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Identification of radical scavenging peptides (<3 kDa) from Burgos-type cheese

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ABSTRACT

The aim of the present study was to identify low molecular weight peptides with radical scavenging activity from cheese made using different types of rennet: batch 1, animal rennet (95% chymosin and 5% bovine pepsin), batch 2, rennet of plant origin (*Cynara cardunculus*) and batch 3, microbial rennet (*Mucor miehei*). After preparation of the peptide extracts (<3 kDa), antioxidant activity was assayed by their DPPH radical scavenging and metal chelating a6ctivity. All of the batches showed antioxidant activity, which could be dependent on the peptides which are present in extracts: Batch 2 and 3 showed the highest values for DPPH inhibition and chelating effect. Fourteen fractions out of the total peptide fractions collected after RP-HPLC analysis showed radical scavenging activity using the DPPH inhibition method. Free amino acids and peptides were identified from these fractions. One of the peptides, derived from α_{s1}-casein, was a potential new antioxidant peptide. These antioxidant peptides were present in a lower content in extracts obtained from animal rennet cheese in comparison with the other extracts.

1. Introduction

It is well known that dietary proteins provide a rich source of biologically active peptides. These peptides are inactive within the sequence of the protein and can be released through hydrolysis by enzymes from different origin (Korhonen & Pihlanto, 2006). Properties of bioactive peptides have been reviewed in many protein food such as meat (Arihara, 2006), milk (Korhonen & Pihlanto, 2004; Otte, Shalaby, Zakora, Pripp, & El-Shabrawy, 2007), potato (Pihlanto, Akkanen, & Korhonen, 2008), egg (Yoshii et al., 2001), soy (Shin et al., 2001), wheat (Pihlanto, Koskinen, Piilola, Tupasela, & Korhonen, 2000) and fish (Curis, Dennes, Waddell, Macgillivray, & Ewart, 2002). Ingestion of bioactive peptides with antihypertensive, antimicrobial, antioxidative, antithrombotic, immunomodulatory and hypocholesterolemic activities may affect the major body systems, namely, the cardiovascular, digestive, immune and nervous systems (Korhonen & Pihlanto, 2006; Shimizu, 2004). The activity is based on their amino acid composition and sequence. The size of active sequences may vary from two to twenty amino acid residues, and many peptides are known to reveal multifunctional properties (Meisel, 2005).

Nowdays, milk proteins are considered as the most important source of bioactive peptides and an increasing number of bioactive peptides have been identified in milk protein hydrolysates, fermented dairy products and cheese (Korhonen & Pihlanto, 2003; Matar, LeBlanc, Martin, & Perdigón, 2003; Otte, Lenhard, Flambard, & Sørensen, 2011; Silva & Malcata, 2005).

Cheese is one of the most important dairy products worldwide. It typically contains numerous peptides that are originated from casein breakdown during production. Proteolysis in cheese is mainly caused by the rennet (Upadhyay, McSweeney, Magboul, & Fox, 2004). Traditional rennet used for cheese making is the one of gastric origin, secreted by young mammals, containing chymosin and pepsin as principal proteinases, with decreasing chymosin percentage as the animal age increases (Upadhyay et al., 2004). In recent years, much effort has been expended on searching for suitable rennet substitutes for cheese making. Several proteinases have been assessed but only bovine pepsin and proteinases from *Rhizomucor pusillus*, *Rhizomucor miehei* and *Cryphonectria parasitica* have been used extensively in commercial practice (Phelan, 1985). Some authors showed that the specificities of these enzymes on the caseins compared to chymosin were clearly very different (Broome, Xu, & Mayes, 2006; Drohse & Foltmann, 1989; Yasar & Guzeler, 2011). On the other hand, the use of plant proteinases as coagulant is interesting in traditional cheeses. In the Iberian Peninsula, some raw ovine and caprine milk cheeses, highly valued by consumers

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because of their sensory characteristics, have been produced with extracts from the flowers of *Cynara cardunculus* as the coagulant agent (Silva, Pihlanto, & Malcata, 2006; Sousa & Malcata, 1998). In this case, milk clotting is caused by the enzymes cardosin and cyprosin, the main aspartic proteases present in this plant rennet (Macedo, Malcata, & Oliveira, 1993; Verissimo, Esteves, Faro, & Pires, 1995). Previous researches compared the proteolytic activity of rennet of plant origin with animal rennet in cheese making, showing an increase in casein hydrolysis using rennet of plant origin, which also had different specificity on caseins (Faccia et al., 2012; Galán, Prados, Pino, Tejada, & Fernández-Salguero, 2008; Pino, Prados, Galán, McSweeney, & Fernández-Salguero, 2009; Prados, Pino, & Fernández-Salguero, 2007; Tejada, Abellán, Cayuela, Martínez-Cacha, & Fernández-Salguero, 2008).

Peptides generated during cheese production contribute to flavour, taste and texture of final product (Alonso, Picon, Gaya, & Nunez, 2013; Galán et al., 2008; Møller, Rattray, & Ardö, 2013), but also different effects have been attributed to these compounds in several studies in cheese such as antihypertensive (Gómez-Ruiz, Ramos, & Recio, 2004; Ong & Shah, 2008; Sagardia, Iloro, Elortza, & Bald, 2013), opioide (Sienkiewicz-Szłapka et al., 2009), antimicrobial (Demers-Mathieu et al., 2013) or antioxidative (Gupta, Mann, Kumar, & Sangwan, 2009; Meira et al., 2012).

