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Screening of whey protein isolate hydrolysates for their dual functionality: Influence of heat pre-treatment and enzyme specificity

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ABSTRACT

Heat pre-treated and non heat pre-treated whey protein isolate (WPI) were hydrolysed using α-chymotrypsin (chymotrypsin), pepsin and trypsin. The *in vitro* antioxidant activity, ACE-inhibition activity and surface hydrophobicities of the hydrolysates were measured in order to determine if peptides with dual functionalities were present. Dual functional peptides have both biological (e.g. antioxidant, ACE-inhibition, opioid activities) and technological (e.g. nanoemulsification abilities) functions in food systems. Heat pre-treatment marginally enhanced the hydrolysis of WPI by pepsin and trypsin but had no effect on WPI hydrolysis with chymotrypsin. With the exception of the hydrolysis by trypsin, heat pre-treatment did not affect the peptide profile of the hydrolysates as analysed using size exclusion chromatography, or the antioxidant activity ($P > 0.05$). Heat pre-treatment significantly affected the ACE-inhibition activities and the surface hydrophobicities of the hydrolysates ($P < 0.05$), which was a function of the specificity of the hydrolysing enzyme. Extended hydrolysis (up to 24 h) had no significant effect on the DH and the molecular weight profiles ($P > 0.05$) but in some instances caused a reduction in the antioxidant activity of WPI hydrolysates. The chymotrypsin hydrolysate showed a broad MW size range, and was followed by pepsin and then trypsin. The bioactivities of the hydrolysates generally decreased in the order; chymotrypsin > trypsin > pepsin. This study showed that by manipulating protein conformation with pre-hydrolysis heat treatment, combined with careful enzyme selection, peptides with dual functionalities can be produced from WPI for use as functional ingredients in the manufacture of functional foods.

1. Introduction

Peptides from milk protein sources are known to have antioxidant activity and ACE inhibitory activity that help protect the human body against negative effects of free radicals and to maintain a normal blood pressure, respectively (Korhonen & Pihlanto, 2006). The potential of these peptides in human nutrition management is increasingly being acknowledged (Lee, Skurk, Hennig, & Hauner, 2007). These peptides have simple structures, and are considered safe and healthy compounds which are easily absorbed by the human body (Li, Le, Shi, & Shrestha, 2004).

Enzymatic hydrolysis of food proteins produces peptides, including antioxidative and ACE-inhibitory peptides (Korhonen & Pihlanto, 2006). Enzymatic hydrolysis has also proven to be the most promising method of producing bioactive peptides from proteins, and in many instances, has been shown to enhance the bioactivity of intact proteins (Pihlanto, 2006). Since enzymes are usually highly specific in their mode of action, careful enzyme selection means they can be used to produce hydrolysates suitable for different food applications. Peptide bioactivity is largely dependent on the specificity of the enzyme used, the protein source and any treatment prior to hydrolysis that modifies the native protein structure (e.g. heat treatment) (Gauthier & Pouliot, 2003).

Heat pre-treatment of proteins prior to enzymatic hydrolysis results in intra- and inter-molecular disulphide interchanges and possible protein conformational changes (Lee, Morr, & Ha, 1992), which will partly dictate the nature of peptides released during hydrolysis, and hence their resultant functionalities. Heat may also induce non-specific peptide bond cleavage and enhance the hydrolysis of some globular proteins, such as β-lactoglobulin (Cheison, Schmitt, Leeb, Letzel, & Kulozik, 2010). The effect of heat pre-treatment on the structural–technological functionalities of milk proteins and their peptides have been extensively addressed over the past decades (Dissanayake & Vasiljevic, 2009; Lee et al., 1992), whereas the influence of heat pre-treatment on the

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biological functionalities of milk proteins have received limited attention. Heat pre-treatment prior to hydrolysis could therefore be used to alter the proportions of different multifunctional peptides generated during enzymatic hydrolysis of milk proteins.

Whey proteins constitute about 20% of the total proteins in milk. With approximately 86 million tonnes produced per year as by products from cheese manufacture, they are readily available, have a high nutritional value owing to a relatively high content of essential amino acids (Custódio et al., 2009), and are easily digested by gastrointestinal enzymes such as chymotrypsin, pepsin and trypsin. The structural and technological functionalities of whey proteins have been extensively studied and well characterised, and have consequently been used as a primary substrate for the generation of multifunctional food peptides in the food industry (Madureira, Tavares, Gomes, Pintado, & Malcata, 2010). Whey proteins have also been shown to contain various bioactive peptides e.g. antihypertensive, antithrombotic, opiate peptides (Lourenco da Costa, Antonio da Rocha Gontijo, & Netto, 2007; Madureira et al., 2010; Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000). These peptides can be released during *in vivo* digestion with enzymes such as chymotrypsin, pepsin and trypsin as well as *in vitro* enzymatic hydrolysis, and once they are released, they may act as regulatory compounds with hormone-like effects (Korhonen & Pihlanto, 2006). *In vitro* digestion offers high controllability over the hydrolysis process and hence can be optimised in order to generate multifunctional peptides for possible inclusion in the manufacture of functional foods.

