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USE OF O-PHOSPHOSERINE (OPS) FOR THE SEPARATION OF PEPTIDES ON IMMOBILIZED COPPER IONS

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Abstract

Recent research into the structure and properties of proteins and peptides as physiologically active diet components has spurred a new interest in the isolation and investigation of bioactive peptides of animal, plant and microbiological origin. The isolation and separation of protein and peptide mixtures requires advanced procedures. It usually involves a multi-stage separation process on chromatographic columns with various packing. Immobilised Metal Ion Affinity Chromatography (IMAC) is frequently used in the complex process of obtaining peptide fractions. Affinity Chromatography (IMAC) relies on the specific interactions between amino acids, their reactive groups in proteins and peptides and „transitory" metal ions, in particular Cu^{2+}. Those ions are immobilised by the chelating compound on the bed, forming specific adsorbents which bind proteins and peptides.

The aim of this study was to determine whether o-phosphoserine (OPS) can be used for the immobilization of copper ions on Sephadex G25 during the separation of peptides and proteins isolated from string beans.

Frozen pods of dwarf, green-podded string bean cv. *Fana* were used in the study. Peptide were extracted from well-homogenized string bean pods with tris-HCl buffer (pH 7.5), from which high molecular weight proteins were isolated with methanol, acetone, 20% trichloroacetic acid and the Magnafloc M-22S cation flocculant. The protein and peptide content of the separated fractions was determined. The peptide content depended on the type of extract from which high molecular weight proteins were isolated. The results obtained by using OPS as a chelating agent in the separation of string bean can be recommended for analysis of plant peptides.

Key words: peptides, IMAC, metal ions, o-phosphoserine.

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WYKORZYSTANIE OPS W PROCESIE ROZDZIA£U PEPTYDÓW NA UNIERUCHOMIONYCH JONACH MIEDZI

Abstrakt

Rozwój nauki o strukturze oraz w³aœciwoœciach bia³ek i peptydów jako fizjologicznie aktywnych sk³adnikach diety przyczyni³ siê do wzrostu zainteresowania izolowaniem i badaniem bioaktywnych peptydów pochodzenia zwierzêcego, roœlinnego i mikrobiologicznego.

Izolowanie i rozdzia³ mieszanin bia³ek i peptydów wymaga zaawansowanej procedury. Stosuje siê zazwyczaj kilkustopniowy rozdzia³ na kolumnach chromatograficznych z ró¿nym wype³nieniem. W tak skomplikowanym procesie otrzymywania frakcji peptydowych szerokie zastosowanie znalaz³a chromatografia powinowactwa na unieruchomionych jonach metali IMAC (Immobilized Metal Ion Affinity Chromatography). Chromatografia powinowactwa wykorzystuje specyficzne oddzia³ywania miêdzy aminokwasami oraz ich reaktywnymi ugrupowaniami w bia³kach i peptydach a jonami metali „przejœciowych", szczególnie zaœ z Cu^{2+}. Jony te s¹ immobilizowane przez zwi¹zek chelatuj¹cy na z³o¿u, i w ten sposób stanowi¹ specyficzne adsorbenty wi¹¿¹ce bia³ka lub peptydy.

Celem pracy by³o zbadanie przydatnoœci OPS (o-fosfoseryny) jako czynnika unieruchamiaj¹cego jony miedzi na z³o¿u w procesie rozdzia³u peptydów i bia³ek wyizolowanych z fasoli szparagowej metod¹ IMAC. Materia³em do badañ by³y mro¿one str¹ki fasoli szparagowej kar³owatej zielonostr¹kowej, odmiany *Fana*. Peptydy i bia³ka izolowano z fasoli szparagowej buforem Tris-HCl, z otrzymanego ekstraktu bia³ka wysokocz¹steczkowe wydzielono: metanolem, acetonem, 20% kwasem trichlorooctowym i kationowym flokulantem Magnafloc M-22S. W otrzymanych frakcjach oznaczono zawartoœæ bia³ka i peptydów. Peptydy obecne w fasoli szparagowej charakteryzowa³y siê zbli¿onym powinowactwem do jonów miedzi. Wykazano, ¿e rozdzia³ peptydów zale¿y w ma³ym stopniu od w³aœciwoœci czynnika zastosowanego podczas usuwania bia³ek z ekstraktu. Przebieg rozdzia³u z wykorzystaniem OPS jako czynnika chelatuj¹cego w technice IMAC z powodzeniem mo¿e byæ stosowany do rozdzia³u peptydów z ekstraktów roœlinnych.

