JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Purification and Identification of Water-Soluble Phosphopeptides from Cheese Using Fe(III) Affinity Chromatography and Mass Spectrometry

MARTIN LUND AND YLVA ARDÖ*

Department of Food Science, Dairy Technology, Centre for Advanced Food Studies, The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

Water-soluble phosphopeptides from cheese were isolated using immobilized metal affinity chromatography (IMAC). Phosphopeptides from aqueous cheese extracts were completely retained on iminodiacetic acid (IDA) Sepharose equilibrated with $FeCl_3$ and subsequently eluted with ammonium dihydrogen phosphate. Peptides in the eluate from the IMAC-Fe(III) column were identified using reversed phase liquid chromatography−electronic spray identification−tandem mass spectrometry. Phosphopeptides from two different cheeses were analyzed using the described method: a 10-month-old semihard Herrgard cheese made with mesophilic starter and a 24-month-old Parmigiano Reggiano cheese made with thermophilic starter. Elution of the IMAC-Fe(III) column with a gradient of ammonium dihydrogen phosphate resulted in three distinct peaks for Herrgard cheese corresponding to peptides carrying one, two, and four phosphorylated serine residues, respectively. Sixty-five different phosphopeptides were identified from the Herrgard, whereas only 9 from the Parmigiano Reggiano.

KEYWORDS: Cheese ripening; proteolysis; phosphopeptides; immobilized metal affinity chromatography; Fe(III) affinity chromatography; mass spectrometry

INTRODUCTION

One of the important nutritional aspects of cheese is the high content of organic phosphate associated with calcium. Digestion of phosphorylated peptides and proteins has been shown to facilitate uptake of calcium and thus contribute positively to stronger teeth and bones (*1*, *2*). During ripening of cheese, casein is hydrolyzed into a large variety of peptides by proteases and peptidases from milk, rennet, starter culture, and secondary microbial flora (*3*). Protein breakdown has several obvious roles in cheese ripening such as the development of cheese texture, background flavor intensity, and the production of flavor precursors in all mature cheese varieties. Proteolysis in cheese during ripening is evaluated by different electrophoretic techniques for the casein components and reversed phase high-performance liquid chromatography (RP-HPLC) for peptide profiles and composition of amino acids. The coupling of tandem mass spectrometry (MS/MS) to HPLC has improved the identification of peptides in cheese (*4*−*6*). However, the number of peptides produced from the breakdown of caseins is very high due to a wide variety of proteolytic and peptidolytic enzymes, and a complete mapping of peptides in cheese will require implementation of multistep purification strategies (*6*). The chemical nature of phosphate groups allows a selective purification of phosphopeptides from cheese. Phosphopeptides are preferably isolated from hydrolyzed milk proteins through interaction with polyvalent metal ions either by precipitation or by chromatographic separation (*7*−*9*). Analytical fractionation of phosphopeptides from cheese has previously been studied by precipitation with barium nitrate (*10*) or by immobilized metal affinity chromatography (IMAC) (*11*, *12*). The combination of analytical IMAC and MS/MS has successfully been employed in the field of proteomics for the identification and characterization of phosphoproteins (*13*−*15*).

Phosphopeptide profiles have mainly been studied in highly cooked, hard cheeses made using a thermophilic starter such as Parmigiano Reggiano (PR) (*4*), Grana Padano (*10*, *16*), and Comté cheese (*17*). The aim of this study was to characterize the composition of water-soluble phospopeptides of a semihard cheese made with mesophilic DL-starter culture. For the first time, the phosphopeptide profile of a semihard cheese, such as Herrgard cheese, is reported. Some of the special characteristics of this cheese are significant residual activity from the rennet enzymes as well as relatively high plasmin activity (*18*). A 10-month-old Herrgard cheese was investigated. For comparison, a 24-month-old PR cheese was analyzed. Ripening of cheese proceeds more slowly in a hard cheese compared to a semihard, and the age of the studied cheeses represents their common age at consumption. In the present work, water-soluble phosphopeptides from cheese were isolated using cation exchange

* Author to whom correspondence should be addressed (telephone +45 35 28 31 93.; fax +45 35 28 31 90; e-mail ya@kvl.dk).

10.1021/jf0401064 CCC: $27.50
Published on Web 09/18/2004

chromatography (CIEC) and IMAC-Fe(III). Phosphopeptides in the eluate from the IMAC-Fe(III) column were subsequently separated and identified using reversed phase liquid chromatography−electrtonic spray identification−tandem mass spectrometry (RP-LC-ESI-MS/MS).

MATERIALS AND METHODS

Cheese Samples. Ten-month-old Herrgard cheese with 45% fat in dry matter was from Skanemejerier, Kristianstad, Sweden. It was shown to be a typical Herrgard by routine organoleptical grading and analyses of casein components, peptide profile, and amino acid composition (data not shown). Twenty-four-month-old Parmigiano Reggiano cheese with 32% fat in dry matter was from Consorzio Del Formaggio Parmigiano-Reggiano, Reggio Emilia, Italy. All reagents employed were of analytical grade.

Extraction. Water-soluble extracts of cheese (*19*) were made with a few modifications. In brief, 6 g of grated cheese was suspended in 30 mL of Milli-Q water, and the suspension was homogenized at room temperature using an Ultra Turrax for 2 × 1 min at 24000 rpm. The homogenate was then stirred for 1 h in a beaker at 40 °C and finally centrifuged for 30 min at 4 °C. The aqueous phase was collected.

