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# Occurrence of the Angiotensin-Converting Enzyme–Inhibiting Tripeptides Val-Pro-Pro and Ile-Pro-Pro in Different Cheese Varieties of Swiss Origin

**U. Bütikofer, J. Meyer, R. Sieber, B. Walther, and D. Wechsler[1]**
Agroscope Liebefeld-Posieux Research Station ALP, Schwarzenburgstrasse 161, 3003 Bern, Switzerland

## ABSTRACT

The contents of the 2 antihypertensive peptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) were determined in 101 samples from 10 different Swiss cheese varieties using HPLC with subsequent triple mass spectrometry. In the category of extra hard and hard cheeses, the Protected Denomination of Origin cheeses Berner Alpkäse and Berner Hobelkäse, L'Etivaz à rebibes, Le Gruyère, Sbrinz, Emmentaler (organic and conventional) and in the category of semihard cheeses, the varieties Tilsiter, Appenzeller ¼ fat and full fat, Tête de Moine, and Vacherin fribourgeois were screened in the study. The average concentration of the sum of VPP and IPP in the screened cheese varieties varied to a large extent, and substantial variations were obtained for individual samples within the cheese varieties. The lowest average concentration of the 2 tripeptides was found in L'Etivaz à rebibes (n = 3) at 19.1 mg/kg, whereas Appenzeller ¼ fat (n = 4) contained the greatest concentration at 182.2 mg/kg. In individual samples, the total concentration of VPP and IPP varied between 1.6 and 424.5 mg/kg. With the exception of a 10-yr-old cheese, VPP was always present at greater concentrations than IPP. Milk pretreatment, cultures, scalding conditions, and ripening time were identified as the key factors influencing the concentration of these 2 naturally occurring bioactive peptides in cheese. The results of the present study show that various traditional cheese varieties contain, on average, similar concentrations of the 2 antihypertensive peptides to the recently developed fermented milk products with blood pressure–lowering property. This may serve as a basis for the development of a functional cheese with blood pressure–lowering property.
**Key words:** angiotensin-converting enzyme-inhibiting peptide, Val-Pro-Pro, Ile-Pro-Pro, Swiss cheese variety

## INTRODUCTION

Angiotensin-converting enzyme (**ACE**)-inhibiting peptide sequences are found in the AA sequence of different proteins, and the peptides can be formed during the fermentation of foods or by digestion of food proteins (Bachmann et al., 2003). These bioactive peptides are of special interest because of their blood pressure–lowering activities. The conversion of angiotensin I to angiotensin II, which causes the contraction of blood vessels and increases blood pressure, is inhibited and the degradation of bradykinin. This, in turn, leads to a dilation of the blood vessels and to a decrease in blood pressure to inactive fragments, is prevented (Li et al., 2004).

Different foods have been shown to contain these ACE-inhibiting peptides and also ACE-inhibiting activity (Li et al., 2004). Water-soluble extracts of several cheese varieties such as Norvegia, Jarlsberg, Cheddar, Blue (Stepaniak et al., 2001), Gouda, Emmentaler, Camembert, Edam, and Havarti (Saito et al., 2000) as well as several Italian cheeses with short and medium ripening times (Smacchi and Gobbetti, 1998) showed large differences in ACE-inhibitory activity in vitro. In addition, ACE-inhibitory potential was measured in the ethanol-soluble fraction of different cheeses such as Gamalost, Pultost, Norvegia, Castello, French Brie, Port Salut, and Kesam (Pripp et al., 2006). Furthermore, a large number of individual peptides with ACE-inhibitory activity could be identified in various cheese varieties. In water-soluble extracts of an 8-mo-old Manchego manufactured from sheep milk, 22 (Gómez-Ruiz et al., 2002) or 75 (Gómez-Ruiz et al., 2004) peptides with ACE-inhibitory activity were identified. These peptides varied in size from dipeptides to decapeptides. Recently, the same research group found 41 ACE-inhibitory peptides (<1,000 Da) in the permeate of different Spanish cheeses analyzed by HPLC-MS/MS and off-line MS/MS, in which all peptides showed moderate or low ACE-inhibitory activity (Gómez-Ruiz et al., 2006). In an 8-mo-old Gouda 2 peptides, $\alpha_{s1}$-casein f(1–9) and $\beta$-CN f(60–68), showed efficient ACE-inhibiting activity (Saito et al., 2000). In cheese, the

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[1]Corresponding author: daniel.wechsler@alp.admin.ch

formation of antihypertensive peptides can be influenced by milk pretreatment, processing, and ripening time (Bütikofer et al., 2007). Cheese made from raw milk showed greater ACE-inhibition than that from pasteurized milk. The ACE-inhibiting activity found in Harz, Camembert, Tilsiter, Edam, Leerdam, Roquefort, Emmentaler, Cheddar, and Parmesan was strongly dependent on the degree of proteolysis and the age of the cheese (Meisel et al., 1997). However, the blood pressure of spontaneously hypertensive rats was significantly reduced to a greater extent after feeding of Gouda cheese aged for 8 mo compared with 24-mo-old Gouda. The younger cheese contained 2 nonapeptides, an undecapeptide, and a tridecapeptide (Saito et al., 2000).

