PURIFICATION OF GTPγS BINDING PROTEINS FROM MEMBRANES OF PORCINE BRAIN USING CONVECTIVE INTERACTION MEDIA (CIM®) SUPPORTS

Aljoša BAVEC[*], Aleš PODGORNIK[**] and Matjaž ZORKO[*]

**Institute of Biochemistry, Medical Faculty, University of Ljubljana, Ljubljana, Slovenia*

***BIA Separations d.o.o., Ljubljana, Slovenia*

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Abstract

Membranes from porcine brain bind relatively large quantities of guanosine 5'-(3-*0*-thio) triphosphate (GTPγS), a poorly-hydrolysable analogue of GTP. Chromatography of solubilized proteins with 1% Sodium cholate using CIM® DEAE column (Convective Interaction Media) revealed two fractions reach in GTPγS binding proteins. In reduced SDS-PAGE analysis only these two fractions showed a protein band with molecular mass 42 kDa which corresponded to α subunits G_s, G_i or G_{11}. Comparison between CIM® DEAE and traditional DEAE-Sephacel methods showed that membrane bound heterotrimeric G-proteins might be isolated by HPLC using CIM® supports on second or minute time scale.

Introduction

Many extracelullar signals are mediated by heptahelical transmembrane spanning receptors coupled to heterotrimeric guanine-nucleotide proteins (G-proteins), important components of cellular signal cascade. An agonist stimulated receptor activates membrane bound heterotrimeric G-proteins, which cycle between an inactive and active configuration by promoting the exchange of GDP bound to G_α subunit for GTP and by catalysing its subsequent hydrolysis into GDP and phosphate.[1] GTP-liganded α subunit activates effectors that regulate a variety of enzymes or ion channels.[2, 3] Instead of GTP, G_α subunit can bind GTPγS (guanosine 5'-(3-*0*-thio), triphosphate) a poorly-hydrolysable analogue of GTP and cause the same effect in cell response as GTP bound G_α subunit.[4,5] Purification of heterotrimeric G-proteins from different types of tissue was studied and well described in literature,[4,5] but all known methods require complex and long

procedure. Since heterotrimeric G-proteins are very unstable,[5] development of fast separation method would be of great interest. In our study we investigated separation of high affinity GTPγS binding proteins (heterotrimeric G-proteins) from plasma membrane of porcine brain by HPLC using CIM® (Convective Interaction Media) supports.

CIM® monoliths are novel chromatographic supports based on a monolithic porous polymer with well-defined bimodal pore size distribution. In contrast to conventional porous particle based chromatographic supports, pore in inside the CIM® monoliths are highly interconnected. During analysis the mobile phase is forced to flow through these channels and the molecules to be separated are transported by convection rather than by diffusion. Since this process is for 2-3 orders of magnitude faster, the overall analysis time can be significantly lowered. Furthermore, separation efficiency as well as dynamic binding capacity becomes flow-independent. Because of these characteristics CIM® supports proved to be an efficient tool for cytosolic protein separation on second or minute time scale.[6, 7]

No study of separation of membrane bound proteins by CIM® supports have been done so far.

Experimental

Plasma Membrane Preparation: Porcine brains were obtained from the heads of freshly decapitated pigs and stored in liquid nitrogen. All further procedures were carried out at 0-4 °C. The whole frozen brain (180 g) was sliced and homogenised in 10 mM potassium phosphate buffer (pH = 7.0) in BRAUN homogenizer and the membranes were collected by centrifugation at 11.000 x *g* for 90 minutes. The membranes were washed at 11.000 x *g* for 90 minutes and then resuspended to a protein concentration of about 20 mg/ml for storage at -70 °C.

Solubilization of Plasma Membrane Proteins: Frozen membranes were thawed and centrifuged at 20.000 x *g* for 1h. The pellet was washed with TED buffer (20 mM Tris-Cl, 1 mM EDTA, 1mM dithiothreitol, pH = 8) containing 100 mM NaCl. The washed membranes were solubilized with TED buffer supplemented with 1% Sodium cholate, which proved to be an efficient solubilizer of GTP-binding proteins from plasma

membrane,[8] and incubated with continuos stirring for 1h at 4 °C. Remaining membrane fragments were removed by centrifugation at 98.000 x *g* for 60 minutes and supernatant containing extracted proteins was used immediately.

Purification of the GTPγS-binding Proteins: Supernatant was applied to an analytical weak ion exchange CIM® DEAE column (BIA Separations, Ljubljana, Slovenia) which had been equilibrated with TED/1% Sodium cholate in HPLC system. G-proteins were eluted from CIM® DEAE column with gradient of NaCl (0-2000 mM) in TED/1% Sodium cholate and identified by their ability to bind GTPγS. Additionally, some preliminary purification with CIM® MELITTIN column in affinity chromatography mode was made. Supernatant was used in similar way as indicated above. All operations were carried out at 4 °C.