S³owa kluczowe: peptydy, IMAC, jony metali, o-fosfoseryna.

INTRODUCTION

Recent research into the structure and properties of proteins and peptides as physiologically active diet components has spurred a new interest in the isolation and investigation of bioactive peptides of animal, plant and microbiological origin. The isolation and separation of protein and peptide mixtures requires advanced procedures. It usually involves a multi-stage separation process on chromatographic columns with various packing. Immobilised Metal Ion Affinity Chromatography (IMAC) is frequently used in the complex process of obtaining peptide fractions. Affinity Chromatography (IMAC) relies on the specific interactions between amino acids, their reactive groups in proteins and peptides and „transitory" metal ions, in particular Cu^{2+}. Those ions are immobilised by the chelating compound on the bed, forming specific adsorbents which bind proteins and peptides. Immobilised metal ions have been successfully applied in separation of the products of proteolysis of leguminous plants seeds proteins (BARANIAK, KRZEPI£KO 2005).

The fresh vegetables and aquatic plants are rich in metal ions, which can be removed by the chelating compound on the bed (without immobilized metal ions) (Bosiacki, Tyksiński 2009, Senze 2009).

The aim of this study was to determine whether o-phosphoserine (OPS) can be used for the immobilization of copper ions on Sephadex G25 during the separation of peptides and proteins isolated from string beans.

MATERIALS AND METHODS

Isolation of peptides from plant material

Frozen pods of dwarf, green-podded string bean cv. *Fana* were used in the study.

Peptides were extracted from well-homogenized string bean pods with Tris –HCl buffer (pH 7.5). Homogenate of 1 g sample of frozen string beans was stirred with a magnetic stirrer with 10 cm^3 of tris–HCl buffer (pH 7.5) for 2 hours at room temperature. The solid fraction was separated by centrifugation at 4,000 rpm for 15 min. The collected extract was lyophilized and used for further determinations. The fractions albumins and globulins dissolved lyophilizes were precipitated by acetone, methanol, 20% TCA and cationic flocculant Magnafloc M-22S. The extracts were separated by immobilized copper ion affinity chromatography.

Gel preparation for peptide separation by IMAC

Sephadex G-25 medium was mixed with a solution containing 0.0375 g $NaBH_4$, 10 cm^3 2 M NaOH and 1 cm^3 of epichlorohydrin. The suspension was mixed slowly for 2 hours at room temperature. Meanwhile, 10 cm^3 of 2 M NaOH and 5 cm^3 of epichlorohydrin solutions were gradually added. The mixture was left overnight to complete the reaction. The gel was washed on a Büchner funnel and dried. The dried gel was mixed with 25 cm^3 of a solution containing 5.3 g Na_2CO_3, 2.5 g *o*-phosphoserine (OPS) and 0.03 g $NaBH_4$. The suspension was left overnight at 60°C and stirred slowly from time to time. After 24 hours, the gel was washed with distilled water followed by a diluted acetic acid and distilled water again, to neutralize the pH. 50 cm^3 of a solution containing copper ions at a concentration of 1 $mg \cdot cm^{-3}$ was added to the gel. Next the bed was transferred to a 1.5 cm diameter and 12 cm long column. The column was equilibrated with 40 cm^3 of a 0.05 M solution of Tris-HCl buffer, pH 7.5. A 2 cm^3 sample was applied to a glass column packed with gel, and fractions 1-36 were eluted with a pH gradient of Tris-HCl buffer: 7.5, 5.5, 4.5 and back to 7.5 (adjusted with EDTA--fraction 37-70). 4 ml fractions were collected at a flow rate of 40 $cm^3 \cdot hour^{-1}$.

Methods for determinations

Protein concentration was determined by using BRADFORD's method (1976) with bovine serum albumin (BSA) as a standard (595 nm). Peptide content was determined spectrophotometrically with trinitrobenzesulfonic acid (TNBS), according to HABEEB's method (1966) modified by ADLER-NISSEN (1979). Leucylglycine was used as standard (340 nm).

