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Comparison of antioxidant capacity, protein profile and carbohydrate content of whey protein fractions

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A B S T R A C T

Whey is used as an additive in food industry and a dietary supplement in nutrition. Here we report a comparative analysis of antioxidant potential of whey and its fractions. Fractions were obtained by size exclusion chromatography, before and after enzymatic digestion with pepsin or trypsin. Superoxide radical scavenging, lipid peroxidation inhibition and cupric ion reducing activities of different fractions were checked. Peptides were detected by SDS–PAGE and GC–MS was used to determine carbohydrate content of the fractions. All samples showed antioxidant activity and the second fraction of the trypsin hydrolysate showed the highest superoxide radical scavenging activity. CUPRAC value of this fraction was two-times higher than that of whey filtrate. The first fraction of the pepsin hydrolysate was the most effective inhibitor of lipid peroxidation. Each sample exhibited a different polypeptide profile. Different percentages of carbohydrates were identified in whey filtrate and in all second fractions, where galactose was the major component.

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1. Introduction

Different commercial whey products, such as whey powder (WP), whey protein concentrate (WPC) or whey protein isolate (WPI), are available in the market. These products are mainly used as a nutritional ingredient of infant formulas and a protein supplement for body builders and athletes (Bayram, Pekmez, Arda, & Yalçın, 2008; Hernández-Ledesma, Recio, & Amigo, 2008). Major components of whey are β-lactoglobulin (β-Lg) and α-lactalbumin (α-La). Bovine serum albumin (BSA), lactoferrin (LF), immunoglobulins (IgA, IgG, IgM), lactoperoxidase, lysozyme, relaxin, cytokines, growth factors such as insulin-like growth factor (IGF-1 and -2), transforming growth factor (TGF-β), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and free amino acids are also found in whey. In addition to the protein, peptide and peptide-related constituents, whey contains carbohydrates (mainly lactose), lipids (fatty acids and phospholipids), minerals and vitamins (Madureira, Pereira, Gomes, Pintado, & Malcata, 2007).

Biological properties and therapeutic potential of whey proteins and peptides have been reviewed in detail (Madureira et al., 2007; Yalçın, 2006). Health-promoting effects of whey are related to antioxidant, acetylcholine esterase (ACE) inhibitory, antimicrobial, immunomodulating, opioid and hypocholesterolemic activities of specific peptides and amino acids, predominantly derived from β-Lg (Hernández-Ledesma et al., 2008). Since oxidative stress is associated with several disease states, antioxidant activity of whey products and hydrolysates has been extensively investigated by various *in vitro* techniques (Table 1). Protective effects of dietary whey have also been examined by *in vivo* techniques. Its wound and burn healing properties have been documented (Jahovic et al., 2005; Velioğlu-Öğünç et al., 2008; Öner, Velioğlu-Öğünç, Cingi, Bozkurt-Uyar, & Yalçın, 2006). Peptides generated from the digestion of milk and whey proteins are reported to have antioxidative effects (Pihlanto, 2006). Moreover, antioxidant activity is elevated by fractionation or hydrolysis of whey proteins (Bayram et al., 2008; Peña-Ramos & Xiong, 2003). Thus, it seems that peptides have priority as functional constituents. Studies on digestion as well as hydrolysis of whey proteins indicated that some peptides, which are inactive within the sequence of intact protein may be activated during gastrointestinal digestion or food processing.

Although it is clear that major whey constituents have significant antioxidant potential, active principles and action mechanisms are not fully understood. In our previous study, β-Lg and α-La could be easily separated by Sephadex G-200 gel filtration chromatography supported with proteolytic cleavages, and free radical scavenging activity of whey and its fractions before and after protease treatment

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Table 1
Literature survey of different antioxidant activity tests used for whey proteins and hydrolysates.

Method	Sample	Reference
Radical scavenging (superoxide)	Whey protein fractions and hydrolysates	Bayram et al. (2008)
Radical scavenging (DPPH, superoxide, hydroxyl)	Whey protein isolate and hydrolysates	Peng et al. (2009)
Lipid peroxidation inhibition (TBARS)	Whey fractions	Tong, Sasaki, McClements, and Decker (2000a)
Lipid peroxidation inhibition (TBARS)	Whey protein concentrate (WPC)	McCarthy, Kerry, Kerry, Lynch, and Buckley (2001)
Lipid peroxidation inhibition (TBARS)	Whey protein isolate (WPI) hydrolysates	Peña-Ramos and Xiong (2001)
Lipid peroxidation inhibition (TBARS)	Whey powder	Coronado et al. (2002)
Lipid peroxidation inhibition (TBARS), radical (peroxyl) scavenging, Iron chelating	Whey	Tong et al. (2000b)
Lipid peroxidation inhibition (TBARS and conjugated dienes)	Whey protein isolate (WPI) and hydrolysates	Peña-Ramos and Xiong (2003)
Lipid peroxidation inhibition (TBARS)	Whey protein isolate (WPI), hydrolysed WPI fractions, commercial WPI hydrolysates	Peña-Ramos et al. (2004)
Lipid peroxidation inhibition	Acid whey (after ultrafiltration, dialysis, heat treatment and chloroform extraction)	Colbert and Decker (1991)

was investigated by diphenylpicrylhydrazyl (DPPH) test (Bayram et al., 2008). It was observed that the DPPH scavenging activity was enhanced more than 11-fold with pepsin cleavage.

