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Isolation and identification of antimicrobial peptides derived by peptic cleavage of whey protein isolate

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ABSTRACT

The antimicrobial potential of whey protein isolate hydrolyzed by gastrointestinal enzymes was determined by attempting to identify and characterize the antimicrobial peptides responsible. While tryptic and chymotryptic hydrolysates did not show antibacterial activity, whey proteins hydrolyzed for 45–90 min by pepsin exhibited significant activity. Fractionation of 60-min hydrolysate by reversed-phase high performance liquid chromatography yielded 5 fractions that were antibacterial, with minimum inhibitory concentrations comprised between 20 and 35 μg/mL. These fractions contained short peptides not previously identified as antimicrobial. Fragment 14–18 (KVAGT) of β-lactoglobulin is very close to a sequence previously identified as antibacterial and is found in antimicrobial sequences of diverse origin. Five other peptides derived from β-lactoglobulin, and one fragment from α-lactalbumin (f117–121, KVGIN), were also identified as antibacterial. The identified peptides do not match pepsin action exactly, indicating modified proteolysis of unknown origin. Protein by-products of the dairy industry offer potential for large-scale production of antimicrobial peptides.

1. Introduction

Milk proteins are the principal source of bioactive peptides encrypted within primary amino acid sequences and released upon enzymatic hydrolysis during gastrointestinal transit or food processing (Gobbetti, Stepaniak, De Angelis, Corsetti, & Di Cagno, 2002; Meisel & Bockelmann, 1999). A growing number of such peptides are being identified in dairy protein hydrolysates and fermented dairy products. Some of these peptides have been shown to possess opioid, immunomodulatory, antimicrobial, antithrombotic, growth-stimulating or antihypertensive properties, as previously reviewed (Choi, Sabikhi, Hassan, & Anand, 2012; Korhonen, 2009; Park, 2009). Despite the notable successes of lactoferricin (LFcin) and kappacin, few studies have addressed the antimicrobial properties of peptides released from dairy proteins by enzymatic hydrolysis. In response to evolving consumer values, the food industry is now showing an increasing interest in milk-derived peptides as alternatives to conventional antimicrobial preservatives. The possibility of adding them to foods as bioactive supplements is also under investigation.

While native proteins such as casein, β-lactoglobulin (β-lg), α-lactalbumin (α-la) and serum albumin (SA) are apparently inactive (Clare & Swaisgood, 2000), a few milk proteins, primarily lactoferrin (LF) and lysozyme, appear to have antimicrobial properties. Meanwhile, numerous reports have confirmed the release of antimicrobial peptides by hydrolysis of the caseins of several animal species (Baranyi, Thomas, & Pellegrini, 2003; López-Expósito, Minervini, Amigo, & Recio, 2006; Zucht, Raida, Adermann, Magert, & Forssmann, 1995). Beside caseins, few works demonstrated the presence of antimicrobial peptides within sequences of whey proteins with

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the exception of LF. Bellamy et al. were the first to describe the antimicrobial peptide LFcin, released from the N-terminal domain of bovine LF hydrolyzed by pepsin *in vitro* (Bellamy, Yamauchi, Wakabayashi, Kawase, & Tomita, 1992) and found later *in vivo* (Kuwata, Yip, Tomita, & Hutchens, 1998). LFcin displays a broad spectrum of activity against bacteria, fungi, viruses and parasites (Bellamy, Takase, Wakabayashi, Kawase, & Tomita, 1992; Jenssen, 2005; Tanaka et al., 1995; Wakabayashi, Hiratani, Uchida, & Yamaguchi, 1996) and is now being used in the food processing industry and cosmetics (Tomita, Wakabayashi, Yamauchi, Teraguchi, & Hayasawa, 2002). The antimicrobial activity of another domain of LF, named lactoferrampin (LFampin), has also been described, but its spectrum differs little from that of LFcin (van der Kraan et al., 2004) and the list of LF-derived antimicrobial peptides is growing and is expected to continue to do so (Elbarbary et al., 2010). In comparison, other whey proteins have so far provided relatively few antimicrobial peptides. Notable works performed by Pellegrini, Thomas, Bramaz, Hunziker, and von Fellenberg (1999), Pellegrini, Dettling, Thomas, and Hunziker (2001) described negatively charged domains isolated from α-la and β-lg using trypsin and/or chymotrypsin and active against Gram-positive bacteria. Finally, some whey-derived peptides that have no demonstrable antimicrobial activity *in vitro* stimulate certain immune system functions *in vivo* and appear thus to increase resistance to gastrointestinal pathogens (Gauthier, Pouliot, &Saint-Sauveur, 2006; Mercier, Gauthier, & Fliss, 2004).

