Unusual Behavior of Natural Polyphosphates during IMAC

MILENA KMÍNKOVÁ, ALEXANDRA PROŠKOVÁ and JIŘÍ KUČERA

Food Research Institute Prague, Prague, Czech Republic

Abstract

KMÍNKOVÁ M., PROŠKOVÁ A., KUČERA J. (2001): **Unusual behavior of natural polyphosphates during IMAC**. Czech J. Food Sci., **19**: 161–165.

The atypical course of IDA-ferric (Imino-Di-Acetyl-agarose saturated with ferric ions) of two polyphosphorylated compounds – phytic acid and Ser(P)-Ser(P)-Ser(P)-Glu-Glu cluster obtained by the way of pancreatic hydrolysis of bovine casein was detected. The spectral data (for phytic acid) and iron content (for polyphosphate cluster) indicate the strong bond of ferric ions to these polyphosphorylated compounds. The data shows these compounds to bind ferric ion stronger even in comparison with IDA (Imino-Di-Acetate) itself. Therefore attention is to be paid to all IMAC (Immobilized-Metal-Ion-Affinity-Chromatography) of natural compounds containing strong chelating groups or several chelating groups in vicinity.

Keywords: phytic acid; Ser(P)-Ser(P)-Ser(P)-Glu-Glu cluster; affinity chromatography; immobilized metal ion; IMAC; IDA-ferric; atypical adsorption

Anticariogenic phosphopeptides, released during the enzymatic hydrolysis of casein by trypsin and pancreatin contain the cluster sequence Ser(P)-Ser(P)-Ser(P)-Glu-Glu which has a commercial potential as toothpaste, mouthwash, and food additives for the prevention of dental caries. The method was developed for the separation of these peptides, using selective precipitation with Ca^{2+} and ethanol from an acid-clarified (pH 4.6) pancreatic casein hydrolyzate (ADAMSON et *al.* 1995). Solubility of calcium salts of these peptides in ethanol/water mixture is nevertheless relatively high, leading to the lower yield in this way. A better way of the separation of phosphopeptides seems to be therefore the utilization of specific chelate-forming effect of Immobilized Metal Ion Affinity Chromatography (IMAC). These sorbents are saturated with ferric ion and the phospho-groups of all phosphate containing compounds are bound to it (ANDERSSON & PORATH 1986; MUSZYNSKA *et al.* 1986, 1992). Such immobilized-metal-ion chromatography is the simplest way of the separation of all the molecules interacting with metal ions to form stable chelates.

Phytic acid, hexaphosphorylated inositol, is also a very important component of human nutrition. This polyphosphate forms complexes with a lot of metal ions influencing their utilization in the human body. It is beneficial in the case of heavy metal (Cd, Hg, etc.) intoxication, but it decreases the utilization of such positively acting metal ions as ferric, calcium etc. The avoidance of phytic acid from the food of plant origin may be advantageous in some diets, and its separation and application may be profitable in the case of heavy-metal intoxication.

Immobilized ferric ion sorbents have shown a selective interaction with phosphate group-containing natural compounds. A strong dependence of the course of adsorption on the character of separated molecules was demonstrated. The strength of interaction between a ferric ion and immobilized ligand is controlled by i) pure electrostatic interactions controlled by charge and ionic radius; ii) overall affinity of the central metal ion to accept electrons from the ligand; and iii) metal ion electric configuration which is capable of stabilization of such chelate due to distortion of octahedral symmetry. Designing of ferric-IMAC adsorption depends on the following factors: i) net charge of the metal-ligand complex, ii) ionic strength of the buffer, iii) presence of surface accessible protein (or other natural compound) phosphate groups, iiii) isoelectric point of the protein or dissociation constant of other phosphate-containing compounds (MUSZYNSKA *et al.* 1986, 1992).

