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# Immobilization of L-glyceryl phosphorylcholine: isolation of phosphorylcholine-binding proteins from seminal plasma

J. Liberda[a,*], P. Maňásková[b], M. Švesták[a], V. Jonáková[b], M. Tichá[a]

[a]*Department of Biochemistry, Charles University, Albertov 2030, 128 40 Prague 2, Czech Republic*
[b]*Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 166 37 Prague 6, Czech Republic*

**Abstract**

The preparation of an affinity sorbent containing immobilized L-glyceryl phosphorylcholine for affinity chromatography of phosphorylcholine-binding proteins from seminal plasma is described. The ligand was coupled either after its maleinylation to poly(acrylamide-allyl amine) copolymer or directly to divinyl sulfone-activated Sepharose. The prepared phosphorylcholine derivative coupled to Sepharose was used for affinity chromatography of phosphorylcholine-binding proteins from bull and boar seminal plasma. Adsorbed proteins were specifically eluted with phosphorylcholine solution. Isolated phosphorylcholine-binding proteins were characterized by SDS electrophoresis and HPLC with reversed phase. Composition of the boar phosphorylcholine-binding fraction obtained by affinity chromatography on immobilized L-glyceryl phosphorylcholine was compared with that eluted from immobilized heparin by the phosphorylcholine solution. No phosphorylcholine-binding proteins were found in human seminal plasma. 

*Keywords:* L-Glyceryl phosphorylcholine; Proteins

## 1. Introduction

A number of different types of phosphorylcholine-binding proteins exist that differ in their properties and functions. The C-reactive protein of human blood serum (CRP) [1], frequently employed as a clinical index of acute inflammation, represents the most studied phosphorylcholine-binding protein. CRP is also found in the invertebrate *Limulus polyphemus* as a constitutive and major component of horseshoe crab hemolymph [2]. Phosphorylcholine-binding proteins were also isolated from Atlantic salmon [3] or hemolymph of the snail *Achatina fulica* [4]. Another type of protein possessing the ability to interact with phosphorylcholine was found among proteins isolated from seminal plasma of different species. Some seminal plasma proteins are known to bind specifically to the sperm surface; in this case an interaction of sperm surface proteins with phosphorylcholine containing-components of the sperm membrane is involved [5]. Interaction of seminal plasma proteins of different species with phosphorylcholine was studied either using affinity chromatography [6,7] or by direct binding assays [8,9].

There exist only a limited number of types of affinity carriers containing immobilized phosphorylcholine: this ligand immobilized on Sepharose via a *p*-amino-phenyl spacer arm, which was used to isolate CRP from human serum [10] or *L. polyphemus* [11], is the only one commercially available. Alternatively, aminohexyl-Sepharose was modified by reaction with phosphorylcholine caproyl *p*-nitro-

*Corresponding author. Fax: +420-2-2195-2331.
*E-mail address:* jirik@natur.cuni.cz (J. Liberda).

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phenyl ester [12] or *p*-diazophenyl phosphorylcholine reacted with tyrosine previously attached to agarose beads [13]. Another possibility was the immobilization of bovine serum albumin containing coupled periodate-oxidized L-glyceryl phosphorylcholine to Toyopearl HW 65 [14]. For the isolation of phosphorylcholine-binding proteins from seminal plasma, the combination of affinity chromatography on immobilized heparin followed by elution of the adsorbed proteins with the phosphorylcholine solution was used [7,15,16].

The present communication describes the preparation of a new type of affinity carrier containing immobilized L-glyceryl phosphorylcholine. The ligand used in our experiments represents a real component of phospholipids present in membranes participating in interactions of seminal plasma proteins in vivo.

## 2. Experimental

### 2.1. Chemicals

Divinyl sulfone and 1-(3-(dimethylamino)propyl)-3-ethyl-carbodiimide were purchased from Fluka (Buchs, Switzerland), and Sepharose 4B from Farmacia Biotech. AB (Uppsala, Sweden).

Heparin immobilized on polyacrylamide was prepared by coupling the ligand to poly(acryl amide-allyl amine) copolymer as described previously [17].

### 2.2. Seminal plasma proteins

Boar and bull ejaculates were obtained from the Veterinary Research Institute, Brno, Czech Republic, and human ejaculates from the Institute of Sexology, Charles University, Praha, Czech Republic. Ejaculates were centrifuged (600 *g*, 20 min, 5°C) to separate plasma and sperm.

Boar and bull seminal plasma was used for the preparation of the delipidated protein fraction: seminal plasma (2.3 ml) was diluted with phosphate-buffered saline (PBS, pH 7.4, 2.7 ml). Diluted plasma was mixed with a methanol+ethanol (3:1) mixture (20 ml) in ice-cooled bath under stirring. After standing for 1 h at 0°C, the suspension was centrifuged (1000 *g*, 20 min, 5°C) and the precipitate was dried under vacuum (SpeedVac).

Human seminal plasma was mixed with benzamidine hydrochloride (16 mg/ml) within 2 h of semen collection. Then ammonium sulfate was added to seminal plasma to reach 80% of saturation. Precipitated proteins obtained by centrifugation (3000 *g* for 20 min) were thoroughly dialyzed using the Spectra/Por CE Membrane (MWCO 2000; Spectrum Medical Industries, Houston, TX, USA) against distilled water and finally against PBS, and stored at −20°C.

