



Degradation of whey from caprine milk by human proteolytic enzymes, and the resulting antibacterial effect against *Listeria monocytogenes*[☆]

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ABSTRACT

Protein degradation of caprine whey by human proteolytic enzymes was studied with regard to antibacterial effect on *Listeria monocytogenes*. The digestion was performed by a two-step degradation-assay, using human gastric juice (HGJ) at pH 2.5, and human duodenal juice (HDJ) at pH 8. Protein profiles were studied by SDS-PAGE after each step and compared with degradation performed by commercial enzymes. Both types of enzymes, both human and commercial, left most of β -LG intact. However, proteins like serum albumin, laktoferrin and immunoglobulins were rapidly degraded. Only minor parts of α -lactalbumin (α -LA) was degraded by human enzymes, while treatment with commercial enzymes gave full degradation of α -LA. The two types of enzymes resulted in different peptide profiles, where the commercial enzymes degraded whey into smaller peptides much more efficiently.

The protein digests produced by HGJ and HDJ were screened for antibacterial effects against *L. monocytogenes*, a food born bacteria responsible for fatal and sometimes deadly infections. Cells of *L. monocytogenes* were strongly inhibited by caprine whey obtained after reaction with both HGJ and HDJ. Undigested caprine whey and the products from the first step of digestion with HGJ demonstrated no significant effect. This indicates that during digestion the antibacterial effect of caprine whey hydrolysates are most effective in the duodenum. This gives a promising opportunity to inhibit listeriosis in humans, and results are also useful for development of dietary supplement, nutraceuticals and functional foods.

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1. Introduction

The prospect for processing whey into an assortment of food products has been extensively studied during the last decades, due to the increasing production of whey as a by-product from the cheese production industry. Both bovine and caprine whey proteins and their enzymatically derived peptides have obtained intensive attention

as precursors of bioactive components, by contributing to particular antimicrobial activities against a broad spectra of bacteria (Meisel and Schlimme, 1996; Pihlanto and Korhonen, 2003). The whey proteins α -lactalbumin (α -LA), β -lactoglobulin, laktoferrin, immunoglobulins, laktoperoxidase and lysozyme all contain specific bioactivity and possess the ability to inhibit or kill a wide range of bacteria (Tomita et al., 1994; Pihlanto-Leppälä, 2000). Many of these microorganisms are important and common sources of food born illness, such as *Escherichia coli*, *Salmonella typhimurium*, *Bacillus cereus* and *Listeria monocytogenes* (Batish et al., 1988; Payne et al., 1990; Almaas et al., 2006a). Especially *L. monocytogenes* is very harmful, and an outbreak of this bacterium among people is often caused by the contamination in different types of food, such as raw vegetables, raw meat products, and raw and pasteurized

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milk and soft cheeses (Adams and Moss, 2004). The bacteria have a quite widespread distribution and have an ability to grow in most non-acid foods, and therefore have plenty of opportunity to enter the food chain and multiply. *L. monocytogenes* causes the disease listeriosis, which is most likely to develop in pregnant women, very young children, elderly or other people with an underlying condition that gives problems with the immune system. For pregnant women listeriosis often results in abortion or premature birth. Detecting natural components that inhibits *L. monocytogenes* could be very valuable, since the mortality rate of adult listeriosis is estimated to be as high as 30% (Griffiths, 1989; Adams and Moss, 2004).

Peptides produced by digestion of caprine proteins using commercial proteolytic enzymes have in several studies been reported to contain antibacterial properties. One of these peptides, lactoferricin C (caprine), was identified as fragment 14–42 of the LF sequence (Recio et al., 2000). Studies done on lactoferricin C produced by commercial enzymes have showed strong antimicrobial activity against various types of bacteria (Kimura et al., 2000; Recio and Visser, 2000).

In the majority of previous studies regarding antibacterial effects of peptides from whey, the peptides have been formed by commercially available proteolytic enzymes as pepsin, trypsin and chymotrypsin from pig pancreas (Tomita et al., 1991). However, if the goal is that these peptides should mimic the gut in human beings, human gastric and duodenal enzymes ought to be used instead (Bellamy et al., 1992). Pepsin, trypsin and chymotrypsin are present in a variety of forms in many different species. Also in diverse isoforms with different catalytic activity within the same specie (Scheele et al., 1981; Dunn, 2002). Consequently porcine and human proteases may produce different peptides from caprine whey.

The general motivation for our studies was primary to develop an *in vitro* procedure to mimic the “human digestion”, by using human gastric and duodenal juice compared to commercial enzymes. Human proteolytic enzymes were obtained from one individual according to Holm et al. (1988). Secondary, to improve gut-health, a screening of the digestion products for antibacterial effects against *L. monocytogenes* was an important task.