CIM® MELITTIN Column Preparation: CIM® epoxy disk was placed in 0.5 M phosphate buffer solution, pH = 8.0, containing 400 μg/ml of melittin and incubated for 4 days at 4 °C. After immobilisation was completed, CIM® disk was inserted in CIM® housing, extensively washed with 0.5 M phosphate buffer, pH = 8.0 and distilled water to remove non immobilised melittin.

Binding Assay: G-proteins were identified by their ability to bind GTPγS as described in the literature,[4] with minor modifications. Briefly, samples containing G-proteins (10 μl) were mixed with a Hepes buffer (30 μl; 10 mM Hepes-KOH buffer (pH 8), 1 mM EDTA, 1 mM dithiothreitol, 0.1 % Lubrol PX), and [^{35}S] GTPγS buffer (40 μl; 2.5 μCi/ml [^{35}S]GTPγS ≈ 100.000 cpm, 2 μM GTPγS, 40 mM $MgCl_2$, 50 mM Hepes-KOH buffer pH=8, 1 mM EDTA, 1 mM dithiothreitol, 200 mM NaCl). The mixture was incubated at 30 °C for 45 minutes. [^{35}S]GTPγS bound to protein was determined by ice-cold rapid filtration of the samples (70 μl) through membrane filters. The filters were washed four times with 2 ml of a washing solution (20 mM Tris-HCl buffer pH=8, 0.1 mM NaCl, 25 mM $MgCl_2$). incubated in 20 ml of Emulssier Safe scintillation mixture (PACKARD), for 24 hours in dark and analysed for radioactivity counting.

SDS-polyacrylamide Gel Electrophoresis: Gel-Electrophoresis of plasma membrane proteins on 12 % polyacrylamide gel was accomplished with the discontinuous system described by Laemmli.[9]

Materials: $MgCl_2$, NaCl and EDTA were from Merck, Darmstadt, Germany; [γ-^{35}S]GTPγS was from Amersham, UK; Emulssier Safe scintillation mixture was from Canberra Packard, USA; CIM® DEAE and CIM® epoxy column were from BIA Separations, Ljubljana, Slovenia. All other chemicals were from Sigma, St. Louis, MO, USA, and were of analytical grade.

Results and discussion

Since G-proteins require presence of detergent in mobile phase to prevent its deactivation and, in addition, to diminish non-specific hydrophobic adsorbtion on the column matrix, the effect of detergent addition was investigated first. In Figure 1 it is shown change of absorbancy when the TED buffer is switched with firstly the TED buffer containing 1% Sodium cholate and secondly with TED buffer containing 0.1% Lubrol PX. Since the absorbency of Lubrol PX was too high all further experiments were done using 1% Sodium cholate in the mobile phase.