In vivo, antihypertensive effects of bioactive peptides were demonstrated in spontaneously hypertensive rats (Nakamura et al., 1995a; Muguerza et al., 2006; Quirós et al., 2007) and in hypertensive human subjects (Hata et al., 1996; Seppo et al., 2002, 2003; Tuomilehto et al., 2004; Mizuno et al., 2005) after administration of *Lactobacillus helveticus*-fermented milk (Nakamura et al., 1995a; Hata et al., 1996; Seppo et al., 2002, 2003; Tuomilehto et al., 2004; Jauhiainen et al., 2005), *Enterococcus faecalis*-fermented milk (Muguerza et al., 2006; Quirós et al., 2007), or a casein hydrolysate prepared using an *Aspergillus oryzae* protease (Mizuno et al., 2005; Sano et al., 2005). The tripeptides valyl-prolyl-proline (Val-Pro-Pro; **VPP**) and isoleucyl-prolyl-proline (Ile-Pro-Pro; **IPP**) from *L. helveticus*-fermented milk were identified as the most potent antihypertensive peptides (Nakamura et al., 1995b). The products given to mildly hypertensive or hypertensive patients contained between 2 and 5 mg of the 2 tripeptides (Hata et al., 1996; Seppo et al., 2002, 2003; Tuomilehto et al., 2004; Mizuno et al., 2005). However, 10-fold-greater doses of 50 mg/d have also been used in clinical studies (Jauhiainen et al., 2005). In a double-blind, placebo-controlled crossover study with 6 healthy subjects, plasma concentrations of IPP were increased after consumption of a lactotripeptide-enriched milk beverage (Foltz et al., 2007).

In a recent study, we quantified the VPP and IPP tripeptides in individual samples of various extra hard, hard, semihard, and soft cheeses with a new method using HPLC coupled with triple MS detection (**HPLC-MS$^3$**; Bütikofer et al., 2007). In the 44 samples we identified 9 different cheese varieties (Berner Hobelkäse, Emmentaler, Gouda, Appenzeller full fat and ¼ fat, Tilsiter, Winzerkäse, Tête de Moine, and Vacherin fribourgeois) that contained VPP and IPP at concentrations above 100 mg/kg. Because these 2 ACE-inhibiting peptides in recently developed fermented milk products have been shown to be effective against hypertension at a dose of about 5 mg/d, the daily consumption of a 50-g portion of cheese containing similar amounts of these peptides could be of interest as a dietary recommendation for individuals with mild hypertension. However, because of the large number of small-scale cheese factories in Switzerland and due to differences in cultures, processing, and ripening conditions from factory to factory, it is difficult to specify the concentration of VPP and IPP for any individual cheese variety. Nevertheless, a better knowledge of the naturally occurring concentrations of VPP and IPP in traditional cheese varieties would be helpful for the development of a reproducible cheese-making process yielding these antihypertensive peptides at high concentrations. The aim of the present work was therefore to measure the concentration of these 2 ACE-inhibitory peptides in a series of traditional Swiss cheese varieties.

## MATERIALS AND METHODS

### *Cheese Samples*

A total of 101 commercial cheese samples from the following Swiss cheese varieties were analyzed: Berner Alpkäse (n = 11) and Berner Hobelkäse (n = 23), L'Etivaz à rebibes (n = 3), Sbrinz (n = 7), Le Gruyère (n = 9), Emmentaler of conventional (n = 12) and organic (n = 8) production, Tilsiter (n = 6), Appenzeller full-fat (n = 6), Appenzeller ¼ fat (n = 4), Tête de Moine (n = 6), and Vacherin fribourgeois (n = 6). With the exception of Appenzeller and Tilsiter, the included cheese varieties are registered as products with Protected Designation of Origin (**PDO**) with well-defined manufacturing procedures. The most important technological characteristics of the cheese varieties are summarized in Table 1. Cheese samples were purchased randomly from local cheese factories or obtained directly from cheese associations. The production date and the approval number of the manufacturer were registered to obtain further information and to determine the exact age of the cheese samples. The cheese samples were stored at −20°C until analysis. All samples were analyzed for moisture, protein, TCA-soluble nitrogen (**TCA-SN**) and fat using standard methods as previously described (Bütikofer et al., 2007). Furthermore, fat in DM and moisture on a fat-free basis were calculated.

### *HPLC-MS$^3$ Determination of ACE-Inhibiting Peptides VPP and IPP*

Analytical grade formic acid and HPLC-grade acetonitrile were purchased from Sigma-Aldrich (Buchs, Switzerland). Deionized water was prepared on a

**Table 1.** Technological characteristics of the investigated cheese varieties

| Cheese variety | Curd firmness | Raw material | Cultures | Scalding conditions: Heating °C | Heating Min | Dry-stirring °C | Dry-stirring Min | Ripening: Minimal ripening, mo | Usual age at consumption, mo |
|---|---|---|---|---|---|---|---|---|---|
| L'Etivaz à rebibes[1] | Extra hard | Raw milk | | 56–57 | 40 | 56 | 2–5 | 34.5 | 34.5–48 |
| Sbrinz[1] | Extra hard | Raw milk | Thermophilic lactic acid bacteria | 57 | 40 | 57–54 | 25 | 16.0 | 16–36 |
| Berner Hobelkäse[1] | Extra hard | Raw milk | Traditional whey culture | 50 | 25–60 | 50 | 5–15 | 18.0 | 18–36 |
| Berner Alpkäse[1] | Extra hard | Raw milk | Traditional whey culture | 50 | 25–60 | 50 | 5–15 | 6.0 | 6–18 |
| Le Gruyère[1] | Hard | Raw milk | Traditional whey culture and cultures of thermophilic lactic acid bacteria | 57 | 40 | 57 | 0–15 | 5.0 | 5–18 |
| Emmentaler[1] | Hard | Raw milk | Cultures of thermophilic lactic acid bacteria, propionic acid bacteria and facultatively heterofermentative lactobacilli | 52–54 | 40 | 53–51 | 30 | 4.0 | 4–18 |
| Appenzeller ¼ fat | Semihard | Raw or thermized milk[2] | Thermophilic and mesophilic lactic acid bacteria | 31–36 | 15–20 | 31–36 | 15 | 3.0 | 3–8 |
| Appenzeller full-fat | Semihard | Raw or thermized milk[2] | Thermophilic and mesophilic lactic acid bacteria | 47 | 25 | 25 | 20 | 3.0 | 3–8 |
| Tilsiter | Semihard | Raw or thermized milk[2] | Thermophilic and mesophilic lactic acid bacteria | 44 | 25 | 25 | 20 | 2.3 | 2.3–6 |
| Tête de Moine[1] | Semihard | Raw milk | Thermophilic lactic acid bacteria | 46–53 | 40 | 53–46 | 20 | 2.5 | 2.5–7 |
| Vacherin fribourgeois[1] | Semihard | Raw or thermized milk[2] | Thermophilic and mesophilic lactic acid bacteria | 30–36 | 10 | 36–30 | 20–50 | 2.3 | 2.3–6 |

[1]Registered as cheese with Protected Designation of Origin.

