Food Research International 43 (2010) 1545–1548

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Short Communication

Identification of bioactive peptides in commercial Cheddar cheese

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ARTICLE INFO

Article history:
Received 6 December 2009
Accepted 1 March 2010

Keywords:
Cheddar cheese
Antimicrobial
Antihypertensive
Antioxidant
Bioactive peptide

ABSTRACT

This study examined the presence of antimicrobial, antioxidant and antihypertensive peptides in three commercially available Australian Cheddar cheeses. Peptide extracts as well as fractionated peptide extracts were examined. Commercial cheese A peptides exhibited the greatest inhibition against *Bacillus cereus* and also commercial cheese A fractionated peptides greater than 10 kDa showed the highest inhibition against *B. cereus*. Commercial cheese A peptides also showed the highest inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH), a free radical used to measure antioxidant activity. All cheese fractionated peptides greater than 10 kDa demonstrated higher inhibition of DPPH after fractionation. Antihypertensive peptides were determined by inhibition of the angiotensin-converting enzyme (ACE). Overall, commercial cheese A had the lowest concentration required to inhibit ACE and commercial cheese A fractionated peptides lower than 5 kDa had the lowest inhibition after fractionation. These preliminary findings suggest that peptide extracts of three commercial Australian Cheddar cheeses exhibit antimicrobial, antihypertensive and antioxidant properties.

1. Introduction

Bioactive peptides are specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts & Weiler, 2003). They affect numerous biological processes including evoking behavioural, neurological, hormonal, gastrointestinal and nutritional responses (Clare & Swaisgood, 2000). They can be from 2 to 20 amino acids in size and many have multifunctional properties (Korhonen, 2009b; Rutherfurd-Markwick & Moughan, 2005).

Bioactive peptides have been isolated from many dairy products including cheese, kefir, milk, and yoghurt. They are inactive within protein molecules and can be released in three ways by enzymatic hydrolysis by digestive enzymes predominantly pepsin, trypsin and chymotrypsin, fermentation of milk with proteolytic starter cultures or proteolysis by enzymes derived from microorganisms or plants (Korhonen, 2009a).

There are few reported studies on the bioactive properties of peptides isolated from commercial Cheddar cheeses (Dionysius et al., 2000; Gupta, Mann, Kumar, & Sangwan, 2009; Haileselassie, Lee, & Gibbs, 1999; Ong & Shah, 2008). In this study, three commercially available Australian Cheddar cheeses were investigated to determine if the peptides had antimicrobial, antihypertensive and antioxidant properties. New bioactive peptides that have more potent activity than previous reported bioactive peptides could be discovered, which could provide greater health benefits to consumers.

2. Materials and methods

2.1. Extraction of cheese peptides and SDS–PAGE

The water-soluble peptides were extracted as per Verdini, Zorrilla, and Rubiolo (2004). Three tubes each containing 100 g of grated cheese (Australian commercial cheese A, B and C) and 300 ml distilled water was homogenised (House and Home Multimixer). The tubes were placed in a 40 °C water bath, shaking at 100 rpm for 1 h and then centrifuged 4250g at 4 °C for 30 min (Sigma Laboratory Centrifuge 6K15, John Morris Scientific Pty Ltd., Chatswood, Australia). The pellet was discarded. The supernatant was re-centrifuged and then filtered through No. 42 Whatman filter paper (Interpath, Heidelberg West, Victoria, Australia) and 0.2 μm syringe membrane filter (Sartorius Australia, Melbourne, Australia). The filtrate was stored at −20 °C until further use.

The concentration of peptide in 100 μl was determined by weighing after freeze-drying (CHRIST Alpha 1–4, B. Braun Biotech International) overnight. A 12.5% SDS–PAGE gel was run to confirm if the peptides had been extracted as per Laemmli, 1970 (data not shown).

2.2. Separation of cheese peptides by RP-HPLC

Extracted peptide samples were separated by an Alltima amino C18 column (Grace Davidson Discovery Science, Deerfield, USA)

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doi:10.1016/j.foodres.2010.03.007

using RP-HPLC (Shimadzu Scientific, Melbourne, Australia). Solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile (LOMB Scientific, Taren Point, NSW, Australia). For each sample, 50 μl was injected and run with a linear gradient 0.2–60% of solvent B (0.1% TFA in acetonitrile) to 60 min followed by 0.2% of solvent B to 71 min at a flow rate of 1 ml/min at room temperature.

The peptides were fractionated by Vivaspin 20 centrifugal concentrators with molecular weight cut-off (MWCO) PES membranes of 5 kDa and 10 kDa (Sartorius Australia, Melbourne, Australia) by centrifugation, 5580*g*, 5 °C, 30 min and ran using the Alltima amino C18 column (Grace Davidson Discovery Science, Deerfield, USA) using the same program as above. For each sample, 50 μl or 100 μl was injected. The flow rate was 1 ml/min.

2.3. Determination of antimicrobial activity

Three bacteria *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538 and *Bacillus cereus* ATCC 11778 were obtained from the University of Western Sydney culture collection, Hawkesbury Campus, Australia.

