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Antibacterial peptides generated by Alcalase hydrolysis of goat whey

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ABSTRACT

Goat whey hydrolysates were produced from goat whey using Alcalase® from *Bacillus licheniformis*. The goat whey hydrolysate (GWH) with the highest degree of hydrolysis was fractionated by size exclusion chromatography (SEC), and the antibacterial activity of the GWH and the obtained SEC-fractions were evaluated using the disc diffusion method. The results showed that the GWH exhibited significantly higher antibacterial activity than the unhydrolysed goat whey. Among all SEC fractions obtained, only SEC-F_2 and SEC-F_3 showed antibacterial activity against the tested bacteria. SEC-F_3 exhibited the highest antibacterial effectiveness against *Escherichia coli* and *Bacillus cereus* with MIC values of 0.09 and 0.03 mg/mL respectively, whereas, SEC-F_3 and SEC-F_2 had comparable MIC values against *Staphylococcus aureus* (~0.02 mg/mL). Transmission electron microscopy showed that the GWH caused changes in the bacterial cells affected. Additionally, LC–MS analysis was used to characterize the peptides of GWH and SEC-F_3. Two major peptides were detected in GWH and SEC-F_3, with higher concentration in the latter, having masses of 730 and 1183 Da, respectively. These results suggest that hydrolysis of goat whey by Alcalase is an easy tool to enhance its antibacterial activities. GWH and its effective antibacterial fractions may be used to control the undesirable bacteria in food and improve its safety.

1. Introduction

The safety and shelf life of foods are of significant concern in the food industry. These are affected by the incidence of pathogenic and spoilage bacteria which can contaminate food through several routes. A broad spectrum of methods have been employed to prevent the growth of such bacteria in food including the use of synthetic and natural antimicrobial agents. However, the use of synthetic agents is precluded owing to the possible negative impact of such chemicals on the environment and human health. So, novel antimicrobial agents from natural sources are highly required. A number of strategies have been suggested to improve the antimicrobial activities of proteins, including chemical modification such as esterification (Sitohy, Mahgoub, Osman, El-Masry, & Al-Gaby, 2013) and enzymatic hydrolysis (using digestive, microbial, or plant proteolytic enzymes; Arruda et al., 2012; Korhonen & Pihlanto, 2006; Kotlar, Ponce, & Roura, 2013; Théolier, Hammami, Labelle, Fliss, & Jean, 2013).

Whey is a major by-product from cheese manufacture and contains 6–7% total solids. Whey proteins represent nearly 20% of total milk proteins (Fox, 1989). The major proteins in whey are beta-lactoglobulin (β-Lg), α-lactalbumin (α-La), immunoglobulins (Ig), and bovine serum albumin (BSA). In addition, whey contains a range of minor proteins including lactoferrin (LF), lactoperoxidase and various growth factors (Park, Juárez, Ramosc, & Haenlein, 2007).

By enzymatic hydrolysis of caprine whey proteins peptides with important biological activities, such as antimicrobial, immunomodulatory, antioxidant, and antihypertensive activities, have been released (De Gobba, Espejo-Carpio, Skibsted, & Otte, 2014; Eriksen, Vegarud, Langsrud, Almaas, & Lea, 2008; Murata, Wakabayashi, Yamauchi, & Abe, 2013). Several studies have investigated the formation of antibacterial peptides by digestion of goat whey proteins using various proteolytic enzymes (reviewed in Atanasova & Ivanova, 2010; Hernández-Ledesma, Ramos, & Gómez-Ruiz, 2011). Digestion of caprine whey proteins by human gastric and duodenal

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juice has produced various antibacterial components that exhibited a strong inhibition of the growth of *Listeria monocytogenes* and other pathogenic bacteria (Almaasa, Berner, Holm, Langsrud, & Vegarud, 2008). Hydrolysis of goat whey proteins with pepsin released peptides with antibacterial activity against some food pathogens (El-Zahar et al., 2004; Théolier et al., 2013). One particular peptide, lactoferricin C (fragment 14–42 of the LF) produced by pepsin and trypsin showed strong antimicrobial activity against various types of bacteria (Kimura et al.,2000; Recio & Visser, 2000).

Alcalase® is an enzyme extract from *Bacillus licheniformis* (Sukan & Andrews, 1982) containing several proteinases with different specificities. Alcalase has been used extensively to prepare soluble hydrolysates of soy protein (Fox, 1989) and fish protein (Rebeca, Peña-Vera, & Díaz-Castañeda, 1991) as well as to produce bioactive peptides (Corrêa et al., 2011; Espejo-Carpio, De Gobba, Guadix, Guadix, & Otte, 2013; Sila et al. 2014; Zhang, Olsen, Grossi, & Otte, 2013). However, to date the antibacterial activity of goat whey hydrolysates produced with Alcalase has not been investigated.

Therefore, the aim of this work was to i) evaluate the antibacterial activity of goat whey hydrolysate (GWH) generated by Alcalase and fractions thereof obtained by size-exclusion chromatography, and to ii) characterize the active fractions. This information is relevant in assessing the potential use of GWH or fractions as bio-preservatives in food.