[2]Thermization conditions: 65 to 68°C for 15 s when using plate heat exchangers or batchwise thermization of the vat milk at 58 to 61°C.

Milli-Q installation from Millipore (Volketswil, Switzerland). Standard peptides Val-Pro-Pro, Ile-Pro-Pro, and Pro-Pro-Pro-Pro (**PPPP**) were purchased from Bachem (Bubendorf, Switzerland).

The concentrations of VPP and IPP were determined in the filtrate of the water-soluble extract of cheese in a single analysis. For analysis, the rind of the cheese samples was first removed. Five grams of each of the grated samples was homogenized in 10 mL of distilled water for 15 s at 17,000 rpm using a Polytron homogenizer from Kinematica AG (Littau, Switzerland) followed by shaking in a water bath for 60 min at 40°C. The samples were centrifuged for 30 min at 4°C and 10,000 × $g$. The aqueous solution was removed from below the fat layer with a syringe and 500 μL was filtered through a Microcon ym-3 filter (Millipore, Volketswil, Switzerland) by centrifugation at room temperature for 99 min at 14,000 × $g$. Filtrates were stored at –20°C until analyzed.

The HPLC separation was performed on a PLRP-S column (1 × 150 mm, 300 Å, 3 μm) from Polymer Laboratories (Ercatech, Bern, Switzerland) with a Rheos 2200 pump (Flux Instruments, Basel, 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 microliters of sample solution and 10 μL of internal standard solution were injected together. The HPLC-MS$^3$ experiments were performed on a Finnigan LTQ linear ion-trap mass spectrometer (Spectronex, Basel, Switzerland). The complete system was controlled by Xcalibur software Version 1.4 (Thermo Electron Corp., Waltham, MA). The following solvent system was used for the analysis: solvent A: 0.5% (vol/vol) formic acid in water and solvent B: 0.5% (vol/vol) formic acid in acetonitrile. The flow was set to 70 μL/min for the whole analysis. The separation of the peptides was performed with a linear gradient of 0 to 40% solvent B over 30 min.

The first 4 min of the eluant flow was directed to waste to reduce salt deposit on the transfer capillary of the MS instrument. The MS$^3$ spectra were recorded from 4 to 15 min. Separate MS experiments were set up for VPP, IPP, and the internal standard PPPP. The retention time of VPP, IPP, and PPPP was 8, 11.5, and 12 min, respectively. For the HPLC-$MS^3$ experiments, the same parameters were used as previously described (Bütikofer et al., 2007).

### Statistical Analyses

The mean values, standard deviations, and medians were calculated with Systat for Windows software (Systat Software, Inc., San Jose, CA). The Shapiro-Wilk test was applied for a check on normality. A comparison of mean values was performed with the Bonferroni test.

## RESULTS

The composition of all cheese samples was analyzed, and the values for protein, fat, and water were compared with the individual specifications of the surveyed cheese varieties. All samples were within the specified limits and a normal symmetric distribution for protein, fat, and water content was found within the individual cheese varieties. The average composition of the investigated cheese varieties is shown in Tables 2 and 3.

### Comparison of Extra Hard and Hard vs. Semihard Cheeses

In the present study, 73 samples belonging to the category of extra hard and hard cheeses and 28 samples of semihard cheese were analyzed for the contents of VPP and IPP. The difference in the content of VPP and IPP was rather small between the 2 cheese categories (Table 4). In the group of extra hard and hard cheeses, the mean concentrations for VPP and IPP were 78.2 and 17.6 mg/kg, respectively, whereas in the group of semihard cheese varieties, similar mean concentrations of 71.8 and 17.6 mg/kg, respectively, were obtained. However, the maximal concentrations of VPP and IPP were considerably greater in the group of extra hard and hard cheeses than in the group of semihard cheeses.

The similar ratio of VPP to IPP indicates that, in both cheese categories, either considerably more VPP is released or VPP is more resistant to further degradation. However, large variations in the ratio of VPP to IPP were found within and between the individual cheese varieties.

### Concentrations of VPP and IPP in Individual Cheese Varieties

The average concentrations of VPP and IPP found in the surveyed cheese varieties are summarized in Tables 2 and 3. Among the different extra hard and hard cheeses with typical ripening periods from 4 mo to 2 yr, the well-known Swiss cheese varieties Emmentaler, Le Gruyère, and Sbrinz as well as the Alpine cheese varieties Berner Alpkäse, Berner Hobelkäse, and L'Etivaz à rebibes were included in this study. Alpkäse is the precursor of Hobelkäse, which is consumed in the form of thin slices prepared with a plane. For the production of Hobelkäse, loaves of smear-ripened Alpkäse are washed at the age of 5 to 7 mo and further dry-ripened at about 12°C and 75% relative humidity to the age of 18 to 36 mo. Among the surveyed extra hard and hard cheese varieties, Berner Alpkäse and Berner Hobelkäse showed the greatest mean values for IPP and VPP, followed by Emmentaler, Le Gruyère, Sbrinz, and L'Etivaz à rebibes (Table 2). The calculated standard deviation for the average concentration of VPP and IPP in Alpkäse and Hobelkäse indicates that there were large variations between individual cheeses samples of the same variety. In individual cheese samples of Alpkäse, the maximal concentrations of VPP and IPP were 333.1 and 97.6 mg/kg, respectively, and similar concentrations of 273.4 and 95.4 mg/kg were obtained in Hobelkäse. However, several cheese samples of the same 2 varieties contained very low amounts (about 10 to 20 mg/kg) of the 2 tripeptides. According to the Shapiro-Wilk test, the contents of VPP and IPP were not normally distributed ($P < 0.001$). In contrast to the other cheese varieties (data not shown), the calculated medians for the concentrations of VPP and IPP in Berner Alpkäse (68.4 mg/kg) and Berner Hobelkäse (56.6 mg/kg) differed greatly from the calculated mean values indicated in Table 2 due to the abnormal distribution.