Isolation of Phosphopeptides. Phosphopeptides were isolated from the water-soluble cheese extract by cation exchange chromatography followed by Fe(III) metal affinity chromatography. The conductivity of the water-soluble cheese extract was lowered to <5 mS by 1:1 dilution with Milli-Q water, and the pH was adjusted to 7.0 by the addition of 1 M NaOH. From the diluted and pH-adjusted extract, 5 mL was loaded to a strong cation exchanger (HiTrap SP FF, 5 mL bed volume, Amersham Biosciences, Uppsala, Sweden) equilibrated with 10 mM HEPES, pH 7.0. The flow-through was collected and used for further purification (starting state elution). The column was eluted with a gradient of 0.5 M NaCl. Phosphopeptides were purified by IMAC-Fe(III) using a 5 mL HiTrap chelating HP column (IDA-Sepharose) from Amersham Biosciences. The column was equilibrated with 2 column volumes of 0.1 M $FeCl_3$, washed with 0.1 M sodium acetate, pH 3.0, and then loaded with 20 mL of the collected flow-through from the cation exchange chromatography with the pH adjusted to 3.0 using 1 M HCl. After washing, the substances were eluted from the column with a gradient from 0 to 0.2 M ammonium dihydrogen phosphate, pH 4.4. Fractions were collected, evaporated to dryness using a vacuum centrifuge at room temperature, and finally dissolved in 0.1% TFA.

Phosphorus Analysis. Total P and inorganic P in cheese extracts as well as inorganic P in chromatographic eluates were measured according to the Fiske−Subbarow method (*20*). Total P content in chromatographic fractions was determined using the sensitive heating method of Bartlett (*21*), which is a modification of the original Fiske−Subbarow method. The second method was more sensitive and used to detect traces of P in fractions that should be essentially free of P.

Identification of Peptides by LC-ESI-MS. Peptides were identified by using an Agilent LC-MSD ion trap system equipped with a Zorbax 300SB-C18 narrow-bore column (2.1 × 150 mm, 5 μm) from Agilent Technologies, Palo Alto, CA. Buffer A was 0.1% TFA and buffer B, 0.1% TFA in 90% acetonitrile. The sample was centrifuged, and 5−40 μL of the supernatant was applied; the column was flushed with buffer A for 5 min before a 40 min linear gradient from 0 to 55% B was applied. The flow was 0.25 mL/min. All separations were performed at 40 °C. The ion trap was operated in positive mode with a nebulizer pressure of 50 psi, a nitrogen flow of 9 L/min, and a drying temperature of 300 °C. The ICC maximum was set at 20000 with an accumulation time of 50 ms. Fragmentation of peptides was made using Smartfrag in the MSD Trap Control software. A fragmentation amplitude of 1.0 V and an isolation width of 3 *m*/*z* were employed. Peptides were identified using the Mascot MS/MS ion search from Matrix Science (www.matrixscience.com) or by comparing MS/MS spectra to theoretical spectra of bovine casein sequences with the precursor ion mass in question. Searches in Mascot were made with the following parameters: Swissprot database, no enzyme, variable modifications phospho (ST) and oxidation (M). A peptide tolerance of 2.0 D and an MS/MS tolerance of 0.8 Da were employed. All experiments reported were repeated a minimum of three times.

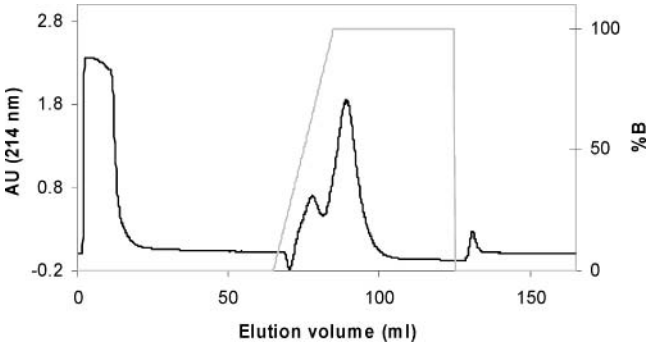

Figure 1. CIEC separation of aqueous extract of Herrgard cheese on SP-Sepharose at pH 7.0. The flow-through containing the phosphopeptides was collected for further purification. In the other peaks (retention volume = 70–100 mL) were the basic non-phosphopeptides α_{s1}-casein f(1–9), α_{s1}-casein f(1–14), and κ-casein f(96–102) detected (data not shown). Buffer B: 0.5 M NaCl.

RESULTS

Purification Strategy. A two-step purification strategy based on the high affinity of phosphate groups to immobilized Fe(III) was employed for the isolation of phosphopeptides from aqueous cheese extracts. The first step, separation on a cation exchanger, was performed to remove certain histidine-rich basic peptides from the Herrgard cheese sample, which also were found to bind to the IMAC-Fe(III) column. In the development of the purification method, it was found that when an extract from the Herrgard cheese was separated on the IMAC-Fe(III) column without a prior purification step, the eluate was dominated by peptides derived from the N terminus of α_{s1}-casein, f(1−9) and f(1−14), and the C terminus of *p*-κ-casein, f(96−102). Because these peptides have a p*I* of 9−11 and the main parts of the phosphopeptides have p*I* values below 5, starting state elution on a cation exchanger at pH 7.0 provided a convenient means to remove the histidine-rich peptides from the sample. Chromatographic separation of an aqueous extract of Herrgard cheese on a strong cation exchanger at pH 7.0 is shown in **Figure 1**.

To prevent binding of peptides that are rich in carboxylic acids to the Fe(III) column, the flow-through from the CIEC was adjusted to pH 3.0 prior to it being loaded to the column. The fraction of the Herrgard cheese sample that bound to the column was eluted in three major peaks when a gradient of 0−0.2 M ammonium dihydrogen phosphate was applied (cf. **Figure 2**). The peptide composition of the PR cheese extract was found to be less complex than that of Herrgard cheese in accordance with previous investigations (*22*). Compared to the Herrgard cheese, only a very small fraction of the PR cheese extract bound to the cation exchanger at pH 7.0. Accordingly, peptides from the PR cheese were purified using a simplified procedure: the CIEC step was omitted, and the phosphopeptides were eluted from the IMAC-Fe(III) column in a single step, which resulted in all of the material bound to the column eluting in only one peak (chromatogram not shown). Pooled fractions from the different purification steps were assayed for total and inorganic P to control potential loss of phosphopeptides during the purification. The content of total P in the water-soluble cheese extracts was close to 300 μg of P/mL for both cheeses. Organic bound P in extracts from Herrgard and PR accounted for 84 and 20%, respectively, of the total P in the extracts. A negligible content of organic bound phosphate was found in

