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Effect of probiotics on antioxidant and antimutagenic activities of crude peptide extract from yogurt

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ABSTRACT

Search for bioactive peptides is intensifying because of the risks associated with the use of synthetic therapeutics, thus peptide liberation by lactic acid bacteria and probiotics has received a great focus. However, proteolytic capacity of these bacteria is strain specific. The study was conducted to establish proteolytic activity of *Lactobacillus acidophilus* (ATCC® 4356™), *Lactobacillus casei* (ATCC® 393™) and *Lactobacillus paracasei* subsp. *paracasei* (ATCC® BAA52™) in yogurt. Crude peptides were separated by high-speed centrifugation and tested for antioxidant and antimutagenic activities. The degree of proteolysis highly correlated with these bioactivities, and its value (11.91%) for samples containing all the cultures was double that of the control. Liberated peptides showed high radical scavenging activities with 1,1-diphenyl-2-picrylhydrazyl and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), IC_{50} 1.51 and 1.63 mg/ml, respectively and strong antimutagenicity (26.35%). These probiotics enhanced the generation of bioactive peptides and could possibly be commercially applied in new products, or production of novel anticancer peptides.

1. Introduction

Chronic diseases and ageing phenomena are relevant to the imbalance of free radical levels in the body. An excess of free radicals can cause lethal cellular damage through oxidising cellular proteins, enzymes, membrane lipids, and DNA; thus arresting cellular respiration (Urso & Clarkson, 2003). When the damage cannot be repaired diseases, such as cancer, cardiovascular diseases and diabetes, can arise (Collins, 2005). Their presence in foods, in addition to lipolytic and peroxidative changes, can cause sensory and nutritional value deterioration (Spitzer, Doucet, & Buettner, 2010). Therefore, it becomes essential to inhibit the peroxidation of lipids and the generation of free radicals in the living cells and foodstuffs.

Although artificial antioxidants, such as butylated hydroxytoluene, butylated hydroxyanisole, and n-propyl gallate, display strong activity against various oxidation systems, the use of these antioxidants in food is prohibited in some countries due to the potential risks to human health (Kahl & Kappus, 1993). This leads to a growing interest towards natural antioxidants for chemotherapeutic and preservation properties. In addition to the functional and physiological properties, antioxidants from protein hydrolysates may confer nutritional value (Pownall, Udenigwe, & Aluko, 2010). Consequently, the search for natural antioxidants becomes a subject of interest to replace artificial antioxidants.

Kudoh, Matsuda, Igoshi, and Oki (2001) identified a κ-casein derived peptide, which displayed 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, from the milk fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus*. Sabeena Farvin, Baron, Nielsen, Otte, and Jacobsen (2010) also found antioxidant peptides comprised of several fragments from β-casein and a few N-terminal fragments of α_{s1}-, α_{s2}- and κ-casein from yogurt. Korhonen and Pihlanto (2006) concluded that most of the peptides are encrypted within the native protein, and can be released during several food processes, or during gastrointestinal activities. Moreover, Gupta, Mann, Kumar, and Sangwan (2009) found that degree of proteolysis, which depends on the strain used in cheese making, is directly related to antioxidant activity. Donkor, Henriksson, Vasiljevic, and Shah (2007) reported that a large number of oligopeptides are generated by extracellular proteinases, and further breakdown into peptides by intracellular peptidases.

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); DPPH, 1,1-diphenyl-2-picrylhydrazyl; WSPE, Water soluble peptide extract; HCA, Hierarchical cluster analysis.

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Nowadays, yogurt consumption has expanded rapidly because of the fact that it fulfils many current nutritional requirements. One distinguishable feature of yogurt is the presence of lactic acid bacteria, which have well developed proteolytic systems enabling them to grow well in milk and release a large number of potentially bioactive peptides. Several studies have been undertaken to confer probiotic effects by incorporating *Lactobacillus* (*L.*) *acidophilus* (ATCC® 4356™), *L. casei* (ATCC® 393™) and *L. paracasei* subsp. *paracasei* (ATCC® BAA52™) into fermented food products (Ortakci & Sert, 2012; Sidira et al., 2013). Additionally, these strains displayed remarkably high proteolytic activities (Donkor et al., 2007; Yeo & Liong, 2010). However, the potential of these strains to release peptides with a particular physiological activity has not been assessed to-date. Thus, the aim of this study was to establish the performance of these strains (individually or in a combination) by co-culturing with yogurt starter culture, in regards to release of bioactive peptides with the antioxidant (free radical scavenging activity) and antimutagenic activities during yogurt manufacturing.

2. Materials and methods

2.1. Substrates and chemicals

Most of the chemicals were supplied by Sigma Chemical Company (St Louis, MO, USA) and used without further purification. Milli-Q water (18.2 MΩ cm) was used to prepare all aqueous solutions. M17 media, de Man Rogosa and Sharpe (MRS) media, and bacteriological agar were purchased from Oxoid (West Heidleberg, Australia). Clindamycin, vancomycin, o-phthaldialdehyde (OPA), trichloroacetic acid (TCA), trifluoroacetic acid (TFA) and serine were purchased from Sigma Chemical Company (St Louis, MO, USA). Instant skimmed milk powder was purchased from a local store (Woolworths Limited, Australia). Acetonitrile was purchased from Merck (Darmstadt, Germany).