In the semihard cheese category, the well-known Swiss varieties of Appenzeller full fat and ¼ fat, Tilsiter, Tête de Moine, and Vacherin fribourgeois were included. With the exception of Tête de Moine, a PDO cheese that has to be made from raw milk, the other 4 varieties can be made from thermized or raw milk. Appenzeller ¼ fat had the greatest mean value of all semihard cheeses, containing, on average, a total of 182.2 mg/kg of VPP and IPP (Table 3). Tête de Moine and Tilsiter also had remarkable amounts of the 2 ACE-inhibiting peptides and contained a total of 105.3 and 96.8 mg/kg, respectively, whereas Appenzeller full fat (61.5 mg/kg) and Vacherin fribourgeois (31.8 mg/kg) contained markedly less. The average composition obtained for Appenzeller ¼ fat and full fat is shown in Table 3. Although the average protein content was only about 42% greater in the fat-reduced Appenzeller, the total amount of VPP and IPP was almost 3 times greater compared with the full-fat variety.

**Table 2.** Composition of cheese and occurrence of Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) in extra hard and hard cheeses of Swiss origin[1]

| Cheese | | VPP | IPP | VPP + IPP | VPP/ IPP | TCA-SN | TCA -SN/TN | TN | Protein | Fat | FDM | Water | MFFB |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | mg/kg | | | mg/mg | g/kg | % | g/kg | | | | | |
| Berner Alpkäse (n = 11) | Mean | 97.9 | 23.9 | 121.9 | 5.5 | 8.6 | 19.7 | 43.6 | 278.2 | 389.6 | 532.2 | 267.5 | 438.5 |
| | SD | 113.0 | 35.3 | 148.0 | 1.4 | 1.1 | 2.6 | 1.8 | 11.6 | 16.8 | 19.7 | 18.6 | 26.2 |
| | Minimum | 9.0 | 1.8 | 10.7 | 3.1 | 7.1 | 15.5 | 41.2 | 262.9 | 367.0 | 498.6 | 237.5 | 408.9 |
| | Maximum | 333.1 | 97.6 | 424.5 | 7.7 | 11.0 | 25.5 | 46.4 | 295.8 | 419.0 | 555.7 | 298.0 | 479.1 |
| Berner Hobelkäse (n = 23) | Mean | 103.3 | 26.5 | 129.8 | 4.8 | 10.4 | 21.8 | 47.6 | 303.8 | 401.3 | 519.5 | 227.2 | 379.4 |
| | SD | 103.1 | 30.3 | 132.0 | 2.0 | 1.2 | 2.9 | 2.1 | 13.3 | 23.3 | 21.3 | 23.6 | 30.1 |
| | Minimum | 4.9 | 1.8 | 6.8 | 2.4 | 7.9 | 15.3 | 44.0 | 281.0 | 331.5 | 458.6 | 184.0 | 330.0 |
| | Maximum | 273.4 | 95.4 | 353.0 | 11.6 | 13.7 | 30.0 | 51.4 | 328.2 | 443.0 | 549.0 | 275.5 | 431.4 |
| L'Etivaz à rebibes (n = 3) | Mean | 16.2 | 2.9 | 19.1 | 6.1 | 15.1 | 31.0 | 48.6 | 310.2 | 383.8 | 503.2 | 237.2 | 384.9 |
| | SD | 8.0 | 1.7 | 9.6 | 1.8 | 1.6 | 2.8 | 0.8 | 5.4 | 22.0 | 17.1 | 17.5 | 14.7 |
| | Minimum | 8.4 | 1.0 | 9.4 | 4.6 | 13.4 | 28.2 | 47.7 | 304.4 | 368.5 | 492.7 | 217.5 | 368.9 |
| | Maximum | 24.4 | 4.3 | 28.6 | 8.1 | 16.7 | 33.8 | 49.4 | 315.1 | 409.0 | 523.0 | 251.0 | 397.8 |
| Sbrinz (n = 7) | Mean | 34.6 | 3.1 | 37.7 | 13.9 | 10.2 | 20.4 | 50.4 | 321.8 | 342.7 | 466.5 | 266.3 | 402.4 |
| | SD | 26.6 | 2.9 | 27.2 | 9.5 | 0.7 | 1.8 | 2.7 | 17.5 | 34.1 | 12.9 | 53.5 | 66.9 |
| | Minimum | 0.7 | 0.9 | 1.6 | 0.8 | 8.9 | 17.8 | 47.9 | 305.4 | 321.0 | 452.8 | 149.0 | 256.0 |
| | Maximum | 87.5 | 9.4 | 90.5 | 28.6 | 10.8 | 22.6 | 55.8 | 355.8 | 419.0 | 492.0 | 297.0 | 440.7 |
| Le Gruyère (n = 9) | Mean | 48.3 | 13.1 | 61.4 | 3.8 | 11.2 | 26.6 | 42.3 | 270.2 | 336.3 | 511.5 | 341.9 | 515.5 |
| | SD | 27.8 | 6.9 | 33.6 | 1.5 | 1.8 | 4.6 | 2.3 | 14.8 | 10.3 | 15.0 | 15.4 | 21.2 |
| | Minimum | 13.7 | 6.3 | 21.6 | 1.5 | 8.4 | 18.2 | 40.0 | 255.4 | 314.0 | 482.3 | 316.0 | 478.9 |
| | Maximum | 102.1 | 26.9 | 129.0 | 5.7 | 13.6 | 33.4 | 46.3 | 295.2 | 348.0 | 530.9 | 358.0 | 536.6 |
| Emmentaler (n = 20) | Mean | 76.4 | 13.3 | 89.6 | 7.5 | 9.6 | 20.9 | 45.7 | 291.5 | 324.6 | 494.0 | 342.3 | 507.3 |
| | SD | 40.7 | 11.4 | 50.9 | 3.2 | 2.6 | 5.1 | 1.7 | 10.6 | 11.7 | 15.1 | 16.4 | 20.9 |
| | Minimum | 27.2 | 3.9 | 31.3 | 3.4 | 6.0 | 13.8 | 43.3 | 276.5 | 303.5 | 464.5 | 310.5 | 466.3 |
| | Maximum | 153.7 | 37.3 | 189.5 | 14.0 | 15.1 | 30.8 | 49.1 | 313.3 | 349.5 | 523.2 | 372.5 | 544.5 |

