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Immobilized Fe^{3+} affinity chromatographic isolation of phosphopeptides

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ABSTRACT

Immobilized Fe^{3+} affinity chromatography is suggested as a means of concentrating phosphopeptides that are present in too low a proportion in a complex mixture to be purified by a single-step method. A high-performance liquid chromatographic system and a chelating Superose HR 10/2 column were used. The chromatographic conditions were optimized using a tryptic hydrolysate of whole casein. The unbound fractions did not contain any phosphorylated peptide. All caseinophosphopeptides were retained. Only four other strongly basic peptides were also retained. The quantitative accuracy of the method was evaluated. This method allowed the isolation of phosphopeptides in gastric effluents of calves fed with milk.

INTRODUCTION

Casein phosphopeptides (CPPs) display the interesting property of being able to form soluble complexes with di- or trivalent metal ions [1,2]. This has led to speculation that CPPs might have a physiological role and could enhance calcium intestinal solubilization and absorption. Naito and co-workers [3,4] reported the occurrence of such phosphopeptides in the distal small intestine of rats during the course of the luminal digestion of a diet containing caseins. Moreover, the amount of soluble calcium in the distal small intestine was higher with caseins than with other dietary proteins [5]. In 1986, Sato *et al.* [6] demonstrated that CPPs enhanced both Ca^{2+} intraluminal solubilization and absorption. Gerber and Jost [7] reported an increase in the calcium bioavailability with CPPs with *in vitro* cultured embryonic rat bone. In contrast, Pointillart and Gueguen [8] demonstrated *in vivo* that calcium absorption and bone parameters of pigs fed CPP-enriched diets were not modified. This Ca^{2+} solubilizing effect of CPPs on its absorption is still much debated. Moreover, the way in which they are introduced to the intestine could also be a determining factor. Hence it would be interesting to know how and when they are released from the stomach after a meal.

The identification of such peptides in the digestive tract is relatively difficult because they are mixed with a number of other peptides and consequently are found in too low a proportion to be purified by a single-step method. Meisel and Frister [9] enriched the CPP fraction of intestinal chyme by affinity chromatography using iron(III) ions immobilized on a chelating Sepharose 6B (Pharmacia–LKB, Uppsala,

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Sweden) column according to the method described by Andersson and Porath [10]. This allowed them to show the occurrence of a phosphopeptide in the chyme from the small intestine of minipigs (Göttinger Miniaturschweine) after ingestion of a casein diet. However, their chromatographic method is time consuming.

In order to study the kinetics of gastric emptying of CPPs in calves fed milk, we developed a faster immobilized metal affinity chromatographic (IMAC) method to concentrate phosphopeptides using a fast protein liquid chromatographic (FPLC) system and a chelating Superose HR 10/2 column (Pharmacia–LKB). The separation of each phosphopeptide was performed in a second step by high-performance liquid chromatography (HPLC) on a C_{18} reversed-phase column. In order to adjust the chromatographic conditions we used a tryptic hydrolysate of bovine whole casein.

EXPERIMENTAL

Chemicals

All buffers were prepared with deionized water purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Sodium acetate, sodium dihydrogenphosphate and iron(III) chloride were purchased from Prolabo (Paris, France). Trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL, U.S.A.) and acetonitrile from Baker (Deventer, The Netherlands). All mobile phases were deaerated with helium.

Preparation of samples

The chromatographic conditions were adjusted using a casein tryptic hydrolysate. This hydrolysate was obtained by incubating a 1% casein solution in phosphate buffer (0.1 *M*, pH 7.4) with trypsin (Sigma, St. Louis, MO, U.S.A.) for 24 h at 37°C. The enzyme/substrate (E/S) ratio was 1:100 by weight. The reaction was stopped by adding HCl to a final pH of 2.0.

Gastric effluents of preruminant calves fed bovine raw skim milk were collected over 7 h after the meal, fractions being taken during 10-min intervals for the first 30 min and then at 30-min intervals. To each sample trichloroacetic acid was immediately added to a final concentration of 12% to stop the enzyme action. After centrifugation, the pellet was resuspended in water and solubilized by increasing the pH to 7.0. An aliquot of this solution was brought to pH 5.0 and centrifuged. The supernatant (100 μl) was applied to the affinity column.