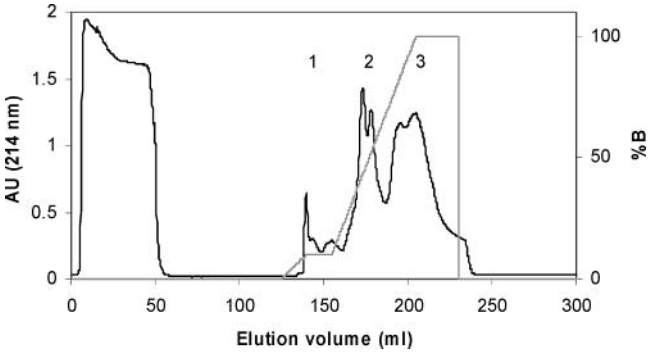

Figure 2. Purification of phosphopeptides of Herrgard by IMAC-Fe(III). Separation of the flow-through from the CIEC (**Figure 1**) on IDA-Sepharose was equilibrated with $FeCl_3$. The column was eluted with 0.2 M ammonium dihydrogen phosphate (buffer B), and the eluate was collected in three pools corresponding to the three numbered peaks in the chromatogram.

the Herrgard eluate from the cation exchanger, which accounted for approximately <1% of the total amount of organic bound P applied to the column. The Herrgard flow-through from the IMAC-Fe(III) column contained a minor amount of organic bound P of 1−2% of the amount found in the applied extract (results not shown).

Identification of Peptides. Peptides in the eluate from the IMAC-Fe(III) column were identified by RP-LC-ESI-MS/MS. **Figure 3** shows RP chromatograms of pooled IMAC-Fe(III) eluates of Herrgard cheese corresponding to the three major peaks observed in **Figure 2**. An MS/MS spectrum of the peak in **Figure 3** pool 3 with retention time of 22.1 min is shown in **Figure 4**, and this was found to match the α_{S2} casein fragment f(6−18)4P. All tandem spectra of peptides retained on the IMAC-Fe(III) column had a characteristic pattern with a very intense peak at a multiple of 98 or 49 Da off the parent ion, corresponding to the meta-stable loss of one or more phosphate groups; see the example in **Figure 4**. All sequence assignments of peptides were based on information from tandem mass spectra. The three peaks in **Figure 2** mainly corresponded to peptides with one, two, or four phosphorylations, respectively (**Table 1**). In summary, 34 monophosphorylated peptides, 12 biphosphorylated peptides, and 19 peptides with four/five phosphorylations were identified from the aqueous extract of Herrgard cheese. A few monophosphorylated peptides were also found in pool 2. The phosphopeptides originated from two parts of β-casein, four parts of α_{s1}-casein, and three parts of α_{s2}-casein (**Figure 5**). The late-eluting high peaks from pool 1 (**Figure 3**) with retention times of ∼40 min could not be identified by MS/MS due to too high masses. On the basis of other results on Herrgard cheese in our laboratory, the intense late-eluting peak at a retention time of 41.7 min with an *m*/*z* value of 1841.5 may correspond to the peptide f(30−93)1P of β-casein A2 with four charges and an average MH^+ mass of 7363 D (*23*).

The RP-LC chromatogram of the only phosphopeptide fraction isolated from the aqueous extract of the PR cheese with all of the phosphopeptides that were retained on the IMAC-Fe column contained only a few peptide peaks (**Figure 6**) of which nine were identified (cf. **Table 2**).

In all analyses, some peaks could not be identified by MS/MS, due to either too high masses or poor quality of the MS/MS spectra. Because of the complex nature of the samples, TFA (0.1%) was used in the eluent to give the best chromatographic separation by suppressing positive ionization of peptides. However, this in combination with the negative charge contribution from the phosphate group does not favor formation of positive ions in ESI.

