Automated Electrophoresis and Antioxidant Activity of Whey and Its Hydrolysates

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Abstract Biological activities of whey and whey peptides resulted from enzymatic hydrolysis of the whey, increase the potential use of whey powder and hydrolyzed whey proteins as natural and functional products in food industry. In this study, automated electrophoresis as an alternative method to conventional electrophoresis was used to analyze whey proteins and peptides produced by pepsin and trypsin cleavage. In addition, antioxidant activity of the samples was evaluated using DPPH and CUPRAC tests.

Different patterns on electropherograms demonstrated that small peptides after protease treatment could be easily detected by automated electrophoresis. Our results confirmed that proteolytic hydrolysis increases the antioxidant activity. Pepsin treatment seemed to be more suitable to obtain small DPPH-scavenging whey peptides whereas trypsin produced more active cupric ion reducing proteins and peptides. Further studies are needed to exert and purify active principle(s) found in whey hydrolysates.

Keywords: whey, whey powder, whey hydrolysates, bioactive peptide, antioxidant activity, automated electrophoresis

Introduction

During cheese production, the liquid remaining after casein precipitation is called whey [1]. Whey is generally converted to whey powder, a by-product, and used as a viscosity, frothiness increaser and as a humility, structural stabilizer in several foods such as baby foods, sausages, salamis, hot-dogs, butter etc. [2].

Whey includes lactalbumin (La), lactoglobulin (Lg), bovine serum albumin (BSA), immunoglobulins (Igs), enzymes, a number of minor proteins and peptides. These proteins and peptides have biological effects including anti-hypertensive, hypo-cholesterolemic, opioidergic, anti-cancer, anti-microbial, antioxidant and anti-viral activities [3-5]. These biological activities, which especially result from the whey hydrolyzed peptides, increase the potential use of whey and whey products as natural and functional food additives. It is estimated that isolated bioactive proteins and peptides can extend the shelf life and increase the quality and stability of food products when they are used as food additives [6].

Our previous study confirmed that protease treatment increases the bioactivity of whey proteins [7, 8]. trypsin hydrolysate is more active in cupric ion reduction [8] whereas pepsin hydrolysate in radical scavenging [7, 8].

In this study, we analyzed whey proteins and peptides by automated electrophoresis, before and after pepsin and trypsin cleavage and measured the antioxidant activity of the samples.

Materials and Methods

Preparation of whey solution

Whey powder was obtained from a commercial milk and milk products company (Malkara Birlik Süt ve Süt Mamulleri-Turkey). Whey powder was suspended in hexane (1:16, w/v) and centrifuged at 3000 x g at room temperature for 10 min to remove lipids. 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 x g at room temperature for 10 min. The clear supernatant filtered through a 0.45 µm membrane filter [8].

**Hydrolysis of whey proteins**

Pepsin (Sigma, P-7125) and trypsin (Sigma, T-4799) were used for the hydrolysis of whey proteins. The pH of the sample was adjusted to 1.5 for pepsin hydrolysis, and to 9.0 for trypsin hydrolysis. Both reactions were carried out at 37°C for 30 min with a protein to pepsin ratio of 1:100 (w/w) or protein to trypsin ratio of 1:50 (w/w). Hydrolysis reactions were stopped by adjusting the pH to 7.8 for pepsin, and to 1.5 for trypsin [8].

**Determination of protein concentration**

Protein concentrations of whey and whey hydrolysates were determined with a modified bicinchoinic acid (BCA) method [9], using BCA kit (Intron, SMART™). Standard protein solutions were prepared with bovine serum albumin.

**Electrophoretic analysis**

The Experion Pro260 analysis kit was used for the analysis of whey proteins and hydrolysates with the Experion automated electrophoresis system (Bio-Rad Experion™) according to the instruction manual of the supplier.

**Superoxide radical scavenging activity test**

Superoxide radical scavenging ability of the samples was determined by a method using a free radical (DPPH, 1,1-diphenyl-2-picrylhydrazyl) [10]. Absorbance of the DPPH solution (0.2 mM) in methanol (MeOH) decreased by antioxidative effect of the sample against control was recorded at a wavelength of 520 nm. Ascorbic acid reference series (5, 10, 25, 50 and 100 µg/mL) was used as a standard. DPPH solution (160 µL) was added to each sample, ascorbic acid reference or blank in a volume of 40 µL in 96 well plates. MeOH was used instead of DPPH in control groups. Microplates were incubated at RT in dark for 10 min and the A₅₂₀ values were measured with a microplate spectrophotometer (µQuant™, BioTek). The analyses were run in triplicates. Superoxide radical scavenging activity was calculated according the following equation:

\[
\text{Scavenging DPPH} \text{ (%) } = \left[1 - \frac{(A_{\text{sample+DPPH}} - A_{\text{sample+ MeOH}})}{A_{\text{blank}}} \right] \times 100
\]