RESULTS AND DISCUSSION

The process of affinity chromatography involving chelate-bound metal ions is conditioned by many factors (UEDA et al. 2003). The type of the applied support, activating factor and metal ion chelating compound also play an important role in the process (ZACHARIOU, HEARN 2000). The possibility of reversible protein-metal ion binding as well as maintaining their permanent bonds with chelating compounds are also important. The effectiveness of different metal ions and chelating factors can be tested reliably only with the use of the same material which is separated under identicalal elution conditions.

Peptide affinity for metal ions is determined by peptide structure, type of metal ions, type of chelating compound, pH, type of solvent, presence of salt and competitive ligands (CHAGA 2001, PORATH, OLIN 1983).

In IMAC, the most popular ligands are iminodiacetic acid (IDA), nitrilotriacetic acid (NTA), carboxymethylated aspartic acid (CM-Asp) and tri(carboxymethyl)ethylene diamine (TED). The effectiveness of OPS as a chelating agent was tested in the present study. This compound has not been used for the isolation of vegetable proteins and peptides to date.

Orthophosphoserine was tested in a model protein study and in the separation of human serum proteins by ZACHARIOU and HEARN (2000). O-phosphoserine is a constituent of biological membranes and it participates in ion transport. It forms complexes with metals and, subject to the degree of dissociation, it may be a tridonor compound similarly to iminodiacetic acid – binding metal ions through oxygen atoms in phosphate and carboxyl groups and nitrogen atoms in the amino group. According to the cited authors, OPS has higher affinity than IDA for iron and aluminium ions and lower affinity for copper ions. The binding capacity of the Cu ion-OPS chelate was comparable to that of chelates formed by hard metal ions (iron and aluminium) and OPS. The effectiveness of copper ions chelated with OPS on the same bed was compared in this study. It was found that OPS, similarly as IDA, forms chelates with the same structure, using three donor sites for this purpose. The effect of chelates other than IDA on the separation process has been also investigated by other authors. In order to compare the

absorption of reference proteins, lysozyme, ovoalbumin, beef albumin, conalbumin and wheat germ agglutinin, on columns packed with Sepharose 6B with copper and nickel ions immobilized with IDA and tris(2-aminoethyl)amine, SHARMA, AGARWAL (2001) described them using four different isotherm models.

Langmuir-Freundlich model was found to be most suitable for explaining cooperativity and quantitative differences in protein binding in all tested systems. The analyzed proteins showed the highest affinity for copper ions chelated with IDA, and their affinity for the other systems varied. CHAOUK and HEARN (1999) reported good results of the separation of human serum proteins in an alkaline environment as Cu ions were mobilized on Sepharose CL-4B with a new tridonor compound, N-2 sodium pyridyl-methylamine acetate. Columns containing TED-Novarose, with chelate-agarose, have been used for the separation of beef calmodulin (CHAGA et al. 1996). Nitrilotriacetic acid has been used to immobilize Ni ions on agarose in the process of purifying proteins obtained by genetic recombination (GLYNOU et al. 2003). Carboxymethyl asparagine has been used for immobilizing cobalt ions on Superflow 6 in order to purify dehydrogenase from chicken breast muscles (CHAGA et al. 1999).

The ligands used in IMAC form tri-(IDA), four-(NTA, CM-Asp), five-donor (TED) complexes (chelates) with metal ions. The number of coordinate bonds between metal ions and chelating compounds determines the general affinity of chelates for proteins and the overall stability of the complex. The higher the donor state, the greater the stability of chelates and the weaker the binding of proteins. In aqueous solutions, chelates undergo strong solvation and all free coordination positions of metals are occupied by water molecules and by the hydroxyl or amino groups of buffer compounds. During separation, these groups are easily replaced by protein, peptide or amino acid molecules.

IDA is commonly used in IMAC. IDA-metal chelates have the highest protein-binding capacity. This ligand forms the most stable complexes with Cu^{2+}, followed by Ni^{2+}, Zn^{2+} and Co^{2+}. Another chelating compound, TED, has been tested with respect to Ni^{2+} and Fe^{3+} ions during the separation of serum proteins (PORATH, OLIN 1983). TED-metal chelates show much lower protein-binding capacity. These compounds are often used for removing metal ions from metalloenzymes. The adsorptive properties of NTA place it between IDA and TED. NTA is particularly effective in Ni^{2+} ion binding.