Table 1. Identified Peptides from the Studied Herrgard Cheese

RT UV (min)	parent ion *m*/*z*	*z*	obsd MH+	calcd MH+	sequence
			IMAC Pool 1		
2.9	670.2	+1	670.2	670.28	αS_1 f(75–79)1P
4.0	671.1	+1	671.1	671.24	αS_1 f(115–119)1P
4.2	807.3	+1	807.3	807.34	αS_1 f(75–80)1P
10.8	485.11	+1	485.10 ± 0.04	485.17	αS_1 f(44–47)1P
13.4	590.1	+1	590.16 ±0.07	590.19	βCN f(33–36)1P
13.8	696.2	+1	696.2	696.26	αS_1 f(112–117)1P
13.9	657.2	+1	657.21 ± 0.06	657.25	αS_2 f(15–19)1P
14.8	835.2	+1	835.21 ± 0.09	835.32	αS_2 f(16–21)1P
15.7	719.2	+1	719.20 ± 0.05	719.23	βCN f(33–37)1P
16.1	975.4	+1	975.29 ± 0.07	975.38	βCN f(32–38)1P
16.1	1103.2	+1	1103.17	1103.41	βCN f(33–40)1P
16.3	981.3	+1	981.34 ± 0.04	981.41	αS_1 f(112–119)1P
17.7	847.3	+1	847.28 ± 0.06	847.32	βCN f(32–37)1P
18.5	788.8	+2	1576.63 ± 0.10	1576.62	βCN f(32–43)1P
18.6	669.3	+1	669.32 ± 0.03	669.29	αS_2 f(14–18)1P
18.6	948.4	+1	948.33 ± 0.02	948.41	αS_2 f(15–21)1P
18.7	1094.41	+1	1094.41	1094.49	αS_1 f(111–119)1P
19.3	770.4	+1	770.26 ± 0.01	770.33	αS_2 f(14–19)1P
19.8	784.3	+1	784.28 ± 0.05	784.32	αS_1 f(115–120)1P
20.5	960.4	+1	960.30 ± 0.09	960.41	βCN f(30–36)1P
20.5	673.4	+2	1345.52 ± 0.04	1345.57	βCN f(30–39) 1P
20.9	1089.5	+1	1089.40 ± 0.09	1089.45	βCN f(30–37) 1P
21.2	910.0	+2	1818.58 ± 0.30	1818.74	βCN f(30–43)1P
21.7	974.0	+2	1946.77 ± 0.05	1946.84	βCN f(29–43)1P
22.0	1061.5	+1	1061.44 ± 0.05	1061.49	αS_2 f(14–21) 1P
23.6	576.2	+2	1151.35 ± 0.14	1151.47	αS_2 f(141–149)1P
24.4	1095.5	+2	2189.9	2188.93	βCN f(30–46)1P
24.6	1159.8	+2	2317.66 ± 0.02	2317.02	βCN f(29–46)1P
25.1	922.2	+1	922.2	922.33	αS_2 f(141–147)1P
			IMAC Pool 2		
14.9	882.3	+1	882.19 ± 0.13	882.25	αS_1 f(44–50)2P
15.6	997.3	+1	997.22 ± 0.14	997.28	αS_1 f(44–51)2P
15.7	825.2	+1	825.22 ± 0.09	825.23	αS_2 f(128–133)2P
15.9	1125.4	+1	1125.29± 0.12	1125.34	αS_1 f(44–52)2P
16.2	997.3	+1	997.19 ± 0.05	997.28	αS_1 f(43–50)2P
16.3	742.9	+2	1484.5	1484.56	αS_2 f(129–140)2P
16.6	1112.3	+1	1112.26 ± 0.04	1112.31	αS_1 f(43–51)2P
16.7	1125.3	+1	1125.35 ± 0.07	1125.38	αS_1 f(42–50) 2P
17.1	1240.4	+1	1240.27 ± 0.11	1240.40	αS_1 f(42–51)2P
18.5	524.7	+2	1048.42 ± 0.09	1048.49	αS_1 f(75–82)1P
18.6	461.2	+2	921.28 ± 0.02	921.38	αS_1 f(115–121)1P
18.8	653.8	+2	1305.55 ± 0.07	1305.62	αS_1 f(75–84)1P
19.1	504.7	+2	1008.40 ± 0.01	1008.42	αS_1 f(115–122)1P
20.1	1327.4	+1	1327.33 ± 0.33	1327.42	αS_1 f(44–54)2P
20.9	785.9	+2	1570.58 ± 0.04	1570.54	αS_1 f(42–54)2P
22.0	634.3	+2	1267.51 ± 0.08	1267.55	αS_1 f(115–124)1P
22.2	941.0	+2	1881.0	1880.57	αS_2 f(5–18)4P
22.3	950.4	+2	1899.71± 0.09	1899.70	αS_1 f(40–55)2P
			IMAC Pool 3		
17.1	1286.2	+1	1286.22 ± 0.09	1286.29	βCN f(13-21)4P
19.6	1486.2	+1	1486.6	1486.41	βCN f(12–22)4P
20.0	1399.6	+1	1399.6	1399.38	βCN f(12–21)4P
20.4	758.3	+2	1515.28 ± 0.13	1515.45	βCN f(15–25)4P
21.5	1528.5	+1	1528.33 ± 0.08	1528.42	βCN f(11–21)4P
21.6	872.3	+2	1743.56 ± 0.12	1743.56	βCN f(13–25)4P
21.9	1481.5	+1	1481.5	1481.43	αS_1 f(64–74)4P
22.1	876.3	+2	1751.72 ± 0.03	1751.53	αS_2 f(6–18)4P
22.3	940.8	+2	1880.68 ± 0.06	1880.57	αS_2 f(5–18)4P
23.0	1167.3	+2	2332.75 ± 0.10	2332.77	αS_1 f(62–79)5P
23.2	1231.8	+2	2462.53 ± 0.06	2461.81	αS_1 f(61–79)5P
23.6	929.0	+2	1857.0	1856.64	βCN f(12–25)4P
24.6	1072.4	+2	2143.8	2143.73	αS_2 f(6–21)4P
24.6	1137.2	+2	2273.4	2272.77	αS_2 f(5–21)4P
24.8	1073.1	+2	2145.4	2144.68	αS_2 f(5–20)4P
26.0	1106.8	+2	2212.6	2211.86	βCN f(12–28)4P
27.0	1170.8	+2	2340.6	2340.91	βCN f(11–28)4P
27.2	1199.9	+2	2398.8	2397.97	βCN f(10–28)4P
28.3	1298.1	+2	2595.2	2594.05	βCN f(8–28)4P