### 2.3. Preparation of affinity carriers

Free L-glyceryl phosphorylcholine was prepared from its $CdCl_2$ complex (500 mg) using AG 501 X-8 (D) mixed-bed ion-exchange resin as described by Stults et al. [14]. The combined filtrate was evaporated to clear syrup (260 mg).

#### 2.3.1. L-Glyceryl phosphorylcholine immobilized on Sepharose 4B

Activation of Sepharose 4B with divinyl sulfone [18] was carried out as follows. Sepharose 4B (10 ml) washed with distilled water (500 ml) was suspended in 0.2 *M* carbonate buffer, pH 10.7 (10 ml) containing divinyl sulfone (1 ml). The suspension was shaken at room temperature for 70 min and then the gel was washed with distilled water.

Coupling of L-glyceryl phosphorylcholine was carried out as follows. Immediately after the activation, the gel of divinyl sulfone-activated Sepharose (5 ml) equilibrated with 0.2 *M* carbonate buffer, pH 10.7, was mixed with the solution of the ligand (260 mg of syrup in 10 ml of the same buffer) and shaken at room temperature for 20 h. The gels were washed first with distilled water and finally with 0.2 *M* carbonate buffer, pH 10.7. Then the gels were suspended in glycine solution (100 mg glycine in 10 ml 0.2 *M* carbonate buffer, pH 10.7), shaken for 2 h at room temperature and again washed with distilled water, 0.05 *M* citrate buffer, pH 3.0, and distilled water.

For preparation of the control gel, 2-mercaptoethanol was coupled to divinyl sulfone-activated Sepharose under the same conditions.

*2.3.2. L-Glyceryl-phosphorylcholine immobilized to poly(acrylamide-allylamine) copolymer*

The syrup of free L-glyceryl phosphorylcholine (206 mg) was dissolved in anhydrous N,N-dimethylformamide (DMF) (15 ml), maleinanhydride (110 mg) was added, and the reaction mixture was shaken overnight at laboratory temperature. Then the solution was mixed with 1-(3-dimethylaminopropyl)-3-ethylcarbodimide (250 mg) and shaken for 3 h. Then the suspension of poly(acrylamide-allylamine) copolymer (10 ml of gel equilibrated with 0.1 *M* Tris–HCl buffer, pH 8.5) was added and shaken for 48 h. Finally, the gel was washed with distilled water, 0.05 *M* citrate buffer, pH 3.0. and distilled water.

*2.4. Affinity chromatography of phosphorylcholine-binding proteins from seminal plasma*

*2.4.1. Affinity chromatography on immobilized L-glyceryl phosphorylcholine*

Delipidated or ammonium sulfate-precipitated seminal plasma proteins (10 mg) dissolved in 2 ml TBS (0.05 *M* Tris–HCl buffer containing 0.15 *M* NaCl, pH 7.4) was applied to a L-glyceryl phosphorylcholine-Sepharose column (1.0×4.5 cm) equilibrated with TBS. The non-adsorbed proteins were eluted with TBS until the absorbance at 280 nm reached the base line. Desorbed proteins were eluted first with 50 m*M* phosphorylcholine and then with 4 *M* urea. Fractions of 3 ml per 20 min were collected. The course of chromatography was followed by measurement of absorbance at 280 nm. Fractions containing proteins were pooled and desalted on Sephadex G-25 in 0.2% acetic acid and lyophilized.

The same procedure was used for the affinity chromatography of seminal plasma proteins on L-glyceryl phosphorylcholine immobilized to polyacrylamide derivative and on control gel containing coupled 2-mercaptoethanol on divinyl sulfone-activated Sepharose.

*2.4.2. Affinity chromatography of seminal plasma on heparin-polyacrylamide followed by elution with phosphorylcholine solution*

The bull seminal plasma (10 ml) diluted with TBS (10 ml) was applied on a heparin-polyacrylamide column (3×15 cm) [17], pre-equilibrated with the same buffer. The non-adsorbed proteins were washed with TBS until the absorbance at 280 nm reached the base line. The adsorbed proteins were first eluted with 10 m*M* phosphorylcholine solution in the same buffer and then with 3 *M* NaCl. Every 20 min, 5-ml fractions were collected. Further procedure was the same as described above for the affinity chromatography on immobilized phosphorylcholine.

*2.5. Reverse-phase high-performance liquid chromatography (RP-HPLC)*

Proteins were subjected to inert HPLC Biocompatible Quaternary Gradient System (Waters, Milford, MA, USA). RP-HPLC was performed using a 218 TP 54 Vydac $C_{18}$ column (4.6×250 mm, 5-μm particle size). A 1-mg of sample in 50 μl of 0.05% trifluoroacetic acid (TFA) was applied and proteins were eluted with a linear gradient of 20–50% acetonitrile (ACN) in 60 min. Protein fractions were lyophilized.