[1]TCA-SN = TCA-soluble N; TN = total N; FDM = fat in DM; MFFB = moisture on a fat-free basis.

**Table 3.** Composition of cheese and occurrence of Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) in semihard cheeses of Swiss origin[1]

| Cheese | | VPP | IPP | VPP + IPP | VPP/ IPP | TCA-SN | TCA -SN/TN | TN | Protein | Fat | FDM | Water | MFFB |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | mg/kg | | | mg/mg | g/kg | % | g/kg | | | | | |
| Tilsiter (n = 6) | Mean | 83.1 | 13.7 | 96.8 | 7.1 | 8.0 | 18.2 | 44.0 | 280.4 | 310.2 | 492.9 | 370.6 | 537.7 |
| | SD | 31.4 | 9.6 | 40.1 | 2.1 | 1.4 | 3.4 | 0.9 | 6.0 | 13.8 | 19.6 | 7.6 | 11.2 |
| | Minimum | 51.0 | 6.2 | 57.2 | 4.0 | 7.0 | 15.4 | 42.8 | 272.9 | 295.0 | 475.8 | 358.0 | 517.3 |
| | Maximum | 120.2 | 30.0 | 150.3 | 9.8 | 10.7 | 25.0 | 45.4 | 289.5 | 332.0 | 523.7 | 379.5 | 547.9 |
| Appenzeller, full fat (n = 6) | Mean | 50.9 | 10.6 | 61.5 | 6.0 | 8.6 | 20.9 | 41.2 | 262.8 | 318.5 | 508.6 | 373.5 | 548.3 |
| | SD | 29.6 | 4.6 | 27.4 | 4.4 | 2.2 | 5.7 | 1.1 | 6.8 | 13.9 | 13.3 | 14.3 | 12.3 |
| | Minimum | 22.0 | 5.6 | 30.5 | 1.5 | 5.6 | 13.1 | 40.2 | 256.4 | 299.0 | 492.6 | 354.5 | 529.1 |
| | Maximum | 88.0 | 17.8 | 96.0 | 11.0 | 11.2 | 27.8 | 42.9 | 273.7 | 337.0 | 530.7 | 392.5 | 561.5 |
| Appenzeller, ¼ fat (n = 4) | Mean | 131.4 | 50.9 | 182.2 | 3.1 | 15.7 | 26.9 | 58.8 | 375.3 | 118.0 | 217.2 | 455.5 | 516.6 |
| | SD | 78.5 | 29.6 | 103.6 | 1.6 | 3.3 | 6.8 | 4.1 | 26.1 | 10.2 | 23.9 | 22.8 | 28.4 |
| | Minimum | 56.0 | 10.5 | 66.4 | 1.5 | 11.2 | 18.3 | 52.9 | 337.7 | 105.0 | 195.9 | 431.0 | 489.2 |
| | Maximum | 238.2 | 79.2 | 317.4 | 5.3 | 18.3 | 34.7 | 61.9 | 394.7 | 130.0 | 251.5 | 483.0 | 555.2 |
| Tête de Moine (n = 6) | Mean | 93.2 | 12.0 | 105.3 | 8.3 | 5.5 | 13.8 | 39.5 | 252.3 | 337.3 | 532.4 | 365.9 | 552.6 |
| | SD | 43.3 | 6.3 | 49.2 | 1.8 | 0.9 | 2.1 | 1.3 | 8.0 | 14.7 | 14.6 | 15.4 | 15.0 |
| | Minimum | 47.3 | 5.2 | 52.4 | 6.3 | 4.3 | 10.9 | 38.0 | 242.4 | 319.0 | 511.2 | 344.0 | 530.0 |
| | Maximum | 169.3 | 20.4 | 189.8 | 11.0 | 6.9 | 16.8 | 41.1 | 262.5 | 351.0 | 552.5 | 386.0 | 569.7 |
| Vacherin fribourgeois (n = 6) | Mean | 20.0 | 11.8 | 31.8 | 2.3 | 6.5 | 16.9 | 38.5 | 245.5 | 303.3 | 518.1 | 414.3 | 595.1 |
| | SD | 21.6 | 17.3 | 38.8 | 0.6 | 1.0 | 2.6 | 1.7 | 10.8 | 16.6 | 20.4 | 11.6 | 7.9 |
| | Minimum | 4.8 | 1.6 | 6.5 | 1.3 | 5.7 | 14.7 | 35.9 | 228.7 | 280.0 | 494.7 | 402.0 | 588.4 |
| | Maximum | 62.1 | 46.7 | 108.8 | 3.0 | 8.3 | 20.4 | 41.0 | 261.5 | 319.5 | 545.1 | 434.0 | 607.4 |

[1]TCA-SN = TCA-soluble N; TN = total N; FDM = fat in DM; MFFB = moisture on a fat-free basis.