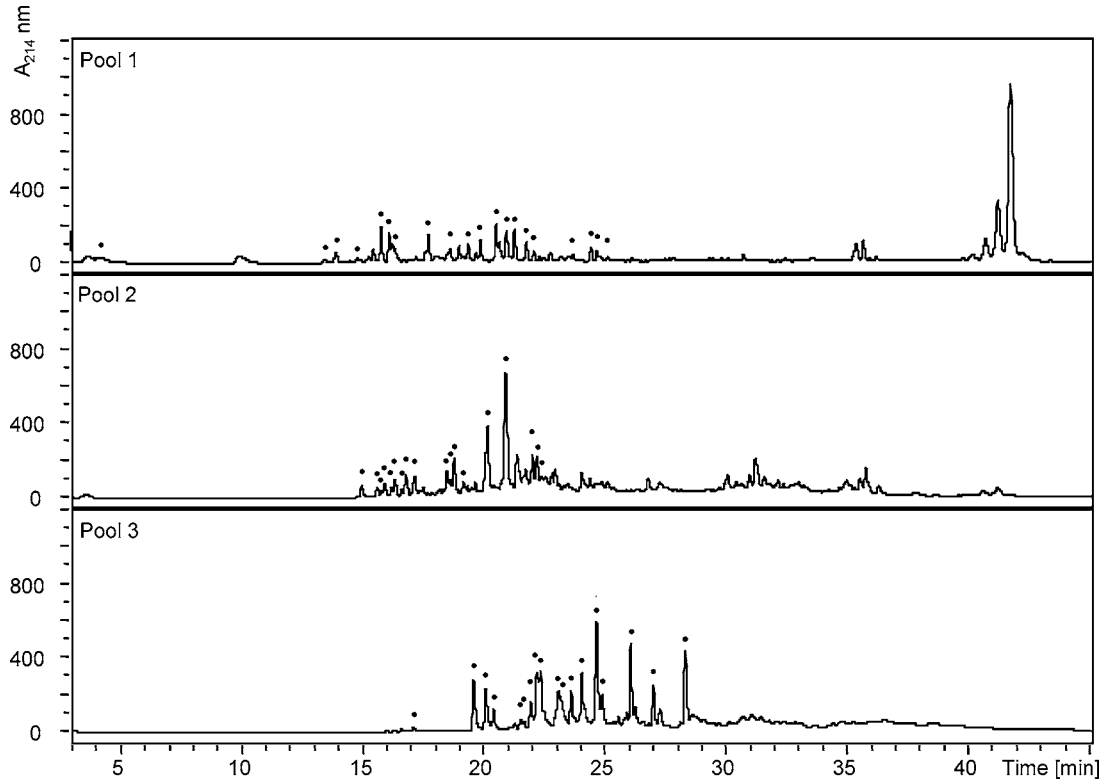

Figure 3. RP-HPLC chromatograms of pools 1–3 from the IMAC-Fe(III) separation of Herrgard cheese in **Figure 2**. Dots indicate the fractions in which peptides were identified as shown in **Table 1**.

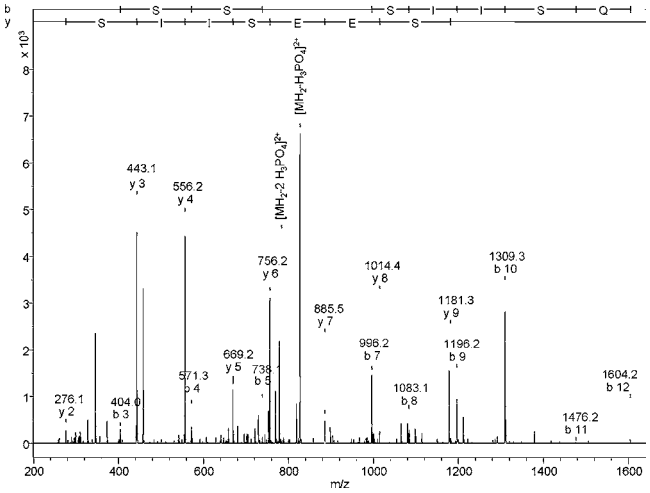

Figure 4. MS/MS spectrum of precursor ion 1751.7 from peak with t_R = 22.1 min in **Figure 3** (pool 3). This peptide was identified as the α_{S2} casein fragment f(6–18)4P.

DISCUSSION

Method Evaluation. Purification of phosphopeptides from aqueous cheese extracts was studied using immobilized metal affinity chromatography and RP-HPLC. It was found that using a system of IDA-Sepharose equilibrated with $FeCl_3$ resulted in a highly selective isolation of phosphopeptides from cheese extracts. Removal of nonphosphorylated peptides with high p*I* from the semihard cheese extract by binding them to a cation exchanger at pH 7.0 practically removed all of them with affinity for the Fe(III) column (**Figure 1**). Otherwise, basic peptides, especially from the N terminus of α_{s1}-casein, dominated the RP-HPLC chromatogram of the Herrgard cheese; this is in agreement with the fact that peptides with two or more histidines in close proximity, such as the N terminus of α_{s1}-casein and the C terminus of *p*-κ-casein, show high affinity for various polyvalent metals. Contrary to the extract of the Herrgard cheese, only a small fraction of the PR cheese extract was retained on the cation exchanger at pH 7.0, in accordance with earlier results of peptide profiling (*22*).

Table 2. Identified Peptides from the Studied Parmigiano Reggiano Cheese

RT UV (min)	parent ion *m*/*z*	*z*	obsd MH+	calcd MH+	sequence
3.4	482.6	+2	963.97 ± 0.07	964.20	αS_2 f(7–13)3P
7.6	978.1	+1	978.06 ± 0.06	978.21	βCN f(16–22)3P
9.8	891.1	+1	891.00 ± 0.11	891.18	αS_1 f(65–70)3P
17.9	461.2	+2	921.4	921.34	αS_1 (115–121)1P
19.5	517.7	+2	1034.4	1034.35	αS_1 (67–74)3P
21.1	741.1	+2	1481.2	1481.43	αS_1 (64–74)3P
21.1	657.7	+2	1314.4	1314.43	αS_1 (65–74)3P
21.3	634.2	+2	1267.32 ± 0.07	1267.55	αS_1 (115–124)1P
21.9	701.2	+2	1401.4	1401.62	αS_1 (64–74)2P

Analysis of P in the flow-through from the IMAC-Fe(III) column revealed that it contained no inorganic P and no phosphopeptides, and it could be concluded that these components were quantitatively retained on the column. In the eluate from the initial CIEC, a minor content of organic bound P was found, which accounted for <1% of the amount applied. This may come from phosphorylated basic peptides in the extract. However, the presence of basic phosphopeptides in cheese extract must be considered to be low due to the nature of O-phosphorylation of casein, which occurs on serine residues adjacent to aspartic acid or glutamic acid (cf. **Figure 5A**−**C**) (*24*). Because of the complex nature of cheese extracts, the IMAC-Fe(III) purification method presented is considered to be a capture step, because column affinity mainly is based on the number of phosphate groups. However, when a gradient of ammonium dihydrogen phosphate was applied to the column,