The presence of a higher number of phosphate groups in the neighborhood is rare in nature. The phosphate

Supported by the National Agency for Agricultural Research (NAZV), Grants No. EP6255 and RE5517.

groups present in the same molecule but at greater distances are more frequent. They do not usually influence each other in such cases. The behavior of highly phosphorylated compounds having the phosphate group in the vicinity has not been described yet. In the present work, therefore, we have studied the way of adsorption of these two polyphosphorylated compounds on imino-diacetyl-agarose (IDA agarose) saturated with ferric ions with the aim to determine how the cooperating phosphate group of separated compounds will affect the course of chromatography.

MATERIAL AND METHODS

Phytic acid and pancreatin were products of Sigma Fine Chemicals. Casein was the product of MILCOM service, Czech Republic.

The sorbent IDA-HEMA (Iminodiacetyl – Hydroxyethylmetacrylate) and Separon SGX C18 (4 × 250 mm, particle size 10 μm) for reverse phase HPLC were products of TESSEK, Prague. The sorbent IDA-agarose (Iminodiacetyl on cross-linked 4% bedded agarose) was a product of Pharmacia.

Ferric phytate was prepared by conductometric titration of phytate solution with ferric chloride solution.

All other chemicals were products of SIGMA Fine Chemicals, reagent grade quality.

Phytic acis was used as a standard for IMAC-ferric chromatography.

Pancreatic casein hydrolyzate for the separation of polyphosphorylated cluster Ser(P)-Ser(P)-Ser(P)-Glu-Glu was prepared according to ADAMSON *et al*. (1995) and crude hydrolyzate was used for chromatography.

Immobilized Metal Ion Affinity Chromatography (IMAC) of phosphorylated casein cluster and phytic acid was carried out principally according to the method of ANDERSSON *et al*. (1986).

Pharmacia FPLC model LCC-500 was used throughout the work.

Philips UV/VIS scanning spectrophotometer model 8730 was used for photometric measurements.

Detection of phytic acid in the effluent from reverse phase HPLC column was carried out by continuous measurement of UV absorption at 254 nm, phosphopeptides at 214 mn, respectively.

The column of total volume 1.8 ml containing IDA-HEMA saturated with ferric ion was used for IMAC of standard samples.

Saturation of Sorbent with Ferric Ions: The sorbent was saturated with ferric ions by the following method: The column of total volume 1.8 ml (2.6 ml) was washed with distilled water followed by an application of 1 ml (2 ml) of 50mM solution of ferric chloride. After the application of ferric chloride the column was washed with distilled water again and then equilibrated with appropriate buffer.

Chromatography of Phytic Acid: 100 μl of the sample (0.8 mg of phytic acid in 100mM acetate pH 3.5 containing 1M NaCl) was applied to the column of IDA-HEMA (total volume 1.8 ml) saturated with Fe^{3+} ions and equilibrated with the same buffer. Unadsorbed material was washed out with the same buffer and phytic acid was then eluted with 0.02M phosphate pH 7.5.

Preparation of Casein Hydrolyzate: Casein (10 g per 100 ml water) was hydrolyzed with 2% of pancreatin calculated per casein dry matter at pH 8.5, 50°C for 2 hours under shaking. The pH value was kept at 8.5 in the course of the reaction. The hydrolyzate was acidified to pH 4.6 using 1M hydrochloric acid, and the precipitate (unhydrolyzed casein) was filtered off (20 min, 12000 g), and pH of the supernatant was adjusted to 8.0 using 1M sodium hydroxide. Ethanol was added to final concentration of 50% (v/v) and solid $CaCl_2$ was added to final concentration of 1%. The precipitate was centrifuged for 20 min, 12 000 g and dissolved in starting buffer.

Chromatography of Casein Hydrolyzate: IDA-ferric affinity chromatography was carried out on the column of IDA-agarose. 100 μl of the sample was applied to the column (total volume 2.6 ml) saturated with Fe^{3+} ion which was equilibrated with 100 mM acetate buffer pH 5.0 containing 0.5M NaCl. The unadsorbed fraction was eluted with the starting buffer and adsorbed peptides were eluted with 100mM Tris-HCl pH 7.5.