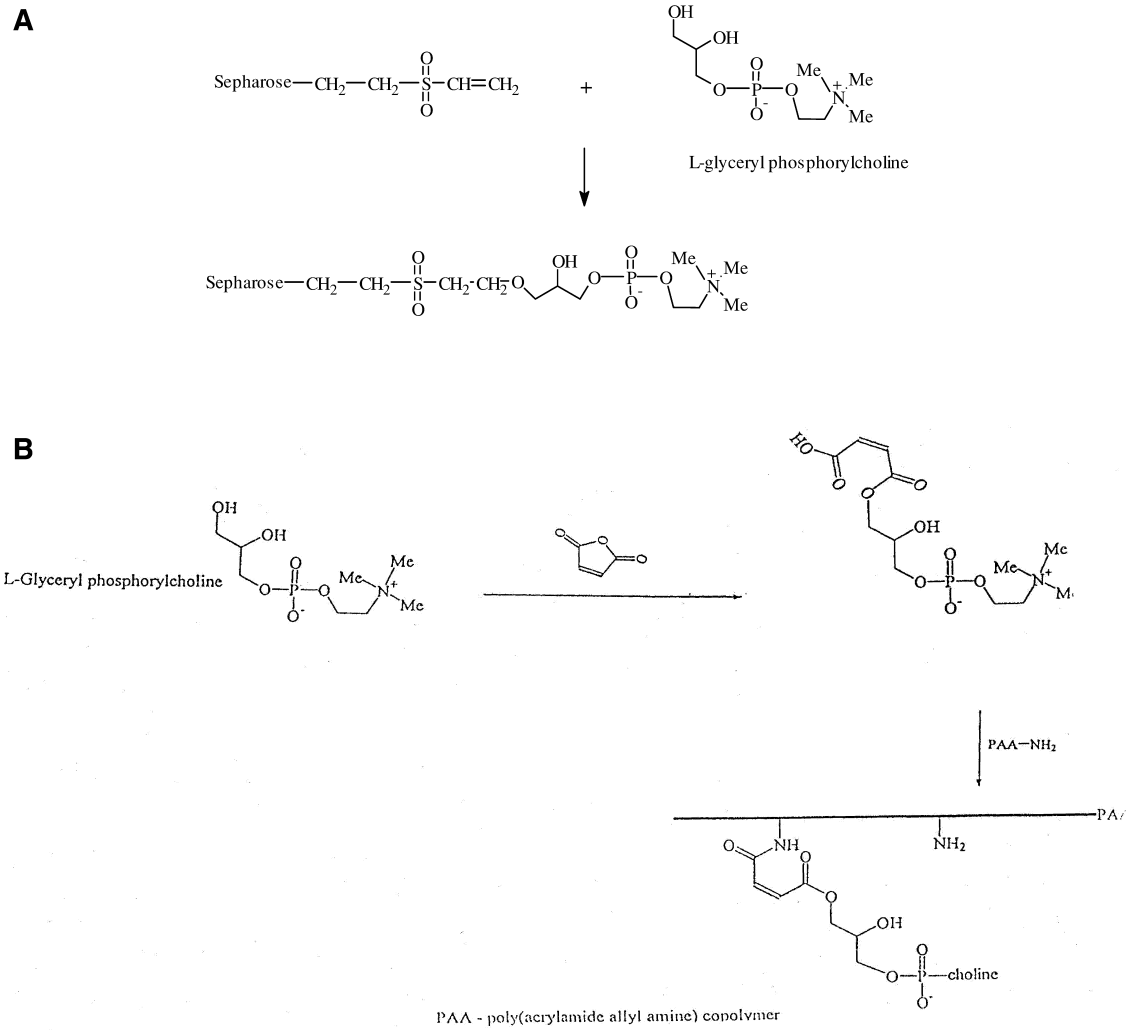
*2.6. SDS electrophoresis*

Polyacrylamide gel electrophoresis in the presence of SDS (SDS–PAGE) was performed according to Laemmli [19] using MiniProtean II (Bio-Rad) in 15% separation gel and 3% stacking gel. Non-reduced samples of seminal proteins and reduced standards were applied. After electrophoresis, the gel was stained with Coomassie Briliant Blue R-250.

## 3. Results

*3.1. Preparation of immobilized L-glyceryl phosphorylcholine*

L-glyceryl phosphorylcholine was coupled to two types of carriers: (i) directly to divinyl sulfone-activated Sepharose (Scheme 1A) or (ii) to poly-(acrylamide-allylamine) copolymer; L-glyceryl phosphorylcholine was maleinylated with maleic anhydride and the carbodiimide reaction was used to couple this derivative to amino groups of the polyacrylamide derivative (Scheme 1B). The content of the coupled ligand was determined using organic phosphate determination after acid hydrolysis [20].




Scheme 1. (A) Immobilization of L-glyceryl phosphorylcholine on divinyl sulfone activated-Sepharose. (B) Immobilization of L-glyceryl phosphorylcholine on poly(acrylamide-allylamine) copolymer.

