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Quantification of the angiotensin-converting enzyme-inhibiting tripeptides Val-Pro-Pro and Ile-Pro-Pro in hard, semi-hard and soft cheeses

Ueli Bütikofer, Jacques Meyer, Robert Sieber, Daniel Wechsler*

Agroscope Liebefeld-Posieux Research Station ALP, Schwarzenburgstr. 161, CH-3003 Bern, Switzerland

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Abstract

A new high performance liquid chromatography with subsequent triple mass spectrometry (HPLC-MS^3) method for the quantitative determination of Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) was applied in a series of 44 traditional cheeses. Additionally, inhibition of the angiotensin-converting enzyme (ACE) was measured in vitro by determination of IC_{50} values. The correlation coefficients between the IC_{50} values and the sum of the two peptides VPP and IPP in hard, semi-hard and in the group of soft cheeses were at $r = -0.797$, -0.580 and -0.357, respectively, suggesting that the high ACE-inhibiting activity found in the water soluble extracts of some investigated hard cheeses was mainly due to the presence of these two tripeptides. Concentrations of VPP and IPP were in the range of 0–224 mg kg^{-1} and 0–95 mg kg^{-1}, respectively, indicating that some cheese varieties contain similar concentrations of VPP and IPP to recently developed fermented milk products with blood-pressure lowering capacity.

Keywords: ACE-inhibiting peptide; Tripeptide; VPP; IPP; Cheese

1. Introduction

During cheese ripening, caseins are degraded by proteinases to large peptides and in a second step by peptidases to smaller peptides and free amino acids. Some of the liberated peptides show biological activity in humans. Depending on the biological effect, the bioactive peptides are classified as casomorphins, angiotensin-converting enzyme (ACE)-inhibiting peptides, phosphopeptides, immunopeptides, antithrombotic or antimicrobial peptides. The amino acid sequence of bioactive peptides is found in the primary structure of various food proteins and they are also found in cheese (Bachmann, Bütikofer, & Sieber, 2003; Rutherford-Marwick & Moughan, 2006).

ACE-inhibiting peptides are of special interest, because they can influence blood pressure. Different foods have been shown to contain such peptides (Li, Le, Shi, & Shrestha, 2004). The degradation of milk proteins with proteinases from *L. helveticus* produced peptides with ACE-inhibiting activity that had a significant antihypertensive effect in spontaneously hypertensive rats (Yamamoto, Akino, & Takano, 1994). The same effect was observed with fermented milk containing *L. helveticus* (Nakamura, Yamamoto, Sakai, Okubo et al., 1995). Two tripeptides valyl-prolyl-proline (Val-Pro-Pro; VPP) and isoleucyl-prolyl-proline (Ile-Pro-Pro; IPP) were identified as the bioactive peptides which were responsible for this effect (Nakamura, Yamamoto, Sakai, & Takano, 1995). A liquid chromatography–mass spectroscopy (LC–MS) method with Ala-Pro-Pro as an internal standard was used for the quantitative determination of these two peptides in casein hydrolysates (Matsuura, Mizuno, Nishimura, Gotou, & Yamamoto, 2005). In several short- and long-term human studies, where VPP and IPP containing fermented milk products were ingested, a blood-pressure lowering effect was observed (Hata et al., 1996; Seppo, Jauhiainen, Poussa, & Korpela, 2003; Seppo, Kerojoki, Suomalainen, & Korpela, 2002; Tuomilehto et al., 2004). The toxicological potential of these tripeptides has been examined in

*Corresponding author. Tel.: +41 31 323 89 00; fax: +41 31 323 82 27.
E-mail address: daniel.wechsler@alp.admin.ch (D. Wechsler).

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extensive studies (see for example Bernard, Nakamura, Bando, & Mennear, 2005).

In vitro measurement of the ACE-inhibitory activity in water soluble extracts of different cheese varieties such as Norvegia, Jarlsberg, Cheddar and Blue (Stepaniak, Jedrychowski, Wroblewska, & Sorhaug, 2001) or Gouda, Emmental, Blue, Camembert, Edam and Havarti (Saito, Nakamura, Kitazawa, Kawai, & Itoh, 2000) showed large differences in the IC_{50} values (concentration of cheese or an individual bioactive peptide that inhibits the ACE activity by 50% in an in vitro assay). Nakamura, Yamamoto, Sakai, Okubo et al. (1995) found IC_{50} values of 9 and 5 μM for VPP and IPP respectively, whereas for Captopril a value of 0.007 μM was reported (Pihlanto-Leppälä, Rokka, & Korhonen, 1998).