**Table 4.** Composition of cheese and occurrence of Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) in extra hard, hard and semihard cheeses of Swiss origin[1]

| Cheese | | VPP | IPP | VPP + IPP | VPP/ IPP | TCA-SN | TCA -SN/TN | TN | Protein | Fat | FDM | Water | MFFB |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | mg/kg | | | mg/mg | g/kg | % | g/kg | | | | | |
| Extra hard and hard cheeses (n = 73) | Mean | 78.2 | 17.6 | 95.8 | 6.5 | 10.2 | 22.1 | 46.2 | 294.5 | 364.2 | 507.7 | 283.1 | 442.5 |
| | SD | 79.5 | 23.7 | 102.2 | 4.4 | 2.1 | 4.6 | 3.1 | 19.5 | 38.7 | 25.6 | 55.9 | 65.7 |
| | Minimum | 0.7 | 0.9 | 1.6 | 0.8 | 6.0 | 13.8 | 40.0 | 255.4 | 303.5 | 452.8 | 149.0 | 256.0 |
| | Maximum | 333.1 | 97.6 | 424.5 | 28.6 | 16.7 | 33.8 | 55.8 | 355.8 | 443.0 | 555.7 | 372.5 | 544.5 |
| Semihard cheeses (n = 28) | Mean | 71.8 | 17.6 | 89.3 | 5.5 | 8.4 | 18.8 | 43.4 | 276.7 | 288.8 | 470.7 | 391.7 | 552.4 |
| | SD | 53.0 | 19.3 | 67.9 | 3.3 | 3.6 | 5.7 | 6.9 | 44.3 | 73.2 | 107.6 | 34.8 | 29.0 |
| | Minimum | 4.8 | 1.6 | 6.5 | 1.3 | 4.3 | 10.9 | 35.9 | 228.7 | 105.0 | 195.9 | 344.0 | 489.2 |
| | Maximum | 238.2 | 79.2 | 317.4 | 11.0 | 18.3 | 34.7 | 61.9 | 394.7 | 351.0 | 552.5 | 483.0 | 607.4 |

[1]TCA-SN = TCA-soluble N; TN = total N; FDM = fat in DM; MFFB = moisture on a fat-free basis.

### Concentrations of VPP and IPP in Emmentaler Cheese Made with a Whey Culture Containing L. helveticus

To study the impact of a whey culture containing *L. helveticus* on the concentrations of VPP and IPP in Emmentaler, we included samples of 3 cheeses aged 4, 9, and 12 mo from an Emmentaler factory still using a traditional whey culture as starter. In addition to the whey culture, customary Swiss cultures of propionic acid bacteria and facultatively heterofermentative lactobacilli were used during manufacture. The occurrence of *L. helveticus* in cheese samples and the whey culture was investigated by PCR as previously described (Fortina et al., 2001; Pillonel et al., 2005). The 3 samples contained, on average, 163.2 mg/kg of the 2 bioactive peptides (Table 5).

### Organic vs. Conventional Emmentaler Cheeses

Within the Emmentaler cheese variety, we measured 8 samples of organic and 12 samples of conventional production. The results indicated that concentrations of VPP and IPP were independent of the type of production system. On average, 74.6 mg/kg of VPP and IPP was found in organic Emmentaler, whereas a mean value of 99.7 mg/kg was calculated in conventional Emmentaler (Table 5). The slightly greater mean value for conventional Emmentaler was influenced by the 3 samples of Emmentaler from the factory using a traditional whey culture containing *L. helveticus*. In the absence of these 3 samples, the mean value of conventional Emmentaler was similar to organic Emmentaler at 78.5 mg/kg.

### Concentration of VPP and IPP in Cheeses at Different Ripening Stages

The degree of proteolysis is usually strongly dependent on the age of the cheese. To investigate the impact of ripening time on the release of VPP and IPP, the age of all cheeses at sampling was recorded. The individual age of the cheeses varied between 84 d and 10 yr. In a Sbrinz cheese, aged exceptionally for 10 yr, only traces of VPP and IPP (1.6 mg/kg) could be found. In contrast to this cheese, the other Sbrinz cheeses were aged between 2 and 3 yr and contained between 23.1 and 90.5 mg/kg of the 2 antihypertensive tripeptides.

The above-mentioned Emmentaler cheeses produced with a traditional whey culture and aged for 4, 9, and 12 mo contained a total of 139.3, 184.6, and 165.6 mg/kg of VPP and IPP, respectively. The other 9 conventional Emmentaler samples contained, depending on age, between 39.9 (4 mo) and 136.5 mg/kg (cave-aged Emmentaler, >12 mo). However, in the group of organic Emmentaler cheeses, there was no correlation between the age of the cheeses and the concentration of the 2 tripeptides ($P > 0.05$, Bonferroni). The data obtained suggested that ripening time was not the only factor determining the release of these 2 tripeptides; other factors such as the composition of raw milk flora and cultures may exert even more pronounced effects on concentrations of VPP and IPP.

## DISCUSSION

Ripened cheese contains high amounts of protein and may serve as a natural source of bioactive peptides. Sufficient proteolysis is required for the release of the physiologically active peptides from the proteins. However, because of further degradation in fermented foods or in the intestinal tract, it is difficult to obtain bioactive peptides in physiologically relevant concentrations. In recent years, a large number of different ACE-inhibiting peptides have been identified in cheese (Gómez-Ruiz et al., 2002, 2004, 2006). Furthermore, the 2 tripeptides VPP and IPP have been identified in fermented milk products as ACE-inhibiting compounds (Nakamura et al., 1995b). In our study we