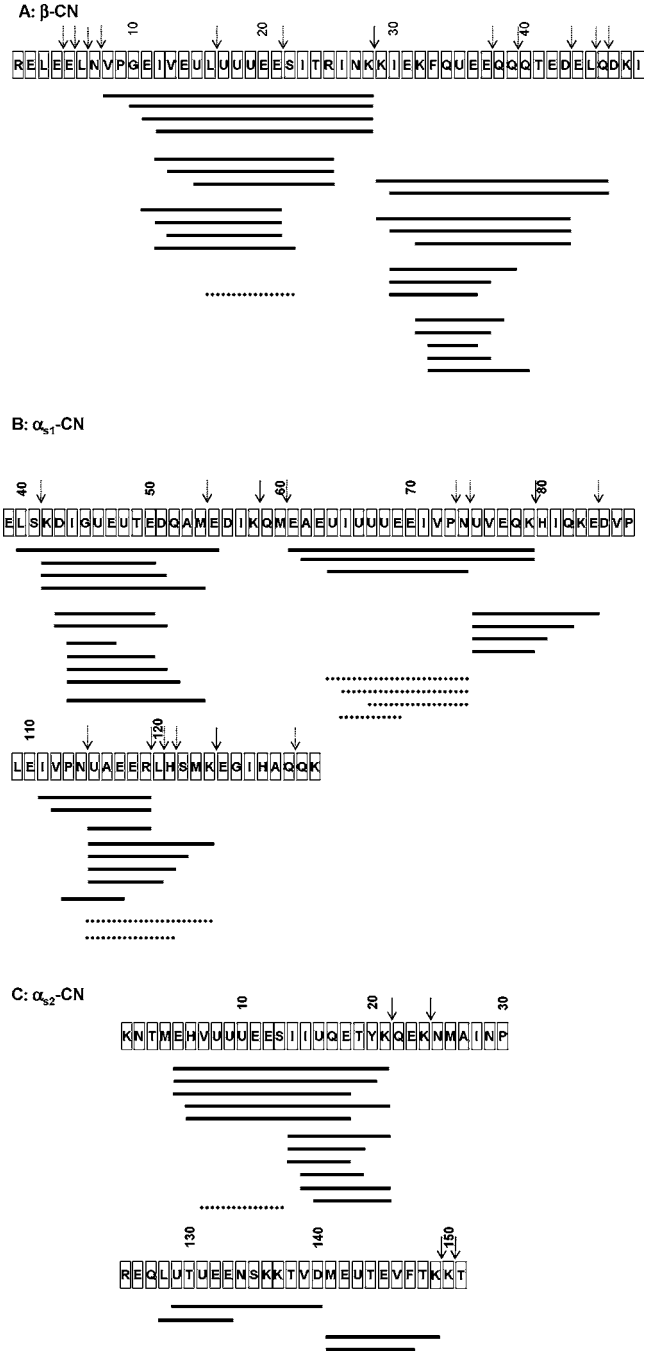

Figure 5. Identified peptides from β-casein (**A**), α_{s1}-casein (**B**), and α_{s2}-casein (**C**) released in aqueous extract of Herrgard cheese (solid line) and Parmigiano Reggiano cheese (dotted line). Single-letter codes used are standard, except for phosphoserine (U). Potential protease cleavage sites found under in vitro conditions adopted from ref *8* are designated by arrows: plasmin (solid arrow) and lactococcal cell envelope protease (dashed arrow).

it was possible to separate the extract in pools of peptides carrying one, two, and four phosphate groups, respectively. The correlation between the number of phosphate groups and affinity of peptides to the Fe(III) column is in good accordance with previous studies on the IMAC-Fe(III) separation of phosphoproteins with different phosphorylations (*25*). The following RP-HPLC allowed a fair separation of pools from the IMAC column into individual peptides; however, some of the peaks from the RP column were found to contain more than one peptide. Furthermore, overlapping of peaks made quantification of peptide content difficult. A better separation may be obtained using two-dimensional liquid chromatography, where cation exchange and reverse phase chromatography are combined (*26*). Compared to precipitation methods (*10*), an advantage of the studied method is that it allows separation of phosphopeptides on the basis of the degree of phosphorylation. By use of a larger column the method can easily be scaled up to allow preparative purification of peptides from semihard cheese with one, two, and four phosphate groups.

Herrgard Cheese. The peptides identified from the Herrgard cheese cover all phosphorylation sites of β-casein, α_{S1}-casein, and α_{S2}-casein, except for the phosphoserines S56, S57, S58, and S62 of α_{S2}-casein. Of the 65 different phosphopeptides found in the 10-month-old Herrgard cheese, α_{S1}-casein and β-casein contributed both with 40%, whereas 20% of the identified phosphopeptides originated from α_{S2}-casein. This is in accordance with the lower abundance of α_{S2}-casein in milk. The identified β-casein-derived peptides indicate the strong contribution of plasmin to the primary proteolysis of β-casein in Herrgard cheese from its cleavage at Lys_{28}-Lys_{29}. Plasmin may also be responsible for the generation of a number of phosphopeptides from its cleavage in α_{s1}-casein at Lys_{58}-Gln_{59} and Arg_{119}-Leu_{120} and in α_{S2}-casein at Lys_{21}-Gln_{22} and Lys_{149}-Lys_{150}. The results are in agreement with other studies on the proteolysis of other cheese varieties with plasmin activity (*4*, *27*, *28*). Several of the found peptides suggest activity of lactocepin (cell envelope protease of *Lactococcus*) on β-casein at Asn_7-Val_8 and Gln_{46}-Asp_{47} and on α_{S1}-casein at Glu_{84}-Asp_{85}. It remains uncertain what activity lactocepin has against α_{S2}-casein. A number of α_{s2}-casein-derived peptides starting with Glu_5 could indicate cleavage of lactocepin at Met_4-Glu_5 of α_{S2}-casein because this cleavage motif is found twice from activity of lactocepin against αs_1-casein (cf. **Figure 5B**). However, this pattern may also be the result of exo-peptidase activity in the N terminus of α_{S2}-casein. Despite a typically high chymosin activity in Herrgard, no primary cleavage stage of chymosin on casein was obviously involved in the generation of the identified phosphopeptides, but it may of course be responsible for the formation of larger peptides that are further cleaved by plasmin and lactocepin. One exception is the peptide f(30−93) from β-casein, which may originate from proteolysis by chymosin, or lactocepin; both may have activity at Met_{93}-Gly_{94} (*27*). Activity of various peptidases on peptides formed from hydrolysis of casein gives rise to the huge number of peptides that have been found in cheese (*28*). Among the different groups of related phosphopeptides found, the ones formed from the degradation of β-casein f(8−28) give the strongest indication of *N*-exopeptidase activity (cf. **Figure 5A**). Some groups of related peptides have the same N terminus, indicating the absence of *N*-peptidase activity. A common feature of these is a phosphoserine in one of the first positions of the N terminus, and this observation is in accordance with other studies on the resistance of peptides to hydrolysis upon serine phosphorylation (*29*). Several groups of related peptides have C termini differing by one or two amino acid residues. Whether this pattern is due to carboxypeptidase activity or random endopeptidase activity remains to be discovered.