The phosphorylcholine content in the prepared affinity carriers was calculated to be 10 μmol/ml of the gel (Sepharose-based carrier) or 0.3 μmol/ml of the gel (polyacrylamide-based carrier). 2-Mercaptoethanol coupled to divinyl sulfone-activated Sepharose (so-called thiophilic gel [21]) was used as a control gel to evaluate the effect of the spacer arm (divinyl sulfone derivative) on the protein interaction.

### *3.2. Affinity chromatography of seminal plasma proteins on immobilized L-glyceryl phosphorylcholine*

The prepared immobilized phosphorylcholine derivatives were used for affinity chromatography of phosphorylcholine-binding proteins from bull, boar and human seminal plasma. The course of the chromatography on phosphorylcholine-Sepharose is

shown in Fig. 1A (bull seminal plasma proteins), Fig. 1B (boar seminal plasma proteins) and Fig. 1C (human seminal plasma proteins). The proteins adsorbed to the affinity carrier were specifically eluted with the phosphorylcholine solution. The following elution with 4 *M* urea did not yield any protein. In the case of human seminal plasma, no proteins were obtained by elution either with the phosphorylcholine solution or with 4 *M* urea. The control gel (containing immobilized 2-mercaptoethanol on Sepharose instead of phosphorylcholine) did not bind any protein from bull or boar seminal plasma. The amount of protein being adsorbed to polyacrylamide-based carrier containing immobilized L-glyceryl phosphorylcholine was about ten times lower compared to the Sepharose matrix.

Isolated phosphorylcholine-binding proteins from bull and boar seminal plasma were characterized by SDS electrophoresis and HPLC with reversed phase. RP-HPLC of the boar and bull phosphorylcholine-binding proteins is shown in Figs. 2 and 3, respectively. SDS electrophoresis of components of bull phosphorylcholine-binding (P+) and non-phosphorylcholine-binding proteins (P−) is shown in Fig. 4A,B. The predominating component of the P+ fraction of bull proteins (Figs. 3B,2 and 4B,$2^{+}$) corresponds to PDC-109, the main BSP protein (BSP, bovine seminal plasma proteins). The identification was based on comparison of the electrophoretic (SDS–PAGE) and chromatographic (RP-HPLC) behavior of the proteins [22,30].

The components of protein fractions isolated from bull seminal plasma using Sepharose or polyacrylamide based affinity carriers did not differ.

### *3.3. Affinity chromatography on immobilized heparin followed by phosphorylcholine elution: comparison of two methods for isolation of phosphorylcholin-binding proteins*

The results of affinity chromatography on L-glyceryl phosphorylcholine-Sepharose were compared with those obtained on immobilized heparin followed by elution with the phosphorylcholine solution, as described for bull or boar proteins [7,15,16]. While in the case of bull and boar seminal plasma, the fraction of phosphorylcholine-binding proteins was obtained by this way, in the case of human seminal plasma, no proteins were eluted under the same conditions with the phosphorylcholine solution.

The composition of the mixture of phosphorylcholine-binding proteins from boar seminal plasma obtained from immobilized L-glyceryl phosphorylcholine described in this paper was compared with the fraction of proteins obtained by phosphorylcholine elution of proteins adsorbed to immobilized heparin [7,15,16]. In the case of boar seminal plasma proteins, phosphorylcholine-binding fractions obtained from both the immobilized phosphorylcholine and the immobilized heparin spermadhesins of AQN family and DQH sperm surface protein predominated (Fig. 2). Identification was based on comparison of the electrophoretic (SDS–PAGE) and chromatographic (RP-HPLC) behavior of proteins [9,16].

## 4. Discussion

Two types of affinity carriers containing immobilized L-glyceryl phosphorylcholine were prepared. In contrast to commercially available Sepharose with coupled *p*-amino-phenyl phosphorylcholine, the ligand used in our experiments represents a real component of membrane phospholipids. Choline phospholipids comprise in some species over 70% of total sperm plasma membrane phospholipids [23]. It has been suggested that these lipids constitute the binding sites for seminal plasma proteins on the sperm surface and thus they are supposed to participate in the formation of protein-coating layers of sperm that are necessary for sperm maturation and capacitation.

L-Glyceryl phosphorylcholine was coupled to two types of inert matrices: poly(acrylamide-allylamine) copolymer or divinyl sulfone activated Sepharose. For preparation of the polyacrylamide-based carrier, an approach analogous to that for the preparation of water-soluble polyacrylamide derivatives of phosphorylcholine [8] was used. Biotinylated water-soluble derivatives of phosphorylcholine were successfully used for the study of the phosphorylcholine-binding activity of isolated boar seminal plasma proteins and their aggregated forms [9,24]. The same derivatives but FITC-labeled were applied to study