Chromatograms developed for protein concentrations determined by measuring absorbance at a wavelength of 280 nm are presented in Figures 1-4. When the ions were immobilized with OPS, the maximum absorbance was observed in fractions 3 or 4, and protein concentrations following the use of copper ions reached 1.365 $mg \cdot cm^{-3}$ for acetone, 2.858 $mg \cdot cm^{-3}$ for the flocculant, 1.598 $mg \cdot cm^{-3}$ for methanol and 0.914 $mg \cdot cm^{-3}$ for 20% TCA. Depending on the precipitating agent used (methanol, 20% TCA and flocculant), additional peaks were detected for fractions 3-7 and 12-13 (Table 1).

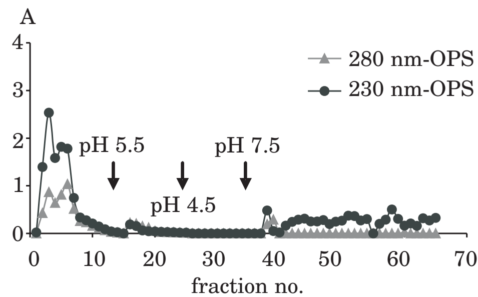

Fig. 1. Elution profiles of the non-bound peptides from IMAC chromatography of extracts from string bean on Cu(II)-OPS-Sephadex (precipitated with methanol after separation in columns)

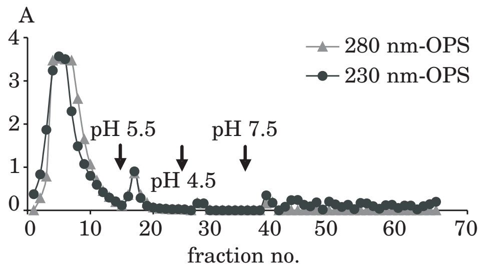

Fig. 2. Elution profiles of the non-bound peptides from IMAC chromatography of extracts from string bean on Cu(II)-OPS-Sephadex (precipitated with acetone after separation in columns)

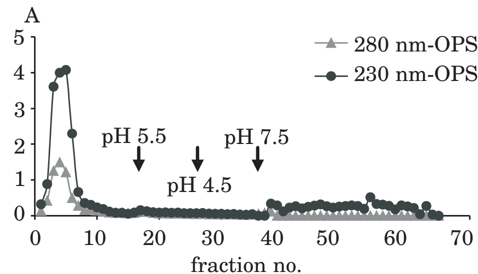

Fig. 3. Elution profiles of the non-bound peptides from IMAC chromatography of extracts from string bean on Cu(II)-OPS-Sephadex (precipitated with 20% TCA after separation in columns)

Chromatograms obtained for peptide levels determined by measuring absorbance at a wavelength of 230 nm are presented in Figures 1-4. As the copper ions were immobilized with OPS, the maximum absorbance was observed in fraction 6, and peptide content was as follows: 0.0261 mg$\cdot$cm^{-3} for acetone, 0.024 mg$\cdot$cm^{-3} for the flocculant, 0.0258 mg$\cdot$cm^{-3} for methanol and 0.0079 mg$\cdot$cm^{-3} for 20% TCA. Smaller peaks were noted for further

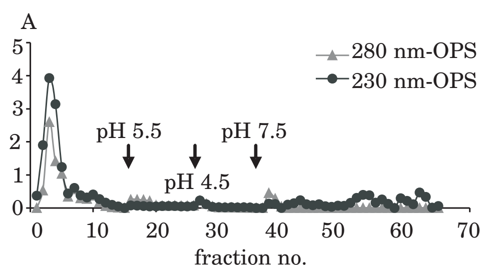

Fig. 4. Elution profiles of the non-bound peptides from IMAC chromatography of extracts from string bean on Cu(II)-OPS-Sephadex
(precipitated with flocculant after separation in columns)

fractions, e.g. 11-15, 18-20, 27-30 or above 36, depending on the precipitating agent used (Table 2). A similar dependency was observed by VANCAN et al. (2002) who tested the affinity of human immunoglobulin IgG for Cu, Ni and Zn ions immobilized with IDA on Sepharose-6B activated with epichlorohydrin. Following the application of a phosphate buffer in the pH gradient in the elution process, as much as 81.4% of protein was removed from the column with copper ions solely in the regeneration process with the use of 50 mM EDTA. When separating raw extract (10% purity) of somatotropin (STH), the human growth hormone, LIESIENÉ et al. (1997) obtained a fraction image identical to that reported in this study after separation in columns with immobilised copper ions on a cellulose bed (Chelat-Carnosel). The highest protein content (measured at A_{280}) was detected in the first few frac-

