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Fractionation and identification of ACE-inhibitory peptides from α-lactalbumin and β-casein produced by thermolysin-catalysed hydrolysis

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Abstract

The thermolysin catalysed hydrolysates of α-lactalbumin and β-casein were fractionated by size-exclusion chromatography (SEC) and reversed-phase high performance liquid chromatography (RP-HPLC) in order to identify the peptides responsible for the high ACE-inhibitory activity of these hydrolysates. The SEC fractionation separated many co-eluting peptides into different fractions allowing individual peptides to be isolated in one or two subsequent semi-preparative RP-HPLC fractionation steps. Five potent ACE-inhibitory peptides from α-lactalbumin were isolated. They all contained the C-terminal sequence -PEW, corresponding to amino acid residues 24–26 in α-lactalbumin, and had IC_{50} values of 1–5 μM. From one SEC fraction of the β-casein hydrolysate two potent ACE-inhibitory peptides were isolated and identified as f58-76 and f59-76 of β-casein A2. They both contained IPP as the C-terminal sequence and had IC_{50} values of 4 and 5 μM. From another SEC fraction a new but less ACE-inhibitory peptide from β-casein was identified (f192–196; LYQQP).

Keywords: Milk proteins; α-lactalbumin; β-casein; Thermolysin; Peptides; ACE inhibition

1. Introduction

Hypertension is a serious condition that affects one out of four adults in the Western world. If left untreated, the condition can lead to atherosclerosis and impaired function of kidneys, vision and heart, and eventually heart failure. Angiotensin converting enzyme (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1), which occurs in many tissues and biological fluids, is a key enzyme in up-regulation of the blood pressure in animals and humans by degrading endogenous peptides like angiotensin I (see Fitzgerald, Murray, & Walsh, 2004; Pihlanto-Leppälä, 2001).

Naturally occurring peptide inhibitors of ACE are present in many food proteins, including milk proteins, from which they are liberated by enzymatic hydrolysis (see Li, Le, Shi, & Shrestha, 2004; Lopez-Fandiño, Otte, & van Camp, 2006). The best known antihypertensive foods are the fermented milk-based products Calpis and Evolus® containing the tripeptides IPP and VPP, which have shown beneficial effects on blood pressure in hypertensive rats and humans (Jauhiainen et al., 2005; Nakamura, Yamamoto, Sakai, & Takano, 1995; see Lopez-Fandiño et al., 2006). In these products, the ACE-inhibitory peptides are formed during fermentation by the proteolytic system of lactic acid bacteria degrading the milk proteins into tripeptides and other peptides with inhibitory activity towards ACE. However, peptides with ACE-inhibitory potential might also be formed by in vitro hydrolysis of milk proteins using microbial and digestive enzymes (see Lopez-Fandiño et al., 2006; Otte, Shalaby, Zakora, Pripp, & El-Shabrawy, 2007). Examples of such hydrolysates are the dodecapeptide (C12; α_{s1}-CN f23–34) enriched hydrolysate produced by tryptic hydrolysis of casein, and Biozate, a whey protein

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hydrolysate, for which the enzyme used was not reported (see FitzGerald et al., 2004; Lopez-Fandiño et al., 2006). Food products containing antihypertensive peptides are highly relevant in the treatment of mild and modeate hypertension since the risk of developing cardiovascular diseases is directly proportional to the level of the blood pressure, the risk being reduced by 16% by a reduction in blood pressure of 5 mmHg (FitzGerald et al., 2004).

In the in vitro hydrolysis approach, the challenges lie in choosing the protein and enzyme combinations that give rise to a high yield of the bioactive peptides, and perhaps to enrich for these. In a recent screening study embracing the hydrolysis of nine milk protein preparations with five bacterial and digestive enzymes, we found that thermolysin was a very good enzyme for the release of ACE-inhibitory peptides from both caseins and whey proteins (Otte et al., 2007). The hydrolysate made from purified α-lactalbumin (α-La) by the action of thermolysin had the highest in vitro inhibitory activity towards ACE among the hydrolysates tested. This hydrolysate contained many peptides, several of which were identified, and based on their primary sequence, some were expected to exert ACE-inhibitory activity. It is not known, however, which of the peptides actually were able to inhibit ACE and to what extent. The purified β-casein A2 (β-CN A2) was the isolated protein that after hydrolysis with thermolysin had the second highest ACE-inhibitory activity (Otte et al., 2007). The peptides in this hydrolysate have not been identified and it is not known which peptides exert the high ACE-inhibitory activity.