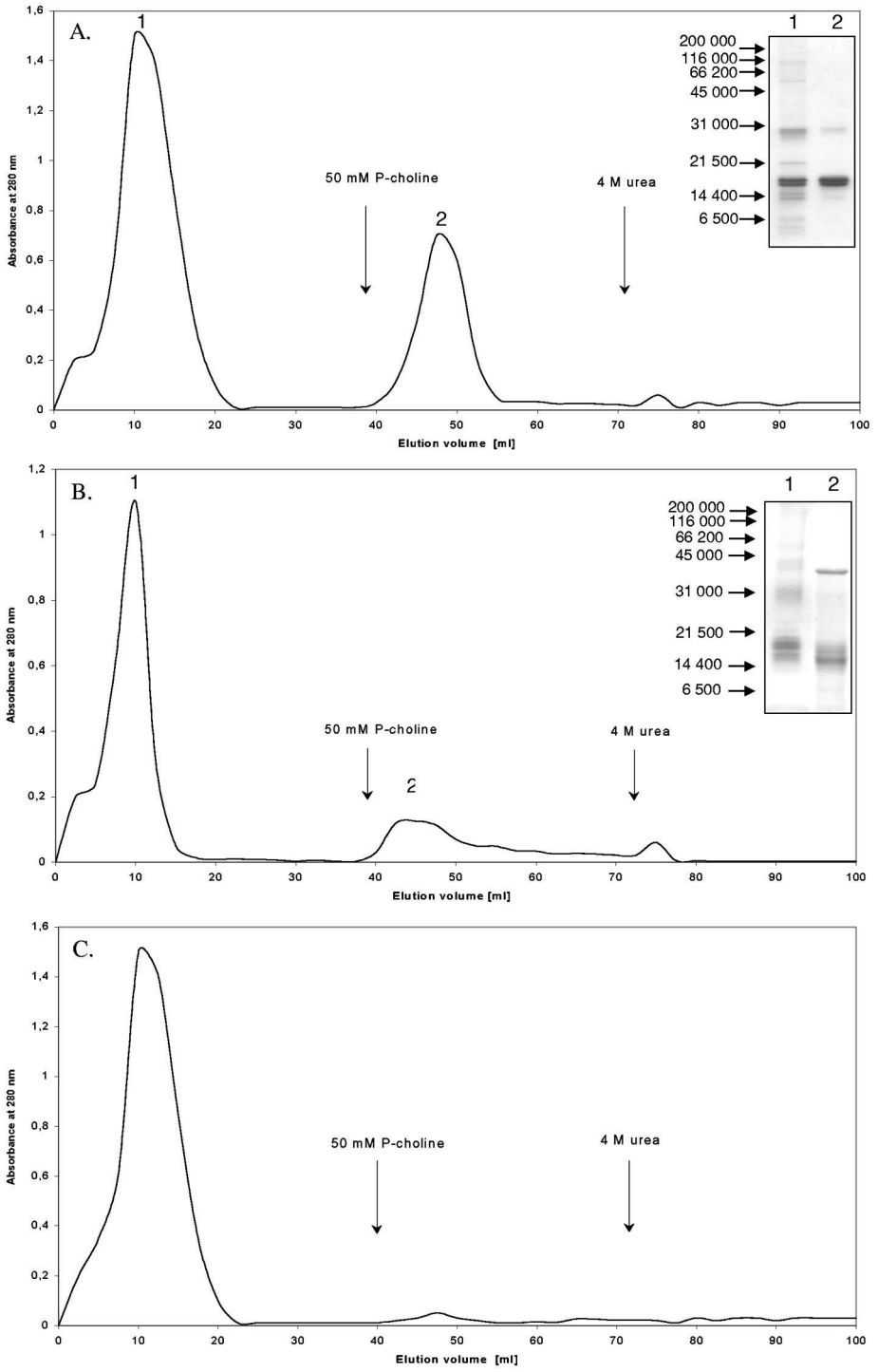



Fig. 1. Affinity chromatography of bull, boar and human seminal plasma proteins on L-glyceryl-phosphorylcholine-Sepharose: (A) bull; (B) boar; (C) human seminal plasma proteins. Inserts A, B: SDS electrophoresis of non-phosphoryl-binding (1) and phosphoryl-binding (2) fractions.