With the growing interest in functional bioactive ingredients from food protein hydrolysate sources, it is necessary to identify peptides that have technological functions in food systems (e.g. nanoemulsification abilities) and also have biological function when consumed (e.g. antioxidant, ACE-inhibition, opioid activities). These peptides may be capable of stabilising nanoemulsions due to the particularly small droplet sizes of this category of emulsions. Thus, in addition to their bioactivities, the emulsifying potentials of these peptides could be exploited in food nanoemulsion systems.

The objective of this work was to generate peptides from WPI that would have their functionality assessed to determine their potential use as dual functional ingredients in food nanoemulsion systems. In this paper, the effect of hydrolysis conditions (heat pre-treatment, enzyme specificity, time) on peptide formation (shown by the degree of hydrolysis) is discussed. The peptides generated were assayed to determine their potential as dual functional ingredients with both technological (molecular weight by size exclusion chromatography [SEC] and surface hydrophobicity and as indicators of emulsifying potential) and biological (antioxidant and ACE-inhibitory properties) functionalities. In addition to obtaining peptides potentially similar to those from *in vivo* action in the gut, this study is aimed at generating both biologically and technologically dual functional peptides for inclusion as functional ingredients in functional food manufacture.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) was purchased from MyoPure Pty Ltd (Petersham, Australia). The digestion enzymes (chymotrypsin, pepsin and trypsin), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-acid (Trolox), *o*-phthaldialdehyde (OPA), dithiothreitol (DTT), bovine serum albumin, ovalbumin, β-lactoglobulin A, cytochome C and cytidine were purchased from Sigma–Aldrich (Sydney, Australia). Fluorescein (FL) and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were obtained from Merck Pty Ltd (Melbourne, Australia). All other chemicals were of reagent grade.

2.2. Preparation of heat pre-treated WPI

WPI at a concentration of 5% (w/v) was heated at 80 °C for 15 min in deionised water. After heating, the suspension was allowed to cool down to room temperature and concentrated by rotary evaporation (Rotavapor R-210 fitted with a vacuum controller V-850, Buchi Labortechnik, Flawil, Switzerland) at 37 °C. The concentrates were freeze dried, homogenised using a blender and then stored at −20 °C for further analyses.

2.3. Enzymatic hydrolysis

WPI hydrolysates were prepared by suspending either the heat pre-treated or non heat pre-treated WPI in 10 mM phosphate buffer, pH 7, at a concentration of 5% (w/v). The suspension was then stirred and allowed to hydrate and equilibrate to the working temperature (37 °C) for 30 min. The suspensions were then adjusted to enzymes working pH (pH 2.6 for pepsin and 7.8 for chymotrypsin and trypsin) with either 2 M HCl or NaOH, respectively. Enzymes were then added at an enzyme: substrate ratio of 1:40 with pH monitoring. The system was stirred throughout the hydrolysis process to avoid sedimentation especially with the heat pre-treated samples. The pH was maintained at the working pH values with 2.0 M HCl (pepsin) or NaOH (chymotrypsin and trypsin) for the first 12 h and left overnight for the 24-h hydrolysis. After the hydrolysis, the pH was adjusted to neutrality with 2.0 M NaOH or 2.0 M HCl accordingly. The hydrolysates were then heated at 90 °C for 15 min to inactivate the enzymes. The suspensions were allowed to cool down to ambient temperature and then freeze dried or kept at −20 °C for further analyses. The degree of hydrolysis (DH) was determined by the OPA method as described by Nielsen, Petersen, and Dambmann (2001).

2.4. Size exclusion chromatography

Molecular weight (MW) distribution of hydrolysates was analysed by gel filtration chromatography under isocratic conditions using a Shodex Protein KW-802.5 column (8.0 mm × 300 mm) fitted with a Protein-Pak 125 Sentry Guard Column (Waters Pty, Sydney, Australia) on an HPLC system. Hydrolysates were dissolved in phosphate buffer saline (50 mM Na_2HPO_4/NaH_2PO_4 and 150 mM Na_2SO_4, pH 7.0) to prepare a 1 mg/mL solution and filtered through 0.22 μm syringe filter with 100 μL being injected into the column. Elution was at room temperature, 0.8 mL/min flow rate and peak absorbance was monitored at 214 nm. Each sample was run in triplicate. Bovine serum albumin (66.0 kDa), ovalbumin (44.3 kDa), β-lactoglobulin A (18.3 kDa), cytochome C (12.4 kDa) and cytidine (0.243 kDa) were run as standards. The percentage abundance (area under peak) of the determined molecular weight was obtained from the HPLC software (Varian STAR chromatography workstation version 6.41, Varian Inc., Victoria, Australia).

2.5. Biological activities

2.5.1. Antioxidant activity

2.5.1.1. Free radical scavenging activity (FRSA). The free radical scavenging activity was carried out using the ABTS decolourising assay according to the method by Re et al. (1999). Briefly, ABTS (7 mM) and potassium persulphate (2.45 mM) were reacted together in 100 mM phosphate buffer saline (PBS, pH 7.4) to generate $ABTS^{\cdot+}$. The reaction solution was kept at the dark for up to 14 h before use. Before each determination, the free radical mixture was diluted with the working buffer to an absorbance of 0.7 ± 0.02 at