Parmigiano Reggiano. The distribution of P and the phosphopeptide profile of the 24-month-old PR cheese differed highly from those of the 10-month-old Herrgard cheese. The contents of total P in extracts of the two cheeses were the same, but organic bound P in the extract of PR accounted for 20% only, compared to 84% for the Herrgard cheese. The lower content of organic bound P and the finding of only nine different phosphopeptides in the PR extract reflect a more extensive

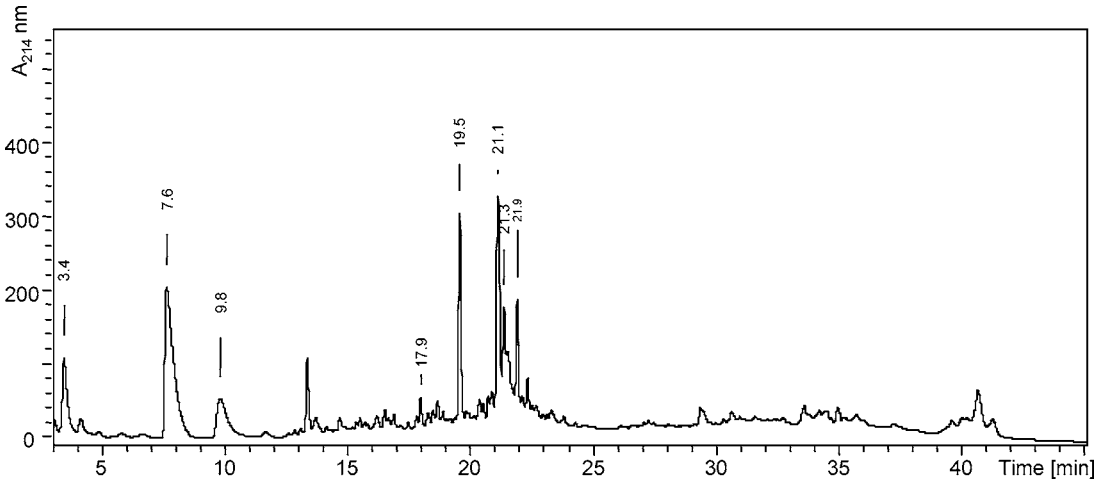

Figure 6. RP-HPLC chromatogram of the eluate of Parmigiano Reggiano extract separated by IMAC-Fe(III). Peptides in peaks marked with retention time were identified by MS/MS.

ripening and the characteristic microflora of this cheese. From the limited number of phosphopeptides found, it is difficult to reveal information about enzymatic cleavage. However, a major difference between the two cheeses besides the inactivation of rennet enzyme is the presence of thermophilic starter bacteria in PR cheese (*3*). Varying results are found in the literature on the phosphopeptide composition of PR and similar cheeses. In a 15-month-old PR cheese, Addeo et al. did not find any phosphopeptides with a mass >500 Da using a chelate column equilibrated with Cu(II) for retention of phosphopeptides and the combination of FAB-MS and amino acid sequencing for identification (*11*). In a study on the development of phosphopeptides in Grana Padano cheese during ripening, Ferranti et al. observed a decrease in the amount of phosphopeptides after 8−14 months, except for the accumulation of β-casein f(16−22)3P, which constituted 25% of all phosphopeptides after 38 months (*10*). The latter is in good agreement with the findings in the present study, where β-casein f(16−22)3P was the only β-casein-derived peptide found and also the dominating peptide peak of the chromatogram with a retention time of 7.6 min (**Figure 6**).

ABBREVIATIONS USED

CIEC, cation exchange chromatography; IDA, iminodiacetic acid; IMAC, immobilized metal affinity chromatography; LC-ESI, liquid chromatography−electronic spray identification; MS, mass spectroscopy; PR, Parmigiano Reggiano; RP, reversed phase.

LITERATURE CITED

(1) Jenkins, G. N.; Hargreaves, J. A. Effect of eating cheese on Ca and P concentrations of whole mouth saliva and plaque. *Caries Res.* **1989**, *23*, 159−164.

(2) Scholz-Ahrens, K. E.; Schrezenmeir, J. Effects of bioactive substances in milk on mineral and trace element metabolism with special reference to casein phosphopeptides. *Br. J. Nutr.* **2000**, *84* (Suppl. 1), S147−S153.

(3) Ardö, Y. Cheese ripening. General mechanisms and specific cheese varieties. *Bull. Int. Dairy Fed.* **2001**, *369*, 7−12.

(4) Gagnaire, V.; Molle, D.; Herrouin, M.; Leonil, J. Peptides identified during Emmental cheese ripening: origin and proteolytic systems involved. *J. Agric. Food Chem.* **2001**, *49*, 4402−4413.

(5) Sforza, S.; Ferroni, L.; Galaverna, G.; Dossena, A.; Marchelli, R. Extraction, semi-quantification, and fast on-line identification of oligopeptides in Grana Padano cheese by HPLC-MS. *J. Agric. Food Chem.* **2003**, *51*, 2130−2135.