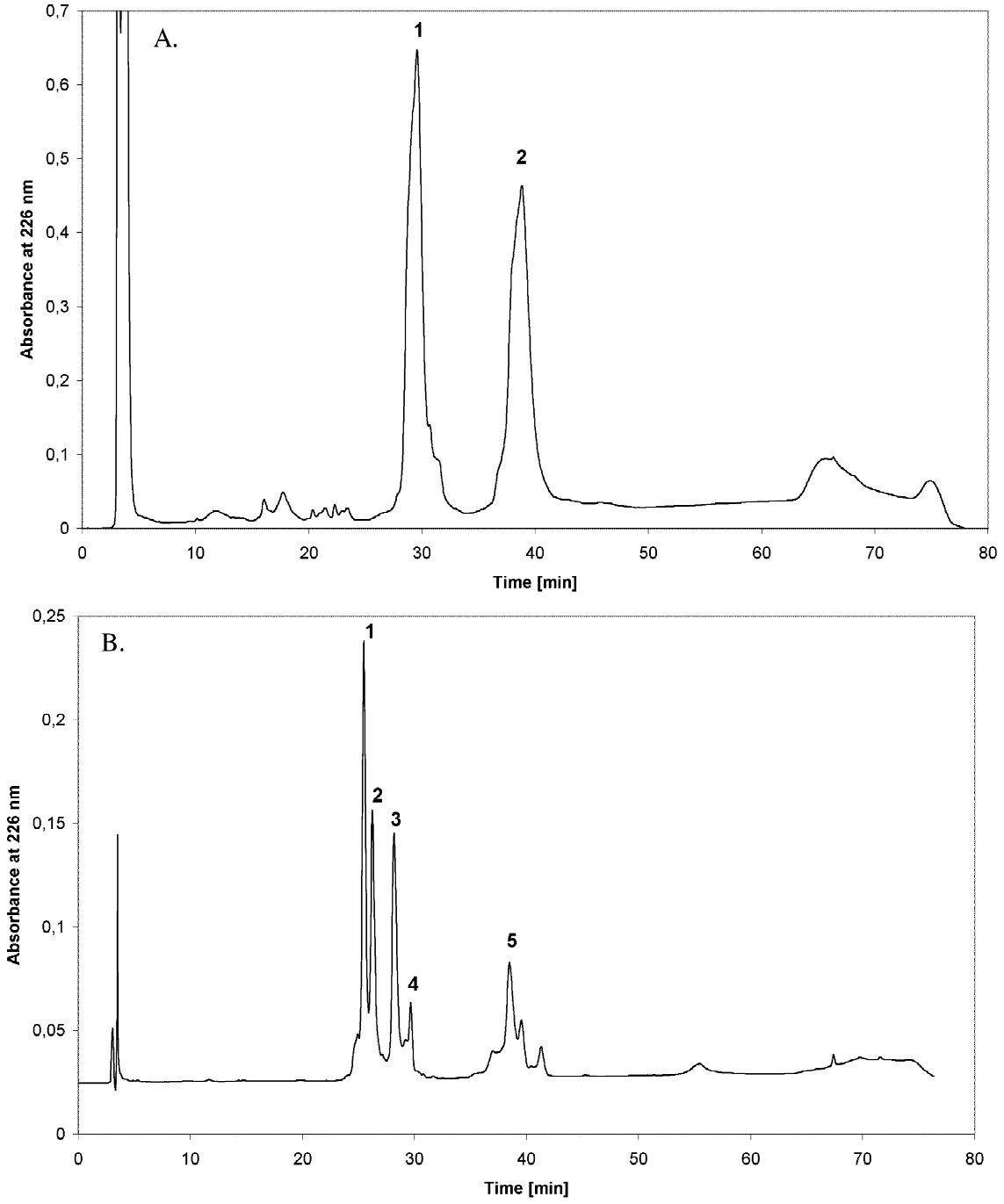



Fig. 2. RP-HPLC of protein fractions obtained by affinity chromatography of boar seminal plasma proteins on L-glyceryl-phosphorylcholine-Sepharose: (A) non-phosphorylcholine-binding proteins; (B) phosphorylcholine-binding proteins. A: 1, PSP I spermadhesins; 2, spermadhesins PSP II and of AWN family. B: 1, DQH sperm surface protein; 2–4, spermadhesins of AQN family; 5, spermadhesins of AWN family.