2. Materials and methods

2.1. Whey protein concentrate from goat

Goat's milk was collected from the university farm. A whey protein concentrate from goats' milk (WPCG) was produced from 570 l of pasteurized (72 °C, 15 s) milk. The milk was treated with a starter culture (CHN-11, Christian Hansen, DK) for 1 h at 32 °C, followed by renneting (25 ml/100 l milk, rennet type SP175, Christian Hansen, DK). The precipitated casein was removed by centrifugation, and the remaining 450 l of whey was reduced to 50 l by diafiltration for 3 h (Alfa Laval UFS4, membrane GR-62-6338/48P, cut-off 20,000 MW). Three kilograms of WPCG was then obtained after spray drying (Minor, Niro Atomizer A/S, DK). The WPCG contained 81% protein as analysed by Kjeldahl (IDF, 1993).

2.2. Human and porcine proteolytic enzymes

Human proteolytic enzymes were obtained in the activated state by collecting gastric and duodenal juice according to Holm et al. (1988). All gastric and duodenal enzymes used in this study were obtained from one

person. In brief, a three-lumen tube enabled simultaneous instillation of saline in the duodenum and aspiration of gastric (HGJ) and duodenal (HDJ) juice. Saline (100 ml/h) was instilled close to the papilla of Vater and duodenal juice aspirated some 18 cm distally. Aspirates were collected on ice and frozen in aliquots at –20 °C to stop the enzymatic reaction. Before further use the individual samples from gastric and duodenal juice were collected in two batches (HGJ and HDJ), to avoid the variations in enzyme activity between the individual samples. Pepsin activity of HGJ was assayed with hemoglobin as a substrate, according to Sanchez-Chiang et al. (1987). Total proteolytic activity of HDJ was assayed with casein as a substrate, according to Krogdahl and Holm (1979). One unit of enzyme activity (U) is defined as the amount of enzyme, which produces an absorbance of 1.0 at 280 nm in 20 min at 37 °C.

The commercial enzymes used were: porcine pepsin A (EC 3.4.23.1), (activity 439 U/mg) obtained from Sigma Chemical, USA and Corolase PP (CPP), an extract from pig pancreas gland, (activity 350 PU/mg) obtained from Röhm GmbH, Germany.

2.3. Digestibility assay

A modified digestibility assay, *in vitro* protein digestion (AOAC Official Method 982.30; Rasco, 1994), was performed in two steps, using human gastric juice (HGJ) and human duodenal juice (HDJ) as illustrated in Fig. 1.

The two digestion steps were performed for 30 min each at 37 °C. Previous results showed that very little if any new peptides were produced with an extended reaction time. In step 1, 10 ml WPCG 50 g/l (sample A) was acidified to pH 2.5 with 2 M HCl and incubated with 50 µl (0.4 U) HGJ. In step 2, pH was adjusted to pH 8.0 with 1 M NaOH, and 400 µl (13 U) HDJ with continuous stirring. 0.5 ml aliquots were taken at different times during the incubation to follow the degradation profile. To stop the reaction, samples were put on ice, frozen and freeze-dried. When commercial enzymes were used, 4 mg pepsin (14 U) and 4 mg CPP (26 U) were used per gram substrate.

2.4. Gel electrophoresis

SDS-PAGE (PhastSystem™, Pharmacia Laboratory Separation Division, Sweden) was carried out to evaluate the resulting protein profile after each step of digestion. The assay was performed according to standard protocols (Laemmli, 1970), using 20% acrylamide gels as described by Almaas et al. (2006b).

2.5. Assay of antibacterial activity against *L. monocytogenes*

L. monocytogenes was obtained from the departmental culture collection at the Norwegian University of Life Sciences. It contained a mixture of three different strains, formerly an extract from the Norwegian food industry, donated from the Norwegian Food Research Institute. The bacteria were cultured in Brain Heart Infusion (BHI)-broth (Oxoid; 37 g/l), at pH 7.4 and 37 °C. Active growing cultures (1%) were used in the growth studies.

Freeze-dried samples of WPCG and hydrolysates were solubilized in water and added to the bacteria culture, resulting in final protein concentrations from 0.3 to 0.6% in each well. Protein concentrations were set according to earlier work (Almaas et al., 2006a). The assay was carried out in microtiterplates, by measuring optical density (660 nm) according to Almaas et al. (2006a). The experiments were repeated three times, with five parallels of each sample.

2.6. Statistics

All the spectrophotometric absorbance measurements were calculated for standard deviation, and results are presented as mean values with $\pm$ S.D. after 10 h (the rest is removed for clarity in the figures). The results obtained from samples containing various sources of whey protein (samples A–C) were also analysed in order to investigate the significance of the difference. A *t*-test (two-sample, assuming unequal variances) was run to compare the different growth-curves based on data obtained after 10 h, when equilibrium in the growth curves was reached. The differences were considered significant when $p < 0.05$.

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