**Table 5.** Composition of cheese and occurrence of Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) in Emmentaler cheeses produced with normal and organic milk as well as with a traditional whey culture[1]

| Cheese | | VPP | IPP | VPP + IPP | VPP/IPP | TCA-SN | TCA-SN/TN | TN | Protein | Fat | FDM | Water | MFFB |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | mg/kg | | | mg/mg | g/kg | % | g/kg | | | | | |
| Emmentaler | Mean | 85.1 | 14.6 | 99.7 | 8.1 | 8.8 | 19.4 | 45.4 | 289.6 | 323.7 | 495.5 | 346.3 | 512.4 |
| conventional[2] | SD | 39.2 | 12.2 | 49.7 | 3.7 | 1.9 | 4.2 | 1.4 | 9.0 | 12.9 | 13.2 | 15.4 | 16.7 |
| (n = 12) | Minimum | 35.5 | 4.4 | 39.9 | 3.4 | 6.1 | 13.8 | 43.3 | 276.5 | 303.5 | 479.5 | 310.5 | 466.3 |
| | Maximum | 147.3 | 37.3 | 184.6 | 14.0 | 11.4 | 26.1 | 48.5 | 309.4 | 349.5 | 523.2 | 365.5 | 525.9 |
| Emmentaler | Mean | 63.3 | 11.3 | 74.6 | 6.6 | 10.8 | 23.2 | 46.1 | 294.4 | 325.9 | 491.6 | 336.4 | 499.5 |
| organic | SD | 41.9 | 10.7 | 52.3 | 2.1 | 3.1 | 5.9 | 2.0 | 12.8 | 10.3 | 18.3 | 17.0 | 25.1 |
| (n = 8) | Minimum | 27.2 | 3.9 | 31.3 | 4.3 | 6.0 | 13.8 | 43.4 | 277.2 | 314.0 | 464.5 | 322.0 | 470.1 |
| | Maximum | 153.7 | 35.8 | 189.5 | 9.6 | 15.1 | 30.8 | 49.1 | 313.3 | 339.0 | 517.6 | 372.5 | 544.5 |
| Emmentaler | Mean | 130.9 | 32.3 | 163.2 | 4.1 | 10.1 | 23.1 | 43.8 | 279.3 | 337.3 | 513.8 | 342.7 | 517.8 |
| produced with | SD | 20.6 | 4.7 | 22.7 | 0.7 | 1.6 | 3.9 | 0.5 | 2.9 | 12.8 | 10.6 | 12.3 | 8.0 |
| traditional whey | Minimum | 107.8 | 28.1 | 139.3 | 3.4 | 8.3 | 18.8 | 43.3 | 276.5 | 324.0 | 502.3 | 330.5 | 509.2 |
| culture (n = 3) | Maximum | 147.3 | 37.3 | 184.6 | 4.9 | 11.4 | 26.1 | 44.3 | 282.3 | 349.5 | 523.2 | 355.0 | 525.1 |

[1]TCA-SN = TCA-soluble N; TN = total N; FDM = fat in DM; MFFB = moisture on a fat-free basis.

[2]Including 3 samples of Emmentaler produced with a traditional whey culture.

focused on these 2 peptides that have been shown to be effective against mild hypertension in several human studies (Hata et al., 1996; Seppo et al., 2002, 2003; Tuomilehto et al., 2004; Mizuno et al., 2005; Sano et al., 2005; Jauhiainen et al., 2005).

In a previous study (Bütikofer et al., 2007), we introduced a newly developed method for the quantitative determination of VPP and IPP in cheese using HPLC-MS[3] and reported the occurrence of these peptides in 44 individual cheese samples. The sum of VPP and IPP was, on average, 100 mg/kg in the group of extra hard and hard cheeses (n = 12), 51.6 mg/kg in semihard cheeses (n = 21), and 3.4 mg/kg in soft and fresh cheeses (n = 11). Due to the very low concentrations of VPP and IPP found in samples of Camembert, Brie, Mozzarella, Gorgonzola, Münster, Limburger, Reblochon, Tomme vaudoise, Vacherin Mont d'Or, and Feta, we excluded soft and fresh cheese varieties from the present study. It was assumed that proteolysis of the soft cheeses is too mild and the ripening time is too short to liberate the bioactive tripeptides.

### *Impact of Cultures and Ripening Time on Concentrations of VPP and IPP in Cheese*

The findings of Addeo et al. (1992), Meisel et al. (1997), and Pripp et al. (2006) confirmed that the ACE-inhibitory potential is correlated to a certain extent with the degree of proteolysis. However, in the present study, there was no general correlation between the individual contents of TCA-SN and the total amount of VPP and IPP (individual data not shown, $P = 0.087$). This implied that the release of VPP and IPP requires specific proteolytic activities, as was demonstrated in fermented milk products with *L. helveticus* (Nakamura et al., 1995a). In Switzerland, the use of cultures containing *L. helveticus* is usually rigorously avoided in production of Emmentaler to prevent the defect of late fermentation and to ensure optimal storage quality (Fröhlich-Wyder et al., 2002). Nevertheless, we included samples of 3 Emmentaler aged 4, 9, and 12 mo from a Swiss Emmentaler factory using a traditional whey culture starter containing *L. helveticus*. Cheeses from this factory are well known for their rapid ripening but also for their poor storage quality and are therefore not adapted for long ripening periods in caves. The average concentration of VPP and IPP determined in these Emmentaler cheeses was 163.2 mg/kg, whereas the average concentration for all other Emmentaler cheese samples included in the study was only 76.6 mg/kg. It is interesting to note that even the 4-mo-aged cheese already contained a total of 139.3 mg/kg of the 2 peptides. In addition, the greater concentration of TCA-SN and the higher ratio of TCA-SN/TN confirmed that whey cultures containing *L. helveticus* accelerated proteolysis (Table 5) and supports the hypothesis that the release of VPP and IPP is strongly related to the proteolytic properties of the strains present in the starter culture.