2. Materials and methods

2.1. Materials and chemicals

Goat milk was obtained from the Food Science Department, Faculty of Agriculture, Zagazig University, Zagazig, Egypt. Sweet whey was prepared from this milk by rennet coagulation as described by Ha, Bekhit, McConnell, Mason, and Carne (2014). The resulting sweet whey was pasteurized at 73 °C for 15 s, cooled, lyophilized, and kept frozen at −20 °C until use. Alcalase 2.4 L (FG; EC 3.4.21.6) from *B. licheniformis* was purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of goat whey hydrolysate (GWH)

Lyophilized goat whey (11% protein) was dissolved in distilled water (100 g/L) and hydrolyzed batch-wise by treatment with Alcalase (E/S ratio of 1:200 “w/w”) at 55 °C and pH 7.8. The hydrolysis was allowed to proceed for 240 min during which the pH was maintained at 7.8 by addition of 1 M NaOH. The degree of hydrolysis (DH) was determined every 60 min during hydrolysis according to Adler-Nissen (1986). At the end of hydrolysis, the enzyme was inactivated by heating at 80 °C for 20 min. The hydrolysate was clarified by centrifugation at 4000 × g for 30 min at 16 °C to remove insoluble substrate fragments, and the supernatant (denoted GWH) was lyophilized and kept frozen at −20 °C until further use.

2.3. Size exclusion chromatography (SEC)

A portion (500 mg) of the lyophilized GWH was suspended in 15 mL of deionized water and separated on a sephadex G-25 gel filtration column (1.6 × 150 cm). Two milliliters were injected and eluted with distilled water at a flow rate of 1 mL/min, and detected at 280 nm. The major peaks were collected and lyophilized to evaluate their antibacterial activity.

2.4. Peptide profiles and LC–MS analysis

Peptide profiles and masses of major peptides in GWH and SEC-F3 were analyzed by LC–MS analysis using an Agilent 1100 LCMSD Trap according to Otte, Shalaby, Zakora, and Nielsen (2007). The lyophilized samples were re-dissolved at 5 mg mL^{-1} in water and 15 μL were injected and separated using a gradient consisting of 100% buffer A (0.1% trifluoroacetic acid (TFA) in water) for 5 min followed by a linear increase from 0 to 55% buffer B (0.1% TFA in 90% acetonitrile) over the next 70 min. On-line MS analysis was performed as described by Otte et al. (2007) with a target mass of 1522 m/z.

2.5. Protein determination

The protein content of the lyophilized goat whey was determined by the Kjeldahl method (AOAC, 1990). The protein contents of the lyophilized GWH and the SEC fractions were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) according to the supplier's instruction.

2.6. Evaluation of goat whey hydrolysate as antibacterial agents

Four bacterial strains including two Gram positive (G^+) bacteria (*Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* ATCC 33018) and two Gram negative (G^-) bacteria (*Salmonella typhimurium* ATCC 14028 and *Escherichia coli* ATCC 8739) were used as indicators to assess the antibacterial activity of the goat whey samples.

Working cultures were obtained by inoculating a loopful of each culture into 50 mL of Mueller–Hinton broth in 100 mL sterilized conical flasks. The flasks were incubated overnight at the optimum temperature (37 °C for *St. aureus, S. typhimurium, E. coli* and 30 °C for *B. cereus*) to activate the growth. After incubation, the cultures were diluted to ca. 6.0 log cfu/mL.

The antibacterial activity of goat whey, GWH and SEC-fractions were evaluated by the disc–diffusion assay (Bauer, Kirby, Sherris, & Turck, 1966). Firstly, the samples were filtered through a cellulose acetate membrane filter with pore size 0.45 μm (ADVANTEC MFS, Inc., Japan). Sterile paper discs (with a diameter of 5 mm) were saturated with 15 μL of test samples (10 mg protein/mL) and placed on the surface of Mueller–Hinton agar plates previously seeded with the indicator strain (10% v/v). The plates were stored at 4 °C for 2 h to permit radial diffusion of the sample, and then incubated for 24 h under optimal growth conditions for the indicator strains. The plates were subsequently examined for presence of inhibition zones indicative of antibacterial activity. The antibacterial activity was compared to that of positive controls; penicillin G against G^+ bacteria, and ampicillin against G^- bacteria.

The antibacterial activity of selected samples was quantified by determination of the minimal inhibitory concentration (MIC) using the critical dilution assay in combination with the disc diffusion assay as described by Yamamoto, Togawa, Shimosaka, and Okazaki (2003). Each sample was serially diluted two fold and saturated discs with each dilution of samples were placed on the inoculated agar medium as mentioned above. The lowest concentration (highest dilution) of the test sample that showed a clear inhibition zone on the Mueller–Hinton agar plates was regarded as the minimal inhibitory concentration (MIC).

2.7. Transmission electron microscopy examination

Transmission electron microscopy (TEM) was used to evaluate the ultrastructure of the bacterial cells as described by Sitohy et al. (2013). Each bacterial type was grown in nutrient broth supplemented with GWH (at a concentration of 2 MIC) and incubated at their corresponding optimum temperature for 24 h. The control samples were prepared with water instead of GWH.