Preparative chromatography

The preparative step of phosphopeptide recovery was carried out with an FPLC system consisting of an LCC 500 controller, two pumps and a chelating Superose HR 10/2 column (2 × 1 cm I.D.). The absorbance was monitored at 220 nm. The chelating ligand of the column is iminodiacetate covalently bound to a Superose 12 matrix. Iron(III) ions were immobilized on this support to give a group-specific sorbent for phosphorylated amino acid side-chains. Iron(III) ions were loaded by applying a 20-m*M* iron(III) chloride solution in water at a flow-rate of 1.0 ml/min until the metal appeared in the eluate. After washing off the unbound metal with water, the column was equilibrated with a loading buffer.

The chelating Superose column was run at 37°C. It was first equilibrated with 10 ml of 20 m*M* sodium acetate buffer (A) adjusted to pH 5.0 at 1.0 ml/min. The tryptic

hydrolysate was then applied and the column was washed with 10 ml of buffer A. The elution was carried out with 10 ml of 100 m*M* sodium dihydrogenphosphate in A at the same flow-rate for 10 min. The column was then re-equilibrated with buffer A for further use.

Analytical chromatography

Analytical chromatography of the chelating chromatographic fractions was carried out with a Waters Assoc. HPLC system on a 10-μm Nucleosil C_{18} column (250 × 4.6 mm I.D.) (SFCC, Neuilly-Plaisance, France). The absorbance was monitored at 220 nm. The column was equilibrated in solvent A (0.115% TFA) and elution was effected with a linear gradient from 0 to 100% solvent B (0.1% TFA–60% acetonitrile). The column and solvents were kept at 40°C. The elution rate was 1.0 ml/min.

Peptide identification

The HPLC fractions were collected and dried with a Speed-Vac evaporator (Savant, France). The peptides were identified from their amino acid composition. These were determined after acid hydrolysis (110°C, 24 h, 5.7 *M* HCl, under vacuum) using the method of Spackman *et al.* [11] with a Biotronik LC 5000 analyser.

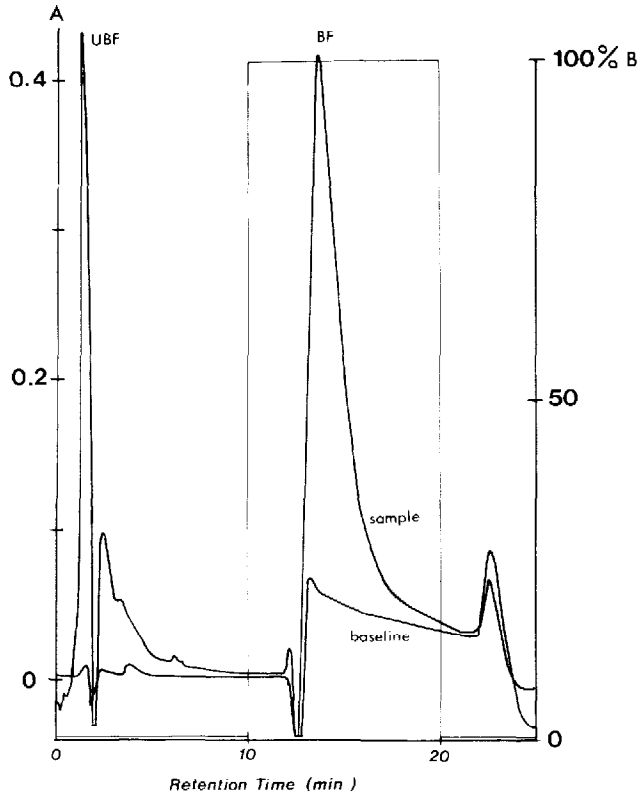

Fig. 1. Preparative affinity chromatography of a tryptic digest of whole casein on Fe^{3+}–chelating Superose HR 10/2. Temperature, 37°C; flow-rate, 1.0 ml/min; detection, UV (220 nm). Eluents: A = 20 m*M* sodium acetate (pH 5.0); B = A + 100 m*M* sodium dihydrogenphosphate.

RESULTS

Fig. 1 shows the IMAC pattern of the tryptic hydrolysate of whole casein. The first peak corresponds to the unbound material and the second peak to the retained material.