Here we analysed antioxidant potential of whey and hydrolysed whey fractions eluted from Sephadex G-50 column and aimed to find out possible active constituents, focusing on polypeptides and carbohydrates. We used three test systems for detecting of antioxidant activity in which superoxide radical scavenging activity, lipid peroxidation inhibition and cupric ion reducing antioxidant capacity (CUPRAC) of the fractions were evaluated. Polyacrylamide gel electrophoresis (SDS–PAGE) was performed to determine polypeptide profiles. Free carbohydrates were detected by gas chromatography–mass spectrometry (GC–MS). The results are expected to contribute to our understanding of which whey fractions are more active and which bioactive constituents are found in native and hydrolysed whey samples after food processing or digestion.

2. Materials and methods

2.1. Chemicals and reagents

Whey powder was obtained from Sütaş Milk and Dairy Products Company (Bursa, Turkey). Pepsin (2500–3500 units/mg), ascorbic acid, 2-thiobarbituric acid (TBA), neocuproine, nitroblue tetrazolium chloride (NBT), riboflavin, ethlyenediaminetetraacetic acid (EDTA), L-α-phosphatidylcholine, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma–Aldrich (Germany). Trypsin (approx. 9000 units/mg), ferrous chloride tetrahydrate and Coomassie brilliant blue R-250 were purchased from Fluka Riedel-deHaën (Germany). Trichloroacetic acid, copper chloride, hydrogen peroxide, methanol, ammonium acetate, sodium carbonate, potassium dihydrogen phosphate, methionine, barium carbonate, hydrochloric acid, hexane, trimethylchlorosilan, hexamethyldisilazan, methanol, benzene, chloroform, pyridine and sodium hydroxide were purchased from Merck (Germany). All solvents used in GC–MS analysis were HPLC-grade. Dipotassium hydrogen phosphate was purchased from BDH (UK). Sephadex G-50 was purchased from Pharmacia (Sweden).

2.2. Whey and whey fractions

Whey powder was suspended in hexane (1:16, w/v) and centrifuged at 3000×*g* at room temperature for 10 min to remove lipid-soluble constituents. Hexane washing was repeated three times. Residues were combined and suspended in ultra-pure water at a concentration of 6% (w/v) by vortexing and centrifuged at 3000×*g* at room temperature for 10 min. The clear supernatant filtered through a 0.45 µm membrane filter was directly applied to a Sephadex G-50 column as whey filtrate (WF), or subjected to protease (pepsin or trypsin) treatment followed by chromatographic separation. Protein concentration of WF and all fractions was determined by a modified Lowry method (Waterborg, 2002).

2.3. Protease treatment

Pepsin and trypsin were used for digestion of whey proteins. The pH of WF was adjusted using 1 M HCl to 1.5 for pepsin digestion or to 9.0 using 1 M NaOH for trypsin digestion. Digestion was carried out at 37 °C for 30 min with a protein to enzyme ratio of 1:100 (w/w) for pepsin, and 1:50 (w/w) for trypsin. For pepsin and trypsin digestions, reactions were stopped by adding 1 M NaOH or 1 M HCl to adjust the pH to 7.8 or 1.5, respectively (Bayram et al., 2008). This was preferred to heating in order to avoid denaturation of small peptides.

2.4. Chromatographic separations

Whey filtrate and pepsin- or trypsin-hydrolysed WF were separated by size exclusion chromatography in a column (1.5 × 30 cm) packed with Sephadex G-50. The column was equilibrated and eluted with 0.02 M phosphate buffer, pH 8.6 at a flow rate of 0.3 mL/min. Fractions of 1–1.5 mL were collected according to their protein content (A280).

2.5. Protein electrophoresis

Polypeptides were analysed by SDS–PAGE in a Bio-Rad mini gel system, as described by Walker (2002). A constant current (3 mA/well for stacking gel and 6 mA/well for running gel) was applied through the gel, and the gels were stained with silver nitrate (Ausubel et al., 1989).

2.6. Carbohydrate analysis

Carbohydrate content was investigated by GC–MS (Ye, Yan, Xu, & Chen, 2006). Briefly, samples were dried under vacuum at 4 °C overnight, dissolved in MeOH (10 mL) and incubated in an oil bath at 95 °C for 6 h under reflux by adding 2 N HCl (10 mL) and benzene (10 mL). After cooling, the acidic hydrolysates were extracted with chloroform and the aqueous phases were neutralised with $BaCO_3$. Then solid particles were removed, aqueous phase was evaporated at 40 °C in a rotary evaporator, dried in a vacuum

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