Chromatography on Reverse Phase HPLC: The reverse phase (RP) HPLC was carried out as follows: The column (Separon SGX C18) was equilibrated with the starting solvent A – 0.1% (v/v) trifluoroacetic acid in water and the phosphopeptide was injected into the column. The elution was effectuated by stepwise gradient elution with the solvent B – 80% acetonitrile in starting solvent. The steps were: i) 7% solvent B, ii) 14% solvent B and iii) 36% solvent B in solvent A, respectively (ADAMSON *et al*. 1995).

The iron concentration in fraction was determined by atomic absorption spectra.

RESULTS AND DISCUSSION

The properties of eluted phytic acid are changed in comparison with the sample injected. The chromatography of standard phytic acid is shown in Fig 1.

The spectral curve in the range of 250 to 330 nm was determined for each product and the same spectrum of ferric phytate was measured for comparison. A small difference was found between the injected and eluted compound. The eluted compound was identified according this spectral comparison as ferric phytate. The differential curves for the product eluted and both the phytic acid injected and the ferric phytate are shown in Fig. 2. The identity of the product eluted from the IDA-ferric column and ferric phytate is seen in this figure.

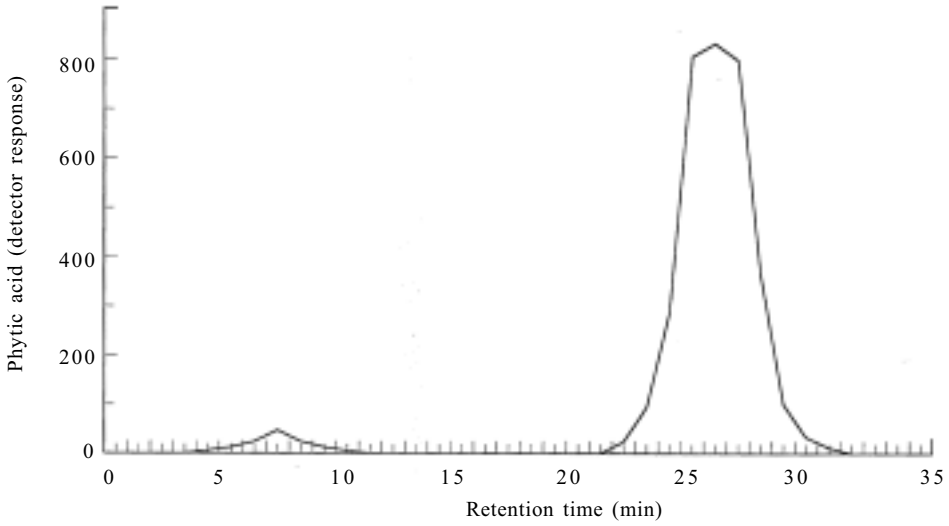

Fig. 1. Typical course of phytic acid-standard sample in IDA-ferric chromatography

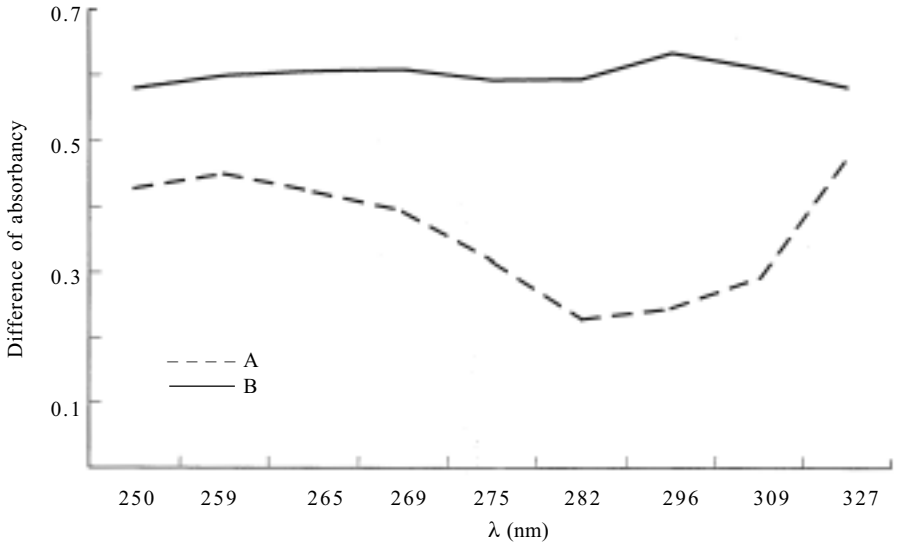

Fig. 2. Differential spectra of phytic acid and eluted fraction (A), and ferric phytate and eluted fraction (B)