E. coli was subcultured on Difco Luria broth (BD, North Ryde, Australia) and both *S. aureus* and *B. cereus* were subcultured on Difco Brain Heart Infusion (BD, North Ryde, Australia). All strains were incubated aerobically at 37 °C for ~18 h. The 0.5 McFarland standard was prepared and used to approximate the concentration of the bacteria (1.5×10^8 cfu/ml). Negative controls contained tetracycline solution (16.12 mg/l) and positive controls contained tryptone water (0.052 mg/l). The samples contained 70 μl of the cheese peptide extract and 130 μl bacterial suspension.

All experiments were carried out in triplicate in a 96-well plate and the absorbance at 595 nm (Bio-rad spectrophotometer, Bio-rad, Gladesville, Australia) was read after 24 h incubation. Percentage of inhibition was determined by:

$$\frac{\left(A^{\text{sample}} - A^{\text{negative control}}\right)}{\left(A^{\text{positive control}} - A^{\text{negative control}}\right)} \times 100$$

2.4. Determination of antihypertensive activity

Antihypertensive activity of the cheese peptide extracts and the fractionated cheese peptides was determined by RP-HPLC using the modified method of Nakamura et al. (1995). This method uses the angiotensin-converting enzyme (ACE) (Sigma-Aldrich, Castle Hill, Australia) and the substrate Hippuryl–Histidyl–Leucine (HHL) (Sigma-Aldrich, Castle Hill, Australia), which produces hippuric acid. The amount of hippuric acid produced was used to determine antihypertensive activity. 5 mM His-Hip-Leu was dissolved in 50 mM HEPES buffer (containing 0.3 M NaCl pH 8.3). 200 μl HHL solution was added to 50 μl peptide or 50 μl distilled water and incubated for 3 min at 37 °C. Then 20 μl 0.1U ACE was added and incubated for 30 min at 37 °C. The reaction was stopped by adding 250 μl 1 M HCl and vortexed. The mixture was filtered using 0.2 μm membrane filter with syringe. The mixture was separated using the Alltima amino C18 column with a 0.4 ml/min flow rate and isocratic program using 50% methanol with 0.1% TFA for 22 min by RP-HPLC. Pure huppuric acid was run as a standard using the same program. The height and retention time of the hippuric acid was measured using class-VP 7.3 software (Shimadzu, Melbourne, Australia) and the percentage of inhibition of ACE was determined by the height of hippuric acid peak in the control sample (ACE, HHL), in the sample containing inhibitor (ACE, HHL and peptide) and in the sample without ACE (HHL, peptide) as follows:

$$\text{percentage of inhibition} = \frac{(\text{control} - \text{inhibitor sample})}{(\text{control} - \text{sample without ACE})} \times 100$$

The concentration required to inhibit 50% of ACE was calculated by the concentration of the peptide in the ACE mixture times fifty divided by the percentage of inhibition.

2.5. Determination of antioxidant activity

The free radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was used to determine if the three cheeses contained peptides that may exhibit antioxidant activity. Antioxidative activity of the cheese peptide extracts was determined by a modified method of Apostolidis, Kwon, and Shetty (2007).

Briefly, three separate ratios of 60 μM DPPH in ethanol to peptide were prepared: 3 ml:250 μl (Apostolidis et al., 2007), 1 ml:500 μl and 1 ml:1 ml. A control of MQ water and DPPH was set up for each ratio before centrifugation at 9470*g* (Mikro 20, Hettich Zentrifugen, Tuttlingen, Germany) for 2 min. The absorbance was read at 517 nm. The percentage of inhibition was calculated by:

$$\frac{A^{\text{control}} - A^{\text{extract}}}{A^{\text{control}}} \times 100$$

2.6. Statistical analysis

The data was analysed using one-way analysis of variance (ANOVA) followed by the Tukey's test to determine significant differences between treatment groups. The analysis was carried out using SPSS version 17.0 (SPSS Inc., Chicago, USA).

3. Results and discussion

3.1. Extraction and separation of cheese peptides

3.1.1. Average peptide concentrations

All three commercial Australian Cheddar cheese peptide extracts had concentrations higher than 10 mg/ml. The commercial cheese B fraction containing peptides larger than 10 kDa was the most concentrated (59.8 mg/ml) and the peptide extract of commercial cheese A had the lowest concentration (10 mg/ml) of peptide.

3.1.2. Separation by RP-HPLC

The peptide profiles of the three cheese peptide extracts showed similarities. However, commercial cheese A has the highest amount of peptide peaks (196 peaks), followed by B (194 peaks), and then C (182 peaks).

3.2. Antimicrobial activity

The peptide extracts were screened for the presence of antimicrobial activity by determining if the growth of three bacteria *E. coli, B. cereus* and *S. aureus* were inhibited. The three cheese peptide extracts inhibit *E. coli* and *B. cereus* when compared with the positive control containing tryptone water. The commercial cheese A peptide extract (~0.245 mg/70 μl) inhibited *B. cereus* the most overall. *E. coli* was inhibited the most by commercial cheese C peptides (~0.588 mg/70 μl).

The results show that *B. cereus* (Fig. 1B) is inhibited the most by commercial cheese A peptides greater than 10 kDa (~1.156 mg/70 μl). *E. coli* is also inhibited the most by commercial cheese A peptides greater than 10 kDa (Fig. 1A); however, the inhibition of *S. aureus* is low for all three fractionated cheese peptide extracts (Fig. 1C). The results for *S. aureus* had no significant differences.