(6) Sommerer, N.; Salles, C.; Prome, D.; Prome, J. C.; Le Quere, J. L. Isolation of oligopeptides from the water-soluble extract of goat cheese and their identification by mass spectrometry. *J. Agric. Food Chem.* **2001**, *49*, 402−408.

(7) Adamson, N. J.; Reynolds, E. C. Characterization of tryptic casein phosphopeptides prepared under industrially relevant conditions. *Biotechnol. Bioeng.* **1995**, *45*, 196−204.

(8) FitzGerald, R. Caseinophosphopeptides (CPPs) as functional ingredients. In *Functional Dairy Products*; Saarela, M., Mattila-Sandholm, T., Eds.; Culinary and Hospitality Industry Publications Services: Weimar, TX, 2003; pp 187−198.

(9) Lin, F. Y.; Chen, W. Y.; Huang, S. Y. Selective separation of caseinophosphopeptides through immobilized metal ion affinity chromatography: effects of pH values, salt concentrations and degree of phosphorylation. *Bioprocess Eng.* **2000**, *23*, 467−471.

(10) Ferranti, P.; Barone, F.; Chianese, L.; Addeo, F.; Scaloni, A.; Pellegrino, L.; Resmini, P. Phosphopeptides from Grana Padano cheese: nature, origin and changes during ripening. *J. Dairy Res.* **1997**, *64*, 601−615.

(11) Addeo, F.; Chianese, L.; Sacchi, R.; Musso, S. S.; Ferranti, P.; Malorni, A. Characterization of the oligopeptides of Parmigiano-Reggiano cheese soluble in 120 g trichloroacetic acid/l. *J. Dairy Res.* **1994**, *61*, 365−374.

(12) Hynek, R.; Kozak, A.; Drab, V.; Sajdoc, J.; Kas, J. Characterisation of casein phosphopeptides in cheese extracts using a combination of immobilized metal chelate affinity and reversed-phase high-performance liquid chromatography. *Adv. Food Sci.* **1999**, *21*, 192−196.

(13) Hart, S. R.; Waterfield, M. D.; Burlingame, A. L.; Cramer, R. Factors governing the solubilization of phosphopeptides retained on ferric NTA IMAC beads and their analysis by MALDI TOFMS. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 1042−1051.

(14) Larsen, M. R.; Sorensen, G. L.; Fey, S. J.; Larsen, P. M.; Roepstorff, P. Phospho-proteomics: evaluation of the use of enzymatic de-phosphorylation and differential mass spectrometric peptide mass mapping for site specific phosphorylation assignment in proteins separated by gel electrophoresis. *Proteomics* **2001**, *1*, 223−238.

(15) Stensballe, A.; Andersen, S.; Jensen, O. N. Characterization of phosphoproteins from electrophoretic gels by nanoscale Fe(III) affinity chromatography with off-line mass spectrometry analysis. *Proteomics* **2001**, *1*, 207−222.

(16) Pellegrino, L.; Battelli, G.; Resmini, P.; Ferranti, P.; Barone, F.; Addeo, F. Effects of heat load gradient occurring in moulding on characterization and ripening of Grana Padano. *Lait* **1997**, *77*, 217−228.

(17) Roudot-Algaron, F.; Lebars, D.; Kerhoas, L.; Einhorn, J.; Gripon, J. C. Phosphopeptides from Comte Cheese−nature and origin. *J. Food Sci.* **1994**, *59*, 544−547.

(18) Antonsson, M.; Molin, G.; Ardo, Y. Proteolysis of the semi-hard cheese Herrgard made at different dairies. Exploratory study. *Milchwissenschaft* **2003**, *58*, 145−148.
(19) Bütikofer, U.; Rüegg, M.; Ardö, Y. Determination of nitrogen fractions in cheese: evaluation of a collaborative study. *Lebensm. Wiss. -Technol.* **1993**, *26*, 271−275.
(20) Fiske, C. H.; Subbarow, Y. The colorimetric determination of phosphorous. *J. Biol. Chem.* **1925**, *66*, 375−400.
(21) Bartlett, G. R. Phosphorus assay in column chromatography. *J. Biol. Chem.* **1959**, *234*, 459−465.
(22) Ardo, Y.; Kristiansen, K. R. Analysis/chromatographic methods. In *Encyclopedia of Dairy Sciences*; Roginski, H., Fuquay, H., Fox, P. F., Eds.; Academic Press: Amsterdam, The Netherlands, 2002; pp 55−62.
(23) Tähtinen, H. M.; Kristiansen, K. R.; Otte, J.; Zakora, M.; Ardö, Y. Identification of characteristic accumulation of large phosphopeptides from beta-casein in semi-hard, long-time/medium-temperature cooked cheese (Herrgard). Unpublished work.
(24) Fox, P. F.; McSweeney, P. L. H. Milk proteins. In *Dairy Chemistry and Biochemistry*; Blackie Academic and Professional: London, U.K., 1998; pp 146−238.
(25) Andersson, L.; Porath, J. Isolation of phosphoproteins by immobilized methal (Fe3+) affinity chromatography. *Anal. Biochem.* **1986**, *154*, 250−254.
(26) Romijn, E. P.; Krijgsveld, J.; Heck, A. J. Recent liquid chromatographic-(tandem) mass spectrometric applications in proteomics. *J. Chromatogr. A* **2003**, *1000*, 589−608.
(27) Boutrou, R.; Mollé, D.; Leonil, J. Action of the lactococcal proteinase during Camembert-type curd making. *Int. Dairy J.* **2001**, *11*, 347−354.
(28) Sousa, M. J.; Ardo, Y.; McSweeney, P. L. H. Advances in the study of proteolysis during cheese ripening. *Int. Dairy J.* **2001**, *11*, 327−345.
(29) Hynek, R.; Zuzalkova, L.; Sikac, Z.; Kas, J. Influence of phosphorylation on proteolytic cleavage. *J. Liq. Chromatogr. Relat. Technol.* **2002**, *25*, 2307−2314.

Received for review March 3, 2004. Revised manuscript received July 6, 2004. Accepted July 29, 2004.

JF0401064