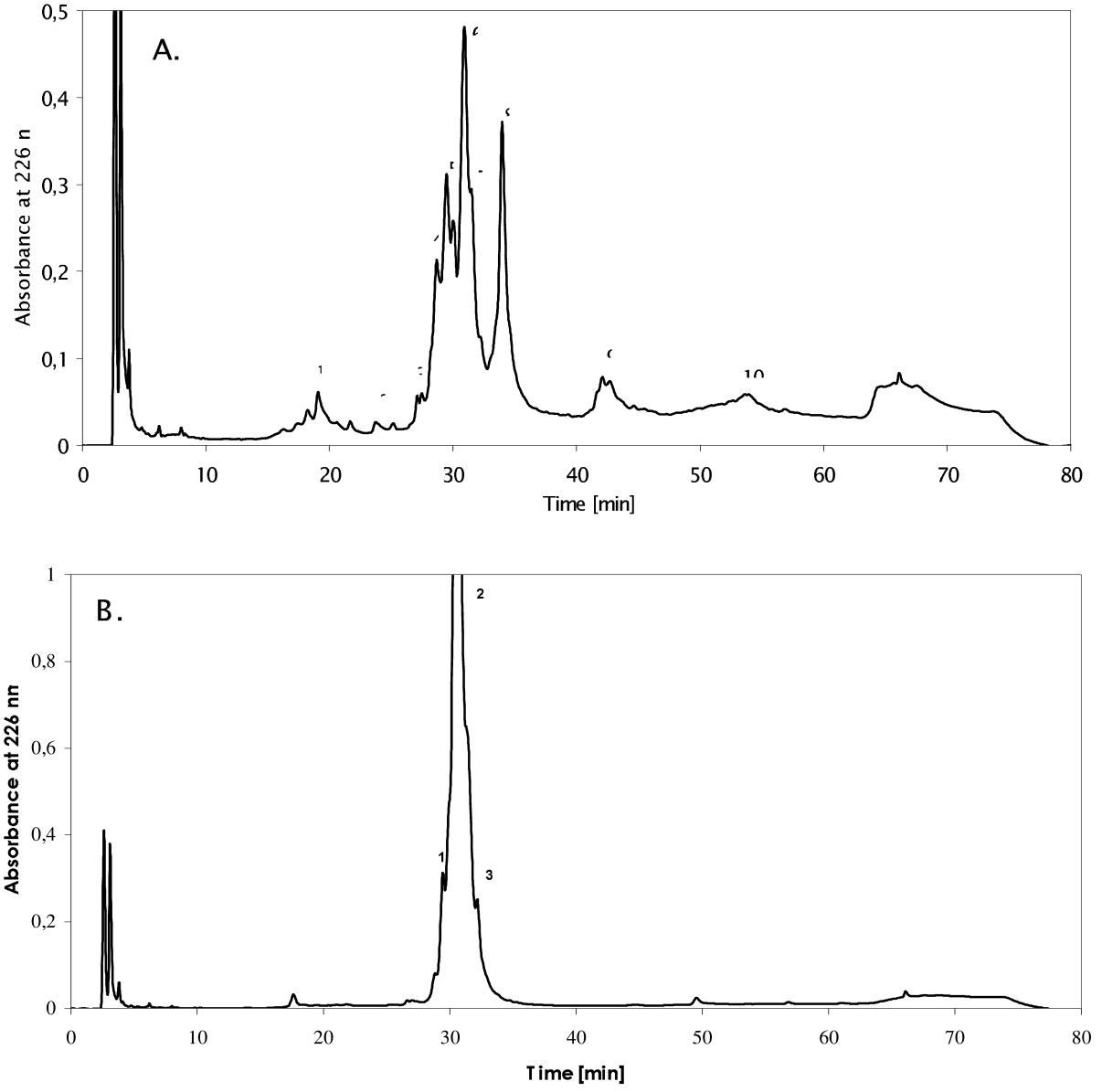



Fig. 3. RP-HPLC of protein fractions obtained by affinity chromatography of bull seminal plasma proteins on L-glyceryl-phosphorylcholine-Sepharose: (A) non-phosphorylcholine-binding proteins; (B) phosphorylcholine-binding proteins. 1, BSP-A2; 2, BSP-A1 (PDC-109); 3, fragment of BSP-30K.

the direct binding of phosphorylcholine to the bull sperm [8]. Divinyl sulfone-activated Sepharose was the second type of inert matrix used for the preparation of immobilized L-glyceryl phosphorylcholine. Divinyl sulfone-activated Sepharose originally developed for cross-linking of agarose gel [18] was found to be a suitable matrix for coupling different types of ligands (e.g. saccharides [25,26], peptides and proteins [27,28] or 3,5-diiodo-L-tyrosine [29]). Besides that, divinyl sulfone-activated Sepharose

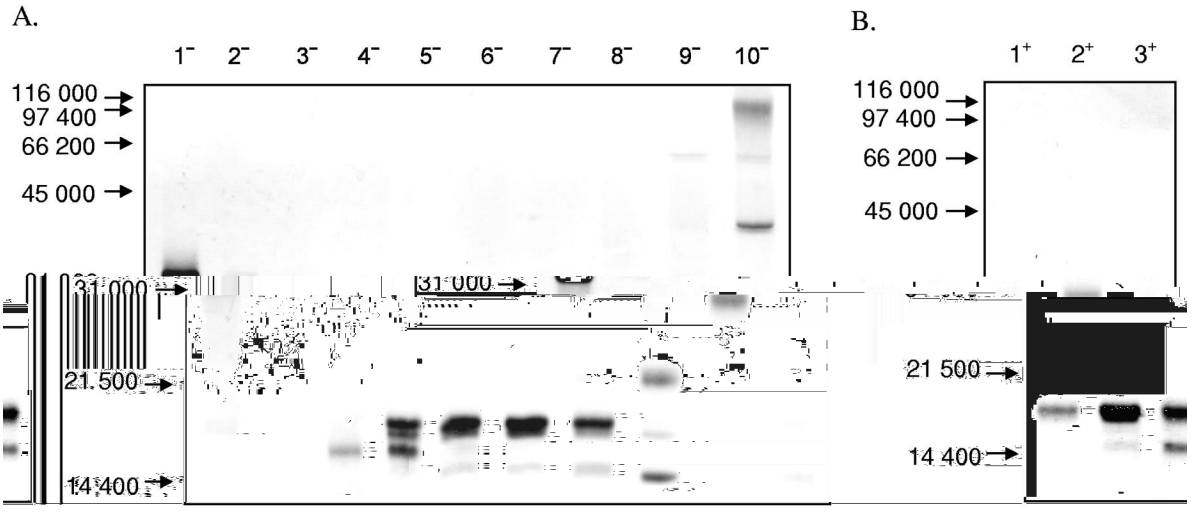



Fig. 4. SDS electrophoresis of RP-HPLC separated components of non-phosphorylcholine-binding (A) and phosphorylcholine-binding (B) fractions of bull seminal plasma. A: $1^{-}$–$10^{-}$; B: $1^{+}$–$3^{+}$ (Fig. 3).

was used for the preparation of thiophilic adsorbents (T-gels) [21].