Table 1

Protein content of extracts precipitated with various agents after separation in OPS columns with immobilised copper ions ($mg \cdot cm^{-3}$)

Precipitated agent	Acetone		Methanol				20% TCA	Flocculant	
Fraction	3-7	12-13	3-7	12-13	27-30	38-39	3-7	3-7	12-13
Cu^{2+}	6.137		6.503				1.459	7.683	0.407

Table 2

Peptide content of extracts precipitated with various agents after separation in OPS columns with immobilised copper ions ($mg \cdot cm^{-3}$)

Precipitated agent	Acetone				Methanol		
Fraction	3-9	11-15	18-20	38-41	3-9	11-15	38-41
Cu^{2+}	0.127	0.048	0.012	0.009	0.118	0.020	0.019
Precipitated agent	20% TCA				flocculant		
Fraction	3-9	11-15	38-41	3-9	11-15	27-30	38-41
Cu^{2+}	0.041	0.007	0.006	0.176	0.015	0.007	0.014

tions eluted with phosphate buffer, pH 7.8 with 0.5M sodium chloride. When the gradually decreasing pH gradient of acetic buffer (pH 6.6 to 4.3) was applied, only a few small peaks with absorbance below 0.1 were detected. In the process of isolating green fluorescent protein (GFPuv) (genetically unmodified), the highest efficiency was reported in respect of copper ions, followed by nickel ions, while the lowest results were obtained for zinc and cobalt ions (Li et al. 2001). Varlamov et al. (1995) recommended the use of IDA-Sepharose with immobilized copper ions at the final purification stage of beta-dopamine hydroxylase from beef bone marrow adrenaline, due to more effective interactions with these ions, compared with copper, zinc and nickel ions.

The cited authors and the authors of this study tested copper ions which form coordinate bonds with electrodonor atoms, prefer bonds with nitrogen, but also interact with oxygen and sulfur. Their efficiency should be determined by the availability of amino acids such as histidine, cysteine or tryptophan. The results of this study suggest that the process of peptide separation is also dependent, although to a lower degree, on the properties of the agent used for protein removal from the extract. In all the cases, the highest peptide content was recorded in fractions 3-9, and additional absorbance maxima (with a substantially lower peptide content) were observed in further fractions (Table 2). A similar fraction image was reported by George et al. (1997), who used copper ions immobilised on Sephadex G-25 in the separation of chick pea protein hydrolysates. Okhubo et al. (1980) observed three maximum absorbance values during the separation of rat liver nucleoside diphosphatase, initially purified in a column packed with DEAE cellulose and Sephadex G-200, on a column with chelated copper ions to epoxy-activated Sepharose 6B. Following the separation of soy beta-conglycinin hydrolysate, obtained through the application of protease S (with *Bacillus* sp.), in a column packed with Sephadex G-25, Chen et al. (1995) noted two maximum values when absorbance was measured at a wavelength of 280 nm and found six peptides in the resulting content. Saito et al. (1991) demonstrated that the yield of caseinoglycopeptides isolated from sweet cheese whey depended on the method of whey protein precipitation.

CONCLUSION

1. Affinity chromatography with the use of metal ions immobilized to OPS-Sephadex G-25 may be successfully used for the separation of peptides isolated from string beans.

2. The results obtained by using OPS as a chelating agent in the separation of string bean peptides were comparable with those reported for IDA, which indicates that OPS can be recommended for analysis of plant peptides.

3. Agents used for removing high molecular weight compounds (soluble at pH 7.5) from extracts of proteins and peptides present in string beans considerably affect the qualitative and quantitative composition of the resulting filtrate.

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