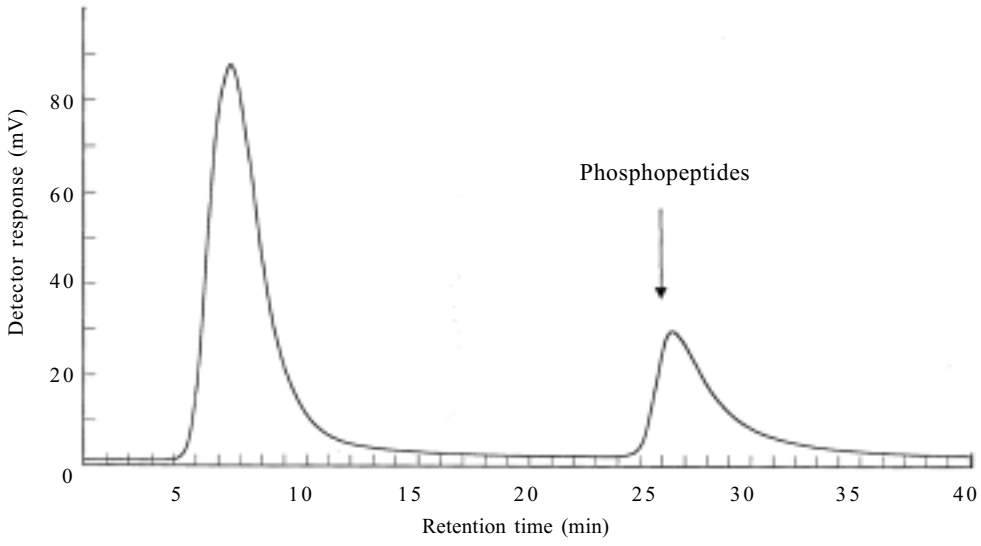

Fig. 3. Immobilized metal ion affinity chromatography (IMAC) of pancreatic casein hydrolyzate on IDA-ferric

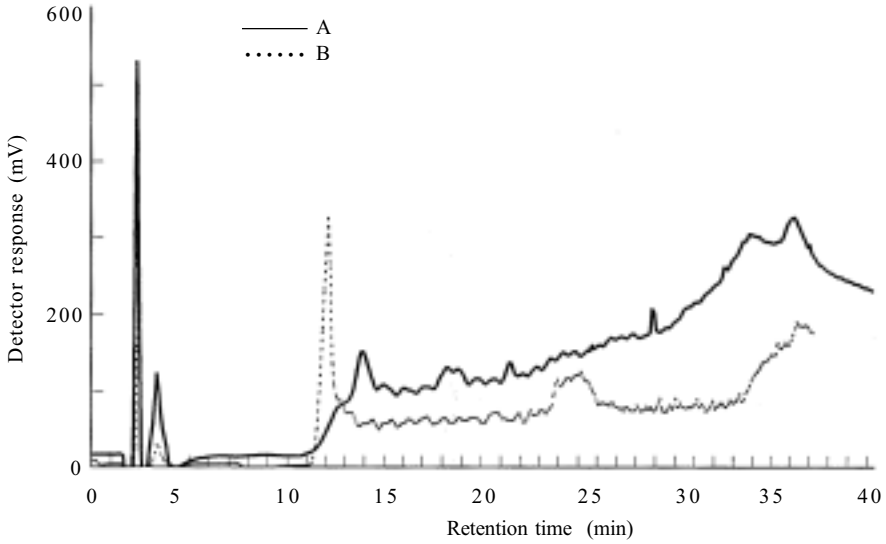

Fig. 4. Reverse phase HPLC of the pancreatic hydrolyzate of casein. Curve A – phosphopeptides separated by IMAC on IDA-ferric column without calcium chloride precipitation. Curve B – the same after calcium chloride precipitation

Typical course of IDA-ferric chromatography is shown in Fig. 3.