The whole casein tryptic hydrolysate (CTH) and the unbound (UBF) and bound (BF) fractions were analysed on the reversed-phase column under exactly the same conditions. The elution patterns are superimposed in Fig. 2. The UBF did not contain any phosphorylated peptide. The peptides of the BF were collected (Fig. 3) and identified by their amino acid composition (Table I). Nine peptides containing phosphoserine residues were found, of which seven could be identified. The amino acid composition of the other two (indicated with asterisks in Fig. 3) did not permit their identification. Peaks 1–4 corresponded to non-phosphorylated small peptides (four or five residues) which contained one His residue.

The quantitative response of the method was tested in each step. In the analytical step (reversed-phase HPLC), the relationship between peak area and amount of phosphopeptides injected was linear up to 10 nmol. In the affinity step the peak area of the BF was also directly proportional to the amount injected up to 1.75 mg of

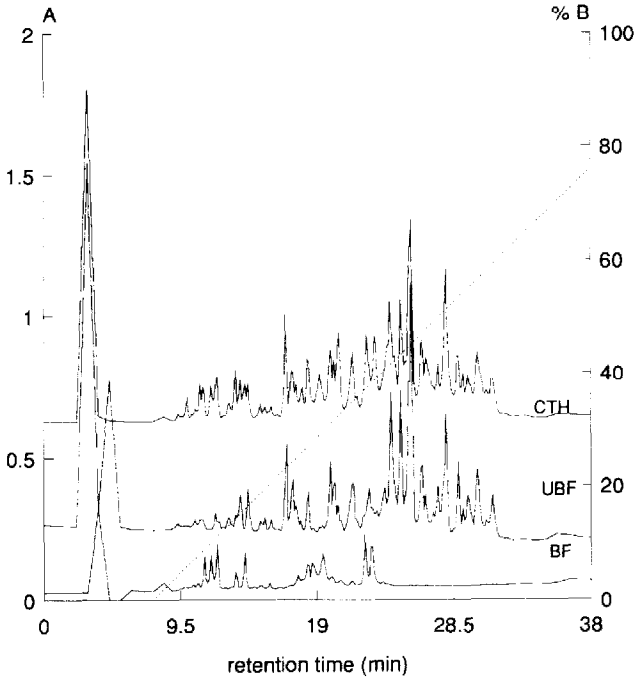

Fig. 2. Superimposition of the chromatograms of the tryptic digest (CTH), the bound fraction (BF) and the unbound fraction (UBF) on an Fe^{3+} affinity column. Column, C_{18} Nucleosil (10 μm); temperature, 40°C; flow-rate, 1.0 ml/min; detection, UV (220 nm). Eluents: A = 0.115% TFA; B = 0.1% TFA–60% acetonitrile. The dotted line indicates the %B gradient.

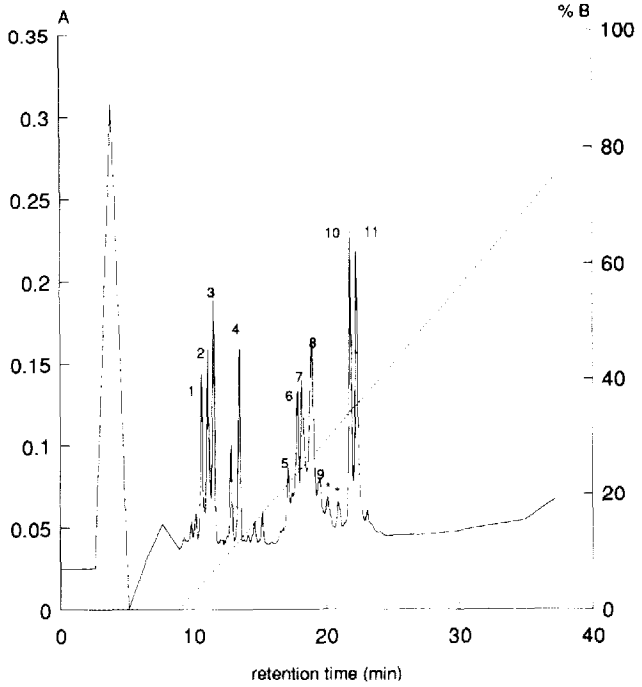

Fig. 3. Analytical RP chromatography of the BF. Conditions as in Fig. 2. For amino acid composition of the peaks 1–11 see Table I.