Despite some studies have showed antioxidant activity of peptides coming from milk protein and digested using different commercial enzymes (Kim, Jang, & Kim, 2007; Rival, Boeriu, & Wichers, 2001; Rossini, Noreña, Cladera-Olivera, & Brandelli, 2009; Tavares et al., 2011), there are very few studies focused on antioxidant peptide generation in cheese made with rennet from different origin. In this sense, Siva et al. (2006) assessed the antioxidant activity of peptides in water-soluble extracts, obtained from raw and sterilized ovine and caprine cheese like systems coagulated with enzymes from the plant *C. cardunculus*.

Thus, the objective of the present study was to obtain peptides with radical scavenging activity, and therefore potential antioxidant effect, from cheese made using rennet from different origin (animal, plant and microbial).

2. Material and methods

2.1. Cheese-making

Burgos type cheese was manufactured in a dairy pilot plant. Three batches were made using 18 l of cow pasteurized milk purchased in a local supermarket distributed into three 6-l vats. Cheeses were manufactured using liquid rennet of different origin: batch 1, 0.06% (v/v) of animal rennet (95% chymosin and 5% bovine pepsin) (Abiasa, S.L., Pontevedra, Spain), batch 2, 0.03% (v/v) of rennet of plant origin (*C. cardunculus*) (Abiasa, S.L., Pontevedra, Spain) and batch 3, 0.034% (v/v) of microbial rennet (*Mucor miehei*) (Lactocyex, S.L., Cáceres, Spain). Milk was heated at 38 °C and 0.075% (v/v) of calcium chloride (Abiasa, S.L., Pontevedra, Spain) was added before addition of liquid rennet. After clotting, coagulum was cut and put into a hoop and whey drainage was carried out during 8 h. Subsequently, cheese was rubbed with salt by hand. The complete set of cheeses (3 batches) was prepared in 3 days and two further replications were produced in the following days. Samples were frozen at −20 °C until extract preparation.

2.2. Preparation of water soluble extracts (WSE) of peptides

WSE were obtained by following the procedure described by Gómez-Ruiz, Ramos, and Recio (2002) with some modifications. Cheese was grated and homogenised with twice its weight of ultrapure water. The homogenate was held for 60 min at 40 °C, and then, centrifuged at 3,800g and 4 °C for 30 min. The resulting supernatant was filtered through a cellulose filter (Ø 5 μm) (Albet, Barcelona, Spain). Permeate was centrifuged at 10,000 *g* and 4 °C for 20 min, and the supernatant was filtered through glass wool and cellulose filter (Ø 0.2 μm) (Albet, Barcelona, Spain). This WSE was ultrafiltered on an AMICON ULTRA tube with 3 kDa pore size (Centripep, Amicon Inc, Beverly, MA, USA). Finally, permeate was freeze-dried, dissolved in 20 ml of ultrapure water and kept at −20 °C until further use.

2.3. Assay of DPPH radical scavenging activity in the extract of peptides

The antioxidant activity of the extracts based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined by the method described by Li, Chen, Wang, Baoping, and Yonnie (2007) with some modifications.

Briefly, a 500 μl aliquot (at 1, 3, 6, 9 and 12 mg/ml) of test sample was added to 500 μl of ethanol (99.5%) (Scharlau (Barcelona, Spain) and 125 μl of ethanol (99.5%) containing 0.01% of DPPH (Sigma–Aldrich, Steinheim, Germany). The mixture was left to stand at room temperature for 60 min in the dark, and its absorbance was read at 517 nm in a Unicam Helios spectrophotometer. The ability to scavenge the DPPH radical was expressed as the inhibition percentage and was calculated using the following formula:

$$\%\,\text{Inhibition} = \left(\left(A_{\text{control}} - A_{\text{sample}}\right) \Big/ A_{\text{control}}\right) \times 100$$

where the A_{control} is the absorbance of the control (DPPH solution without sample), the A_{sample} is the absorbance of the test sample (DPPH solution plus test sample).

2.4. Assay of metal chelating activity in the extract of peptides

The Fe^{2+}-chelating ability of the extract was estimated by the method of Li et al. (2007) with some modifications. Briefly, an 800 μl aliquot (at 1, 3, 6, 9 and 12 mg/ml) of test sample was added to 10 μl of a solution of 1 mM $FeCl_2$ (Panreac, Barcelona, Spain) and 20 μl of 2.5 mM ferrozine (Panreac, Barcelona, Spain) and the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine–Fe^{2+} complex formation was calculated from the equation:

$$\%\,\text{chelating activity} = \left(\left(A_{\text{control}} - A_{\text{sample}}\right) \Big/ A_{\text{control}}\right) \times 100$$

where the A_{control} is the absorbance of the control (without sample), the A_{sample} is the absorbance of the test sample ($FeCl_2$ and ferrozine solutions plus test sample).

2.5. Separation and collection of peptides by RP-HPLC

Peptide separation was performed on an Agilent Technologies HP1100 equipped with a C18 column (4.6 × 250 mm, 5 μm, GL Science, Tokyo, Japan). Solvent A was a mixture of water-trifluoroacetic acid (1000:1) and solvent B contained a mixture of acetonitrile-trifluoroacetic acid (1000:0.8) (Quirós et al., 2007). Trifluoroacetic acid was obtained from Sigma (St. Louis, MO, USA) and acetonitrile was from Scharlau (Barcelona, Spain). Elution was performed with a linear gradient of solvent B in A from 10% to 40% B in 50 min at 35 °C and a flow rate of 1 ml/min. Absorbance of the eluent was monitored at 214 nm. Sample concentration was 20 mg peptide/ml and the injection volume was 50 μl. Fractions from the