**Cupric ion reducing antioxidant capacity test**

The samples were checked for their cupric ion reducing antioxidant capacity (CUPRAC) using the method described by Apak et al. (2006) [11]. One molar ammonium acetate, 7.5x10⁻³ M Neocuprin (NC, in 96% ethanol) and 10⁻² M CuCl₂ solutions were prepared. Trolox solution (1x10⁻³ M) in 96% ethanol was used as reference antioxidant. Each well of 24 well-microplates on a shaker was loaded with 250 µL CuCl₂, 250 µL NC, 250 µL ammonium acetate buffer, 25 µL sample and 250 µL ultra-pure water. The assay mixture was kept at room temperature for 30 min and absorbance at 450 nm against a reagent blank was measured in a microplate reader. CUPRAC value was calculated as mmol trolox equivalent per mg protein. In our assay system, the molar absorptivity of trolox (ε_Trolox) was = 1.74x10⁴ L/mol/cm.

**Statistical analyses**

Statistical analyses were performed using one-way ANOVA followed by Tukey’s Multiple Comparison Test using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA (www.graphpad.com). The probability values of P<0.05 were considered as statistically significant.

**Results and Discussion**

**Protein concentrations**

Protein concentration of whey samples depends on the commercial source and its manufacturing process as well as hydrolysis methods. Protein concentrations were found as 4.45±0.003 mg/mL, 1.43±0.02 mg/mL and 2.58±0.012 mg/mL for whey solution, trypsin hydrolysate and pepsin hydrolysate, respectively. As expected, protein concentration decreased after protease treatment. This decrease can be explained by the low interaction between hydrolysis products (peptides) and the BCA kit reagents. Similar results have been obtained in previous studies [6, 7].
Electrophoretic analysis

In this study, electropherograms were obtained from BioRad Experion™ automated electrophoresis system, which uses Labchip microfluid technology. Different patterns on electropherograms were detected for different samples (Fig 1). Molecular weight of bioactive peptides after hydrolysis of the whey is proposed to be usually under 10 kDa [12]. The signals belonging to these small peptides were prominent in hydrolysates, although some of them were regarded as noise.

Antioxidant activity tests

DPPH assay conducted with whey samples, each in a protein concentration up to 1 mg/mL, revealed that antioxidant activities of hydrolysates were higher than whey solution (Table 1). Moreover, radical scavenging activity of pepsin hydrolysate was the highest one. Same concentration of ascorbic acid caused 96.3% DPPH inhibition. Thus, pepsin treatment seemed to produce more active peptides in radical scavenging, although whey solution and hydrolysates were not as effective as ascorbic acid. Trypsin hydrolysate was found to be more effective in CUPRAC test (Table 1). Enzymatic hydrolysis resulted in approximately 2 fold increase in TEAC value of trypsin hydrolysate in comparison to whey solution.

Our results were correlated with previous reports [13] exhibiting that proteolytic cleavages increase bioactivity of whey proteins due to production of active peptides.

Table 1: DPPH scavenging activity and TEAC (“Trolox Equivalent Antioxidant Capacity”) values of whey solution and hydrolysates.

<table>
<thead>
<tr>
<th>Samples</th>
<th>DPPH Inhibition (%)</th>
<th>TEAC (mmol Trolox/mg)</th>
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<tbody>
<tr>
<td>Whey solution</td>
<td>15.5±0.01</td>
<td>0.07±0.005</td>
</tr>
<tr>
<td>Trypsin hydrolysate</td>
<td>17.7±0.003</td>
<td>0.13±0.011</td>
</tr>
<tr>
<td>Pepsin hydrolysate</td>
<td>22.19±0.02</td>
<td>0.10±0.02</td>
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* Concentration of all samples was 1 mg/mL.

Conclusion

It is possible to obtain small peptides from whey solutions effectively after protease treatment [13], but it is extremely difficult to detect these small peptides with manual electrophoretic methods in laboratory conditions. Recently, more efficient methods like LC-MS and IEF have been used for the analysis of bioactive peptides from whey [14, 15].

In this study, whey solution and its hydrolysates obtained by enzymatic hydrolysis with trypsin or pepsin were analyzed by BioRad Experion™ automated electrophoresis system. Electropherograms exerted different patterns for different samples, quite enough to distinguish them.

It is well known that whey proteins and hydrolysates have antioxidant activity, and whey hydrolysates including small peptides have higher antioxidant activity than whey solution. Our results were in accordance with related previous reports...
Trypsin and pepsin treatment is thought to increase total antioxidant activity by increasing the number of small peptides.

Whey is a valuable by-product including small bioactive peptides. Further studies are needed to develop new production/separation/purification strategies in order to use these bioactive small peptides in food industry as food additives. The interaction of bioactive peptides with other food components such as carbohydrates and lipids should also be investigated.

The beneficial effects of these bioactive peptides should be demonstrated in human cell culture models and should be supported by clinical trials.

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References