The phosphopeptides separated by IDA-ferric chromatography and those precipitated first with calcium chloride and then further purified by the chromatography on IDA-ferric were compared by means of reverse phase HPLC. The elution curve of both samples differs remarkably. The typical elution profile of RP HPLC is shown in Fig. 4 for both samples. The differences seen in Fig. 4 were believed to be due to the fact that the phosphopeptides saturated with ferric ion are eluted from IDA-ferric column under elution conditions in case the calcium-chloride precipitation does not take place. When the sample after calcium-chloride precipitation was applied, the phosphate groups were transformed to their calcium salt and the interaction with ferric ion was looser. This fact was verified by the determination of the iron content of eluted material. The iron concentration in fraction was determined by atomic absorption spectra to be 0.1674 μg Fe per ml of eluate. As the eluate outside of the phosphopeptide peak has zero concentration of iron, the iron present in the phosphopeptide fraction is believed to be the ferric ions bound to phosphate groups.

As a result of the above experiments the vicinal polyphosphates are supposed to compete for ferric ions with the IDA groups under elution conditions. This ability is believed to be due to stronger interactions of a set of vicinal phosphate groups with ferric ions compared with the IDA itself. Consequently, attention should be paid to this fact in chromatography of natural compounds with strong chelation ability.

References

ADAMSON N.J., REYNOLDS E.C. (1995): Characterization of multiply phosphorylated peptides selectively precipitated from a pancreatic casein digest. J. Dairy Sci., **78**: 2653–2659.

ANDERSSON L., PORATH J. (1986): Isolation of phosphoproteins by immobilized metal (Fe^{3+}) affinity chromatography. Anal. Biochem., **154**: 250–254.

MUSZYNSKA, G., ANDERSSON L., PORATH J. (1986): Selective adsorption of phosphoproteins on gel-immobilized ferric chelate. Biochemistry, **25**: 6850–6853.

MUSZYNSKA, G., DOBROWOLSKA G., MEDIN A., EKMAN P., PORATH J.O. (1992): Model studies on iron(III) ion affinity chromatography. II. Interaction of immobilized iron(III) ions with phosphorylated amino acids, peptides and proteins. J. Chromatogr., **604**: 19–28.

Received for publication August 5, 2000
Accepted for publication December 15, 2000

Souhrn

KMÍNKOVÁ M., PROŠKOVÁ A., KUČERA J. (2001): **Neobvyklé chování přírodních polyfosfátů během IMAC**. Czech J. Food Sci., **19**: 161–165.

Byl shledán atypický průběh chromatografie fosfopeptidu Ser(P)-Ser(P)-Ser(P)-Glu-Glu získaného pankreatickou hydrolýzou kaseinu a kyseliny fytové na imobilizovaných železitých iontech (IMAC). Stanovením obsahu železa a stanovením diferenciálního

spektra eluovaného materiálu bylo prokázáno, že obě látky tvoří pevnější chelátovou vazbu s železitými ionty než samotná imino-diacetylová skupina (IDA) vázaná na agarose. Tento neobvyklý průběh chromatografie ukazuje, že je třeba dbát zvýšené opatrnosti při separacích silně chelátotvorných látek metodou IMAC.

Klíčová slova: kyselina fytová; Ser(P)-Ser(P)-Ser(P)-Glu-Glu fosfopeptid; afinitní chromatografie; imobilizované železité ionty

Corresponding author:

Ing. MILENA KMÍNKOVÁ, Výzkumný ústav potravinářský Praha, Radiová 7, 102 31 Praha 10-Hostivař, Česká republika
tel.: + 420 2 72 70 23 31, fax: + 420 2 72 70 19 83, e-mail: m.kminkova@vupp.cz