The potential of bovine β-lg and α-la to yield antimicrobial peptides appears under-exploited. Whey protein isolates (WPI) are widely used as dietary supplements and functional ingredients in food products and are composed mainly of these two proteins. They are inexpensive and thus appear suitable as starting material for the production of natural bioactive agents. The objective of the present study was to identify and characterize potentially antimicrobial peptides derived from whey proteins by hydrolysis using digestive enzymes and to describe the kinetics of their release.

2. Materials and methods

2.1. Materials and microorganisms

A commercial WPI from dairy cows (Bipro) was obtained from Davisco Foods International (Le Sueur, MN, USA). Its protein portion consisted primarily of β-lg (74.0%, w/w), α-la (12.5%, w/w), bovine SA (5.5%, w/w) and immunoglobulin (5.5%, w/w). The powder contained 93.4% (w/w) protein and 0.12% (w/w) calcium. HPLC-grade acetonitrile, analytical grade trifluoroacetic acid (TFA), porcine pepsin A (570 units/mg), trypsin (16700 units/mg) and chymotrypsin (⩾40 units/mg) were all purchased from Sigma (St. Louis, MO, USA). Microbial growth inhibition assays were performed using one Gram-positive (*Listeria ivanovii* HPB28), and one Gram-negative (*Escherichia coli* MC4100). Both bacteria strains were grown aerobically in Tryptic Soy Broth with 0.6% yeast extract (w/v) (TSB; Difco Laboratories, Sparks, MD, USA). *L. ivanovii* was incubated at 30 °C and *E. coli* was incubated at 37 °C.

2.2. Hydrolysis of whey proteins

The method described in a previous paper was used (Vermeirssen, van der Bent, Van Camp, van Amerongen, and Verstraete, 2004). A 4% (w/v) solution of WPI was prepared and held at 37 °C for 15 min before use. The pH was then adjusted to 2.0 prior to adding pepsin or to 6.5 for trypsin and chymotrypsin. Enzymes were added at a final enzyme/substrate ratio of 1:250 (w/w) and hydrolysis was conducted at 37 °C with stirring. Samples were taken at the start and then every 15 min for a total of 2.5 h. Hydrolysis was stopped by heating at 85 °C for 10 min. The samples were then filtered on Whatman No. 41 paper (Whatman International, Ltd., Maidstone, UK) using a Buchner funnel. Samples were then stored at −20 °C for subsequent analysis. For each sample, degree of hydrolysis (DH) was determined using the o-phthaldialdehyde method described previously (Church, Swaisgood, Porter, & Catignani, 1983).

2.3. Purification of peptides

The samples of hydrolyzed WPI were filtered on Centricon membrane (10 kDa molecular mass cut-off; Millipore, Billerica, MA, USA), followed by adjustment to pH 7.0 using 0.5 M NaOH. Protein concentrations were determined in triplicate using the Dumas method using a Leco FP-528 device (Leco Corp., St. Joseph, MI, USA). All samples were stored at −20 °C until testing. Peptides were separated using a Gold HPLC system (Beckman-Coulter, Brea, CA, USA) with an analytical reversed-phase C18 column (150 × 4.6 mm; Phenomenex, Torrance, CA, USA). Linear gradients were conducted from 2% to 60% solvent B (0.1% TFA in acetonitrile) over solvent A (0.1% TFA in water) in 60 min with a flow rate of 1 mL/min. Elution was monitored by UV absorbance at 214 nm. Concentrations of protein in the fractions were estimated by the bicinchoninic acid (μBCA) method (BCA, Pierce Biotechnology, Rockford, IL, USA).

2.4. Identification of peptides by ESI mass spectrometry

The mass spectra of the compounds were recorded with an electrospray ionization mass spectrometer (Agilent series 1100, Agilent Technologies, Inc., Loveland, CO, USA) connected to the reversed-phase chromatography unit. The ESI spectrometer was operated in positive ion mode. Conditions were spray voltage of 4.5 kV; sheath gas (nitrogen) flow rate of 1.05 L/min; capillary voltage of 14.0 V; heated capillary temperature of 190 °C; tube lens offset voltage of 35.0 V; octapole 1 offset of −3.0 V; octapole 2 offset of −5.0 V; lens voltage of −16.0 V; octapole RF amplitude of 400.0 V; trap DC offset of −10.0 V. The fractions were dissolved in a 4:2 (v/v) blend of acetonitrile and 50% formic acid and were injected into the electrospray source using a syringe pump at a flow rate of 5 μL/min. Within the ion trap, dry nitrogen was used as the nebulizing gas at 12 psi, 4 L/min and 325 °C.

Each fraction was analyzed in MS/MS full scan acquisition mode to determine major peptides with *m/z* range scanned from 150 to 3000. Since many sequences corresponded to a specific *m/z* ratio, a second run discriminated each ratio of