2.2. Propagation of cultures

Streptococcus thermophilus 1275 (*S. thermophilus*) and *Lactobacillus delbrueckii* ssp. *bulgaricus* Lb1466 (*L. bulgaricus*) were obtained from the Victoria University Culture Collection (Werribee, Australia). *Lactobacillus acidophilus* ATCC 4356 (*L. acidophilus*), *Lactobacillus casei* ATCC 393 (*L. casei*) and *Lactobacillus paracasei* subsp. *paracasei* ATCC BAA52 (*L. paracasei*) were purchased from Cell Biosciences Pty Ltd (Heidelberg, Victoria, Australia). The strains were stored at −80 °C in 40% glycerol in MRS broth. The strains were activated by triplicate sequential transfer, by inoculating 1% (v/v) of each strain in MRS broth (except *S. thermophilus*, which was inoculated in M17 broth) and incubating for 18 h at 42 °C for *L. bulgaricus*, 37 °C for *S. thermophilus*, *L. acidophilus*, *L. casei* and 30 °C for *L. paracasei*. The activated strains were inoculated at 1% (v/v) into 10 ml of sterile reconstituted skimmed milk (RSM, 14%, w/v) containing glucose (2%) and yeast extract (1%). Finally, the cultures were inoculated at 1% (v/v) into sterile RSM and incubated at 37 °C for 20 h to obtain approximately 10^8 colony-forming units (CFU) per millilitre. The final step was performed so that yeast extract was not carried over during yogurt manufacturing, which might interfere with results (Donkor, Henriksson, Singh, Vasiljevic, & Shah, 2007).

2.3. Yogurt preparation

Yogurt was prepared as described by Donkor et al. (2007). Briefly, yogurt mix was prepared by heat treating reconstituted skimmed milk (140 g/l) at 85 °C for 30 min followed by cooling to 45 °C, and aseptically inoculating with 1% (v/v) of each of *L. bulgaricus* and *S. thermophilus*. The inoculated milk was divided into eight equal portions; one portion was used as a control, while the other portions were further inoculated with 1% (v/v) of each probiotic cultures (*L. acidophilus*, *L. casei* and *L. paracasei*) separately, two in- combination and all three in- combination as shown in Table 1. The mixes were poured into polystyrene cups aseptically and incubated at 42 °C until the required pH of 4.5 ± 0.1 was reached. Cooling to 4 °C was done to halt further acidification. The pH of the heat treated RSM was adjusted to 4.5 ± 0.1 by using aqueous 1 M HCl.

2.4. Selective enumeration of probiotic and yogurt strains

Cell populations of *S. thermophilus*, *L. bulgaricus*, *L. acidophilus*, *L. casei* and *L. paracasei* were determined using the pour plate technique as described by Donkor et al. (2007) with a few modifications. Briefly, the samples (10 g) weighed aseptically into sterile stomacher bags were diluted with sterile (0.15%, w/v) peptone (Oxoid) water to 100 g and homogenised using a Stomacher (Stomacher400, John Morris Scientific Pty Limited). The resulting diluted sample (10^{-1}) was serially diluted in sterile peptone water, and 1 ml of appropriate dilutions were used for enumeration by the pour plate technique. *S. thermophilus* was selectively enumerated using M17 medium, supplemented with lactose under aerobic incubation at 45 °C for 24 h; *L. bulgaricus* using MRS agar (pH adjusted to 5.2 using 1 M HCl) at 45 °C for 72 h anaerobically and *L. acidophilus* using MRS-clindamycin (pH 6.2; 0.5 ppm clindamycin) at 37 °C for 72 h anaerobically (Casteele et al., 2006). Both *L. casei*/*L. paracasei* together or alone were selectively enumerated from other probiotic and yogurt cultures using MRS-vancomycin agar (pH 6.2; 1 ppm vancomycin) at 37 °C for 72 h anaerobically (Sakai et al., 2010). Plates containing 25–250 colonies were considered for enumeration and the results were reported as log CFU/g.

2.5. Determination of degree of hydrolysis

Degree of hydrolysis (DH) was analysed using the OPA method described by Nielsen, Petersen, and Dambmann (2001) and Donkor et al. (2007) with a few modifications. The OPA reagent was prepared daily by combining 25 ml of sodium tetraborate buffer (100 mM; pH 9.3), 2.5 ml of sodium dodecyl sulphate (20%, w/w), 40 mg of OPA (dissolved in 1 ml of methanol), and 100 μl of β-mercaptoethanol and diluting to 50 ml with water. The serine standard (0.9516 meqv/l) was prepared by dissolving 50 mg serine (Sigma Chemical Company) in 500 ml deionized water. Three millilitres of 0.75 M TCA were added to 3 g aliquots of yogurt samples; and incubated for 10 min at room temperature. The mixture was centrifuged at 4000×*g* for 30 min at 4 °C. The supernatant was passed through a 0.45 μm syringe filter and stored at −20 °C until assayed.

Small aliquots (400 μl) of the samples were added to a test tube containing 3 ml of the OPA reagent and mixed gently for 5 s. The absorbance (A_{sample}) was measured at 340 nm using a Nova-Spec®-II Spectrophotometer (Pharmacia, England, UK) after exactly two minutes of incubation at room temperature. The experimental steps were repeated with Milli-Q water as a blank (A_{blank}); and with the serine solution as standard ($A_{standard}$). Degree of hydrolysis was determined by using Eq. (1).

$$DH(\%) = \frac{h}{h_{tot}} \times 100 \quad (1)$$

where, h_{tot} was the total number of peptide bonds per protein equivalent; for casein, the h_{tot} value was 8.2 mEq/g protein (Nielsen