The purpose of this study was to identify the peptides in the thermolysin-catalysed hydrolysates of α-La and β-CN A2 responsible for the high ACE-inhibitory activity of these hydrolysates. The hydrolysates were fractionated by size-exclusion chromatography (SEC) followed by semi-preparative reversed-phase high performance liquid chromatography (RP-HPLC). The ACE inhibitory activity of fractions and isolated peptides was assayed using microtiter plates as previously described (Shalaby, Zakora, & Otte, 2006), and peptides were identified by means of liquid chromatography-tandem mass spectrometry (LC-MS/MS) and N-terminal sequencing.

2. Materials and methods

2.1. Materials and chemicals

The α-La and β-CN A2 substrates as previously described (Otte et al., 2007) were used. Thermolysin (EC. 3.4.24.4), from *Bacillus thermoproteolyticus*, was obtained from Boehringer Mannheim GmbH, Mannheim, Germany. Angiotensin-I-converting enzyme (ACE; EC 3.4.15.1, from rabbit lung, 0.25 units mL^{-1}) was from Sigma Chemical Co., St. Louis, MO, USA. The substrate *N*-[3-(2-Furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) and ammonium hydrogen bicarbonate were from Fluka, Sigma-Aldrich, Brøndby, Denmark. Tris-HCl (Tris (hydroxy methyl) amino methane-hydrochloride), and trifluoro acetic acid (TFA; for spectroscopy) were from Merck KGaA, Darmstadt, Germany. Acetonitrile (HPLC gradient grade) was from Fisher Scientific UK Limited, Leicestershire, UK. Reagents used for sequencing were purchased from Applied Biosystems (Warrington, UK). All solutions were prepared with highly purified water (Milli-Q PLUS, Millipore Corperation, Billerica, MA, USA).

2.2. Preparation of protein hydrolysates

Substrate solutions containing 2% protein (w/w on protein basis) were made in water and kept at 5 °C overnight for rehydration. The pH was adjusted to 8.0 by a few drops of 0.1 M NaOH. The thermolysin solution (80 mg mL^{-1} water) was prepared immediately prior to use. To 10 mL of protein solution, 25 μL of the enzyme solution was added (giving 1% w/w enzyme with respect to protein), and after thorough mixing, the reaction mixture was incubated at 40 °C for 3 h. The reaction was stopped by heating at 90 °C for 15 min, and the mixture was cooled in an ice–water bath for 20 min, and centrifuged at ~11,150 × *g* for 3 min (Sigma centrifuge-113, VWR International Aps, Albertslund, Denmark) to remove insoluble material. The peptide profile and ACE-inhibitory activity of the resulting hydrolysates were measured as described below before fractionation was initiated.

2.3. Fractionation by SEC

Fractionation of peptides was performed using an FPLC system (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) mounted with a column packed with Superdex^{TM} 30 prep grade gel filtration resin (2.6 × 61 cm; Amersham Biosciences, Hillerød, Denmark). Samples (5 mL) were injected, and eluted with 0.1 M ammonium hydrogen carbonate, pH 8.0, at a flow rate of 2.5 mL min^{-1}. The effluent was monitored at 280 nm with the absorbance range set to 2.0. Fractions of 7.5 mL (3 min) collected from 40 to 170 min after sample injection were pooled according to the elution profile (The collected SEC fractions from α-La are named *αSEC* followed by a number referring to those in Fig. 2(A); correspondingly the SEC fractions from β-CN are named *βSEC* and a number referring to Fig. 2(B)). The peptide profiles and ACE-inhibitory activity of the pooled fractions were determined as described below. Fractions with the highest ACE-inhibitory activity were frozen and vacuum concentrated overnight. They were then redissolved in 1 mL of water and concentrated again, and kept at −20 °C until use. Samples originating from SEC fractions containing 7.5 and 15 mL were redissolved in 250 and 500 μL of 0.1% TFA, respectively, before further fractionation by semi-preparative RP-HPLC.