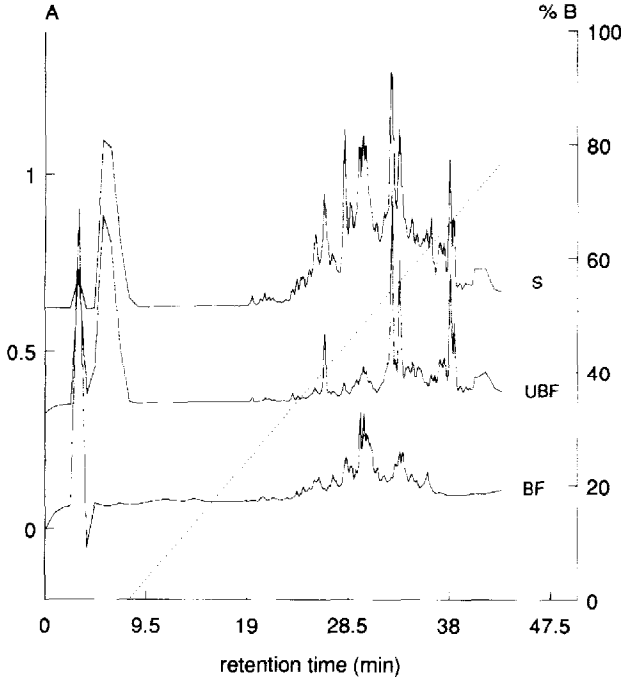

Fig. 4. Superimposition of the chromatograms of a gastric effluent (S), the bound fraction (BF) and the unbound fraction (UBF) on an Fe^{3+} affinity column. The amounts injected for the three chromatograms were different. Conditions as in Fig. 2 except for the gradient. The dotted line indicates the %B gradient.

TABLE I

AMINO ACID COMPOSITION (in mol/mol) OF THE PEPTIDES NUMBERED IN FIG. 3

The theoretical composition of the identified peptide is given in parentheses. Ide. = Identification; Phos. = phosphate.

Amino acid	Peak No.										
	1	2	3	4	5	6	7	8	9	19	11
Asx					1.54(2)	1.78(2)	1.23(1)	2.40(3)	3.40(4)	1.40(1)	1.15(1)
Thr	–				0.75(1)	0.80(1)		1.05(1)	0.92(1)	0.21(0)	0.91(1)
Ser				0.87(1)	1.20(1)	3.40(5)	4.10(5)	2.20(2)	2.60(3)	1.14(1)	4.91(5)
Glx	0.85(1)	1.06(1)			9.50(9)	7.89(8)	6.76(7)	4.80(4)	7.34(5)	4.20(4)	6.68(7)
Pro			0.95(1)				0.91(1)			2.02(2)	1.09(1)
Gly	0.30(0)					1.82(1)		0.63(1)	0.74(1)	–	1.26(1)
Ala			–		0.38(0)	2.21(3)	0.91(1)	0.80(1)	1.04(1)	1.00(1)	–
Cys	–				–	–		–	–	–	
Val			–			1.43(2)	0.87(2)	0.40(0)	1.12(1)	1.30(2)	1.33(2)
Met	–	–		0.56(1)	–		0.87(1)	0.75(1)	1.02(1)	–	
Ile		0.82(1)	0.85(1)			0.94(1)	1.32(2)	1.70(2)	1.60(2)	0.66(1)	1.31(2)
Leu				0.84(1)	0.68(1)	–	–	–	0.78(1)	1.09(1)	2.70(2)
Tyr	0.54(1)	–	–			0.48(1)	–	–		0.69(1)	–
Phe		–	–		0.56(1)	–	–	–		–	
His	0.95(1)	1.02(1)	1.04(1)	0.90(1)	–	–	–	–	–	–	
Lys	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.15(1)	–
Arg	–	–	–	–	–				–	0.88(1)	1.71(2)
Ide.	77–80 α_{s_2}	80–84 α_{s_1}	4–7 α_{s_1}	120–124 α_{s_1}	33–48 β	46–70 α_{s_2}	59–79 α_{s_1}	43–58 α_{s_1}	37–58 α_{s_1}	104–119 α_{s_1}	1–25 β
Phos.	0	0	0	0	1	3	5	2	2	1	4

phosphopeptide. However, during this step the recovery was not the same for all the tested phosphopeptides, *i.e.*, 65% for peptides 6, 7 and 8 and 80% for peptide 11.

