inactivated (15 min, 95 °C) enzyme (E/S 1/100, v/v) served as control.

Nitrogen contents of the hydrolysates were measured using the combustion or Dumas method (AOAC, 1995) with a NA 2100 Protein nitrogen analyser (CE Instruments, Milan, Italy). A $6.38 \times N$ conversion factor was used to convert nitrogen content to protein content. The hydrolysates were stored at 4 °C before further analysis.

2.3. Aggregate formation in the WPI hydrolysates

WPI hydrolysates (DH 3.2%, 5.3% and 6.8%) and the WPI control solution, containing 10 mg of proteinaceous material were adjusted to pH 7 with 0.25–2 M NaOH solutions and to ionic strengths of 0.020, 0.075 and 0.200 M with a 2 M NaCl solution. The total mass of each sample was set to 1 g with Millipore water. Each sample was divided into two parts. After incubation for 1 h at 20, 40 or 60 °C, one part was centrifuged (20 min at 19,000 *g*, at 20 °C) while the other part was not centrifuged. In order to determine the extent of aggregation, the nitrogen content (N) of the supernatant and the non-centrifuged hydrolysate (N_0) were determined using the Dumas method. The proportion of aggregation was defined as $(1-N/N_0) \times 100\%$. Aggregated material was defined as the material removed using the centrifugation and solvent conditions applied. The experiment was performed in duplicate.

The pellets, containing the aggregates, were washed twice with 0.020, 0.075 or 0.200 M NaCl solutions at 20, 40 or 60 °C and analysed by size exclusion chromatography.