The results obtained for Berner Alpkäse and Berner Hobelkäse also indicated that the composition of the starter culture is a key factor for the release of VPP and IPP. According to the list of requirements for these Alpine PDO cheeses, home-made whey cultures have to be used during manufacture and a minimal scalding temperature of 50°C must be applied for 25 to 60 min. Because more than 560 Alpine farms are producing these PDO cheeses, large variation in the concentration of VPP and IPP was expected due to heterogeneous processing parameters and cultures. However, one group of samples contained low amounts of the 2 antihypertensive peptides in the range of 0 to 100 mg/kg, whereas a second group of samples showed high concentrations >200 mg/kg. In contrast to the overall evaluation, a significant correlation between individual concentrations of TCA-SN and the total amount of VPP and IPP was found for Berner Alpkäse and Berner Hobelkäse ($P < 0.05$, Bonferroni). The abnormal distribution of the individual concentrations of VPP and IPP and the greater degree of proteolysis found in the group of cheeses with high amounts of VPP and IPP suggest that home-made whey cultures of some of the producers contained *L. helveticus*. It seems that the presence or absence of *L. helveticus* or other lactic acid bacteria is somehow causing an "all-or-nothing" effect leading to 2 groups of cheeses containing either high or low concentrations of VPP and IPP. Depending on the casein content of the included cheese varieties, a total of 2.3 to 3.6 g/kg of VPP and IPP could theoretically be released. Because the terminal sequence Xaa-Pro-Pro is resistant to further degradation by aminopetidases (including X-prolyl dipeptidyl aminopeptidase), the accumulation of VPP and IPP is favored (Christensen et al., 1999). Nevertheless, it is likely that degradation of VPP and IPP also occurs during ripening.

### *Impact of Processing on Concentrations of VPP and IPP in Cheese*

Due to the predominant effect of the culture used for manufacture on the extent of proteolysis and specific degradation products, only imprecise estimations can be made for the concentrations of VPP and IPP for an individual cheese variety. In the present study, only small differences in the average concentration of VPP and IPP were found between the group of semihard cheeses and the group of extra hard and hard cheeses (Table 4), underlining the fact that ripening time is not the determining factor for maximal concentrations of VPP and IPP in cheese.

The results obtained suggest that, in addition to the type of culture and ripening time, milk pretreatment and processing also exert an influence on the average concentration of VPP and IPP in the individual cheese varieties. In the group of extra hard and hard cheeses the average concentration of the 2 bioactive tripeptides varied from 19.1 mg/kg for L'Etivaz à rebibes to 129.8 mg/kg for Berner Hobelkäse, whereas in the group of semihard cheeses, concentrations ranged from 31.8 mg/kg for Vacherin fribourgeois PDO cheese to 182.2 mg/kg for Appenzeller ¼ fat. As indicated in Table 1, there are differences in the heat treatments applied during the manufacture of the surveyed cheese varieties. All traditional extra hard and hard cheeses without exception were made from raw milk but the conditions applied for scalding the curd were specific for each variety. The results obtained suggest that scalding conditions also influence the average concentrations of VPP and IPP. Low or moderate concentrations of VPP and IPP were obtained in cheese varieties such as L'Etivaz à rebibes (19.1 mg/kg, minimum age 34.5 mo), Sbrinz (37.7 mg/kg, minimum age 16 mo) and Le Gruyère (61.4 mg/kg, minimum age 5 mo), which require intense scalding conditions (40 to 60 min at 56 to 57°C) for manufacture. Slightly greater concentrations were found in Emmentaler (89.6 mg/kg; minimum age 4 mo), in which scalding is typically carried out for 40 to 70 min at 53°C; the greatest concentrations were obtained, on average, in Berner Alpkäse (121.9 mg/kg; minimum age 6 mo) and Berner Hobelkäse (129.8 mg/kg; minimum age 18 mo) with mild scalding conditions (40 to 70 min at 50°C). The conditions for scalding applied in extra hard and hard cheese are similar to those applied for the batchwise thermization of milk and lead to a strong reduction in the raw milk flora that are not adapted to such high temperature treatments (Casey et al., 2006). The partial elimination of the raw milk flora seems to limit the specific release of VPP and IPP, probably because of the inactivation of strains of *L. helveticus* present in raw milk. However, the mean values obtained for the hard cheese varieties implied that long ripening periods (>18 mo) may lead to a decrease in VPP and IPP due to further degradation of VPP and IPP with ongoing proteolysis.

A similar trend was observed in the category of semihard cheeses. High concentrations of VPP and IPP were found in Tête de Moine (105.3 mg/kg), the only variety that is made from raw milk, whereas the lowest concentrations were obtained for Vacherin fribourgeois (31.8 mg/kg) which, with rare exceptions, is made from thermized milk. For Tilsiter and Appenzeller, thermization is often applied on only a fraction of the cheese milk. In most factories only the evening milk is thermized and then combined with freshly delivered raw milk in the morning. But there are also producers that either apply complete thermization or process only raw milk.

In our previous study (Bütikofer et al., 2007), we included 4 samples of industrial semihard cheeses such as Edamer, Manchego, Raclette, and St. Paulin that were made from pasteurized milk. In all samples the total concentrations of VPP and IPP ranged from 1.0 to 1.3 mg/kg, underlining the fact that pasteurization and the use of direct starter cultures with limited lactic acid bacteria strain diversity leads to an almost complete reduction in the release of the 2 antihypertensive peptides.

## CONCLUSIONS

In the present study, the contents of the 2 antihypertensive peptides VPP and IPP were determined in 101 samples of different Swiss cheese varieties using HPLC-$MS^3$. In individual samples of Berner Alpkäse and Berner Hobelkäse, a maximum concentration of 300 to 400 mg/kg of VPP and IPP was found. Lower maximum concentrations were found in other hard and extra hard cheeses such as Emmental (190 mg/kg), Gruyère (130 mg/kg), Sbrinz (<100 mg/kg), and L'Etivaz (<30 mg/kg). In the group of semihard cheeses, only Appenzeller ¼ fat showed maximum concentrations of >300 mg/kg, whereas in the individual samples of the other varieties (Tilsiter, Appenzeller full fat, Tête de Moine, Vacherin fribourgeois) maximum concentrations of 100 to 190 mg/kg of VPP and IPP were found. The detection of physiologically relevant concentrations of VPP and IPP in ripened cheese is a promising feature with regard to nutrition and consumption of cheese. However, the individual results of the 101 samples and the evaluation of the 10 surveyed cheese varieties indicated that large variation exists between samples of the same variety as well as between different varieties. On the basis of the obtained results, milk pretreatment, cultures, and scalding and ripening times were identified as critical parameters that determine the concentrations of VPP and IPP in cheese.

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