Both types of the prepared affinity carriers containing immobilized L-glyceryl phosphorylcholine were used for the separation of phosphorylcholine-binding proteins from boar and bull seminal plasma. The adsorbed proteins were eluted specifically with the phosphorylcholine solution. No proteins of bull seminal plasma interacted with the matrix containing the spacer arm formed by the divinyl sulfone derivative. The Sepharose-based affinity matrix was characterized by higher capacity than that of the polyacrylamide derivative; this fact is related to the substitution degree of the adsorbents. The results of SDS electrophoresis and RP-HPLC showed that the composition of phosphorylcholine-binding protein fractions from boar seminal plasma was the same as that obtained by a commonly used procedure (elution of proteins adsorbed on immobilized heparin with phosphorylcholine solution) [7,15,16]. No proteins of human seminal plasma were adsorbed to L-glyceryl phosphorylcholine-Sepharose. The absence of phosphorylcholine-binding proteins in human seminal plasma was confirmed by affinity chromatography on immobilized heparin followed by phosphorylcholine elution.

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## References

[1] S.P. Ballou, I. Kushner, Adv. Intern. Med. 37 (1992) 313.
[2] N.Y. Nguyen, A. Suzuki, S.M. Cheng, G. Zon, T.Y. Liu, J. Biol. Chem. 261 (1986) 10450.
[3] V. Lund, J.A. Olafsen, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 149 (1998) 471.
[4] C. Mandal, M. Biswas, A. Nagpurhor, S. Mookerjea, Dev. Comp. Immunol. 15 (1991) 227.
[5] L. Desnoyers, P.J. Manjunath, J. Biol. Chem. 267 (1992) 10149.
[6] L. Leblond, L. Desnoyers, P. Manjunath, Mol. Reprod. Dev. 34 (1993) 443.
[7] J.J. Calvete, M. Raida, M. Gentzel, C. Urbanke, L. Sanz, E. Topfer-Petersen, FEBS Lett. 407 (1997) 201.
[8] J. Liberda, T. Trnka, J. Sejbal, V. Jonáková, M. Kraus, M. Tichá, Chimia 53 (1999) 528.
[9] P. Maňásková, J. Liberda, M. Tichá, V. Jonáková, Folia Biologica (Praha) 46 (2000) 143.
[10] E.B. Oliveira, E.C. Gotschlich, T.Y. Liu, J. Immunol. 124 (1980) 1386.

[11] F.A. Robey, T.Y. Liu, J. Biol. Chem. 256 (1981) 969.
[12] T.F. Spande, J. Org. Chem. 45 (1980) 3081.
[13] L.M. Martin, Tetrahedron Lett. 37 (1996) 7921.
[14] N.L. Stults, Y.C. Lee, C.A. Hoppe, K. Kawaguchi, S. Kohda, I. Takagahara, T. Koishi, T.Y. Liu, Anal. Biochem. 161 (1987) 567.
[15] J.J. Calvete, K. Mann, L. Sanz, M. Raida, E. Topfer-Petersen, FEBS Lett. 399 (1996) 147.
[16] V. Jonáková, M. Kraus, L. Veselský, D. Čechová, K. Bezouška, M. Tichá, J. Reprod. Fertil. 114 (1998) 25.
[17] M. Tichá, B. Železná, V. Jonáková, K. Filka, J. Chromatogr. 656 (1994) 423.
[18] J. Porath, T. Lass, J.C. Jansen, J. Chromatogr. 103 (1975) 680.
[19] U.K. Laemmli, Nature (London) 227 (1970) 580.
[20] P.S. Chen, T. Toribara, H. Warner, Anal. Chem. 13 (1956) 1756.
[21] J.A. Scoble, R.K. Scopes, J. Chromatogr. A 787 (1997) 47.
[22] J.J. Calvete, P.F. Paloma, F. Varela, L. Sanz, A. Romero, K. Mann, E. Topfer-Petersen, Protein Expr. Purif. 8 (1996) 48.
[23] P. Watson, in: G. Morris, A. Clarke (Eds.), Effects of Low Temperature on Biological Membranes, Academic Press, London, 1981, p. 189.
[24] V. Jonáková, P. Maňásková, M. Kraus, J. Liberda, M. Tichá, Mol. Reprod. Dev. 56 (2000) 275.
[25] M. Fouillet, M. Levi-Strauss, V. Gindicelli, D. Lutomski, D. Bladier, M. Caron, R. Jourbet-Caron, J. Chromatogr. B 706 (1998) 167.
[26] S.K. Nadimpalli, V.L. Zerramalle, A. Hille-Rehfeld, K. von Figura, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 123 (1999) 261.
[27] P. Pauw, C. Neyt, E. Vanderwinkel, R. Wattiez, P. Falmagne, Protein Expr. Purif. 6 (1995) 371.
[28] J.D. Cornillot, M. Caron, R. Jourbet-Caron, D. Bladier, Int. J. Biochem. 24 (1992) 1585.
[29] H. Arnoštová, Z. Kučerová, I. Tišlerová, T. Trnka, M. Tichá, J. Chromatogr. A 911 (2001) 211.
[30] J. Liberda, M. Kraus, H. Ryšlavá, M. Vlasáková, V. Jonáková, M. Tichá, Folia Biologica (Praha) 47 (2001) 113.