This method was then applied to gastric effluents of calves fed bovine raw skim milk. The three chromatograms of the sample (S), the BF and UBF are superimposed in Fig. 4. A group of three peptides was particularly retained on the chelating Superose column. They were eluted at a retention time of 30 min on the C_{18} column. The amino acid compositions of these peptides are similar and correspond approximately to the sequence 110–145 from α_{s_1}-casein. Their N- and C-terminal residues have to be cidentified in order to establish the exact sequence of these peptides.

DISCUSSION

This study demonstrated that a chelating Superose gel loaded with Fe^{3+} can be employed successfully to concentrate phosphopeptides from a mixture in which they are present in too low a proportion to be purified by a single-step method. This technique has the advantage of being faster than that used by Meisel and Frister [9], only 30 min, including the equilibration time, being required. Only one run on each of the two chromatographic systems is necessary to collect enough peptide for identification. The column can be used immediately after a run since regeneration is not necessary. No modification of retention was observed after repeated separations.

Chromatographic conditions were chosen according to the conditions described previously [10]. Phosphoserine was strongly retained at pH 3.1 or 5.0 and recovered from the Fe^{3+} column with 20 m*M* phosphate. The loading was therefore performed at pH 5.0. Some peptides were not desorbed by 50 m*M* sodium dihydrogenphosphate in sodium acetate (pH 5.0). The phosphate concentration was too low and 100 m*M* phosphate was necessary for complete desorption. A gradient of phosphate concentration gave a poor separation. Desorption with EDTA was possible but the column had to be reloaded with iron(III) ions between each run.

All peptides were eluted independently of their phosphate content and their strength of binding on the column and the analytical step in the reversed-phase mode allows their separation. Eleven phosphopeptides were theoretically present in the tryptic hydrolysate. Seven phosphopeptides were identified: all phosphopeptides from α_{s_1}- and β-caseins were found and one phosphopeptide from α_{s_2}-casein was also identified. Two others phosphopeptides were found but have not been completely identified. One of them was probably the phosphopeptide NH_2-terminus of α_{s_2}-casein, but it was not pure. Two small phosphopeptides (twelve and thirteen residues) were not found, probably because the proportion in the mixture was too small and their absorbance at 220 nm too low.

The observed recoveries vary with the phosphopeptides. The phosphate content of the peptides might be responsible for these differences, as Muszynska *et al.* [12] showed with IDA–Fe^{3+} gel that the strength of binding was dependent on the phosphate content: peptide 11 (80% recovery) contains four phosphate groups whereas peptide 7 contains five phosphate groups and was obtained with 65% recovery. The recoveries for peptides 6 and 8, containing three and two phosphate groups, respectively, were the same as for peptide 7. Because of these differences in recovery, an internal standard of each peptide to be analysed must be used in quantitative assays.

The IMAC step provides a good enrichment of the phosphopeptide fraction. Phosphopeptides represented 12% by number of the peptides present in the casein tryptic hydrolysate. In the enriched fraction, they were predominant. Moreover, they were eluted later than the non-phosphorylated peptides in HPLC. This made their purification easier.

The non-phosphorylated peptides retained on the Chelating Superose column were small peptides containing one His and one Lys residue. Some tests were performed by adding 10 or 20 m*M* imidazole (Kodak, Rochester, NY, U.S.A.) to the loading buffer (the pH being readjusted to 5.0 with acetic acid). No change was observed and His-containing peptides and phosphopeptides were always retained on the chelating Superose gel. This result shows that binding is not due to coordination via histidine. Other tests were also made by adding 100 m*M* NaCl sodium chloride to the loading buffer. His-containing peptides were not retained on the chelating Superose but the retention of some phosphopeptides slightly decreased. These observations could be consistent with electrostatic interactions. His- and Lys-containing peptides (strongly basic) could interact with the iminodiacetate groups of the chelating Superose. Binding of phosphopeptides probably occurs via the oxygen of the phosphate group by coordination with Fe^{3+}. Secondary, electrostatic interactions can also occur and explain the different recoveries of the phosphopeptides.

The proposed method could be optimized by coupling the two columns via a six-way valve as described by Rybacek *et al.* [13]. Some tests were made using the chelating Superose column and a pepRPC column (Pharmacia-LKB), but the major problem was the different pressure resistances of the two gels and consequently the columns cannot be used in series.

The method described is suitable for concentrating phosphopeptides from complex mixtures and for identifying them in gastric effluents of animals. It is therefore now possible to study the kinetics of the gastric emptying of phosphopeptides.

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