In further studies, a large number of individual peptides with ACE-inhibitory activity were isolated from cheese: a total of 22 and 75 peptides from a water-soluble extract of an eight month old Manchego manufactured from sheep milk (Gomez-Ruiz, Ramos, & Recio, 2002, 2004), 41 ACE-inhibitory peptides in the permeate >1000 Da of different Spanish cheeses analysed by HPLC-MS/MS and off-line MS/MS (Gomez-Ruiz, Taborda, Amigo, Recio, & Ramos, 2006) and two of four peptides (α_{s1}-casein f(1–9) and β-casein f(60–68) in an eight month old Gouda (Saito et al., 2000). Milk pre-treatment, processing and ripening are to be considered as important key factors for the formation of such peptides: the ACE inhibition was stronger in cheese from raw milk than in cheese from pasteurised milk (Gomez-Ruiz et al., 2002) and it was strongly dependent on the degree of proteolysis and the age of the cheeses (Meisel, Goepfert, & Günther, 1997). Also, Gouda aged for 8 months resulted in a significantly stronger reduction of the blood pressure of spontaneously hypertensive rats than 24 month old Gouda (Saito et al., 2000). However, a close correlation cannot be expected between the ACE-inhibitory activity of a cheese variety in vitro and its blood pressure lowering effect in vivo. On the one hand, most of the peptides released during the ripening of cheese are further degraded during gastrointestinal digestion. On the other hand, new ACE-inhibiting peptides can be liberated during the same process as shown with Emmental using a simple in vitro protocol simulating gastrointestinal digestion (Parrot, Degraeve, Curia, & Martial-Gros, 2003).

According to Saito et al. (2000) the systolic blood pressure was lowered in spontaneously hypertensive rats fed with isolated ACE-inhibiting peptides of various cheese varieties. In several human studies, a dose dependent blood pressure lowering effect of the two tripeptides IPP and VPP has been proven (Hata et al., 1996; Seppo et al., 2002, 2003). The quantification of these two well characterised ACE-inhibiting peptides is therefore a promising approach in order to assess the potential of a cheese variety for lowering blood pressure in humans. In contrast to fermented milk products, the use of HPLC-UV for separation and quantification of such peptides in cheese cannot be used because of the more complex matrix. Nevertheless, the identification of traditional cheese varieties with a naturally high content of physiologically active ACE-inhibiting peptides might be of interest for dietary recommendations in case of high blood pressure. The aim of the present work was therefore to determine the ACE-inhibiting activity of the most popular traditional Swiss cheese varieties compared with some non-Swiss cheeses and to quantify their content of VPP and IPP with a new method using liquid chromatography with subsequent triple mass spectrometry ($LC\text{-}MS^3$). The application of this method allows the recording of specific fragments of these two peptides that can be used for quantification in case of limited chromatographic resolution of complex peptide mixtures. To our knowledge, this is the first survey quantifying the amounts of the two blood pressure lowering peptides in a large number of cheese varieties.

2. Material and methods

2.1. Choice of cheeses

A total of 36 cheese samples of Swiss origin were purchased randomly at local cheese shops. Additionally, eight non-Swiss cheese varieties such as old Gouda, Allgäuer Limburger, Münster, Reblochon, Gorgonzola, Roquefort, Manchego and Feta were included in the study in order to have a representative range of the most popular cheese varieties consumed in Switzerland. All 44 cheese samples were analysed for moisture (IDF, 1982), protein, trichloroacetic acid soluble nitrogen (TCA-SN) (Collomb, Spahni-Rey, & Steiger, 1990) and fat (IDF, 1987) using common standard methods. Furthermore, fat in dry matter (FDM) and moisture on fat-free basis (MFFB) were calculated.

2.2. $HPLC\text{-}MS^3$ determination of ACE-inhibiting peptides VPP and IPP

2.2.1. Materials

HPLC grade acetonitrile and analytical grade formic acid were purchased from Sigma-Aldrich (Buchs, Switzerland). Deionised water was prepared on a Milli-Q installation from Millipore (Volketswil, Switzerland). Standard peptides VPP, IPP and Pro-Pro-Pro-Pro (PPPP) were purchased from Bachem (Bubendorf, Switzerland).

2.2.2. $LC\text{-}MS^3$ analysis

The concentrations of VPP and IPP were determined in the filtrate of the water soluble extract (see 2.3.3) in a single analysis. The HPLC separation was performed with a Rheos 2200 pump (Flux Instruments, Basel, Switzerland) on a PLRP-S column 1 × 150 mm (300 A, 3 μm) from Polymer Laboratories (Ercatech, Bern, Switzerland). The temperature of the column was maintained at 25 °C in a column oven LC-Pelcooler (Labsource, Reinach, Switzerland). A PAL HTS Autosampler (CTC Analytics, Zwingen, Switzerland) was used for automatic injection and the sample vials were kept at 8 °C. Ten μL of sample solution and 10 μL of internal