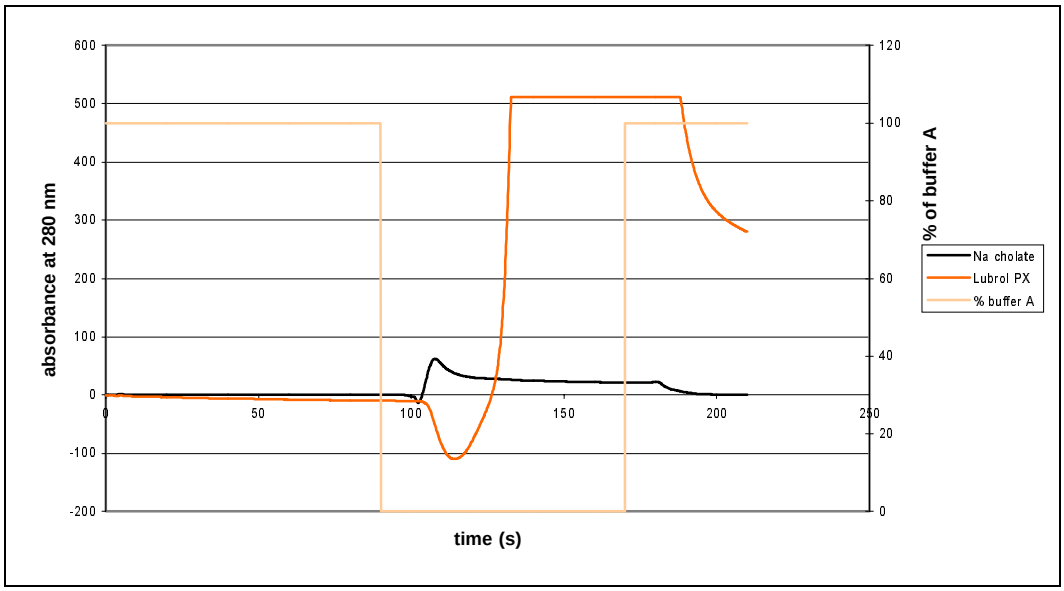

Figure 1: Baseline absorbency of TED buffers with detergents Sodium cholate and Lubrol PX. Buffer A: TED, pH = 8.0; buffer B: buffer A+1% Sodium cholate. Buffer B_2: buffer A+0.1% Lubrol PX, pH = 8.0; Gradient: 100% A in 90s, 0% A for 1 min, 100% A for 1 min; Flow rate: 4 ml/min. Detection: UV-VIS at 280 nm.

For the separation of solubilized proteins gradient method using weak ion-exchange CIM® DEAE column was applied. Many chromatographic analyses were needed to find the optimal gradient shape. However, since the analysis time of a single run in the case of CIM® supports is very short (less then 3 minute) the entire optimisation procedure was completed in less than one hour. Optimised chromatographic method revealed two fractions (peaks 2 and 3 in Figure 2) reach in GTPγS-binding proteins (Table 1). In reduced SDS-PAGE analysis only these two fractions showed a protein band with an apparent molecular mass ≈ 42 kDa which corresponded to α subunits G_s, G_i or G_{11} [1, 10, 11] of G-proteins (lane 2 and 3 in Figure 3).

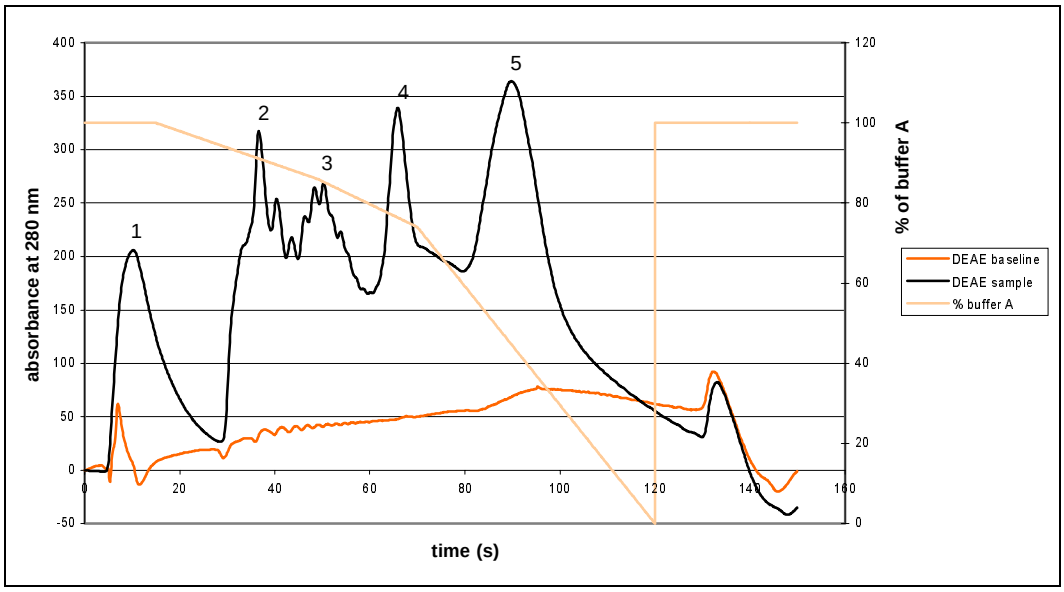

Figure 2: Separation of GTPγS-binding plasma membrane proteins by HPLC on CIM® DEAE disk. Buffer A: TED/1% Sodium cholate, pH = 8; buffer B: buffer A + 2 M NaCl; gradient: 100% A in 15 s, 0-14% B for 35 s, 14-26 B for 20 s, 26-100 B for 50 s, 100% A for 30 s; flow rate: 4 min/ml. Detection: UV-VIS at 280 nm.

Table 1: Analysis of GTPγS-binding proteins after CIM® DEAE chromatography.

FRACTION	PROTEIN CONC.	PROTEIN	GTPγS BINDING	GTPγS BINDING	SPECIFIC ACTIVITY
	mg/ml	*μg*	*cpm*	*pmol*	*pmol/mg*
1	0.246	2.153	139	0.093	43.1
2	0.328	2.625	2182	1.47	560.1
3	0.262	2.293	588	0.396	172.8

4	0.283	2.476	56	0.0377	37.7
5	0.213	1.863	112	0.0754	75.4

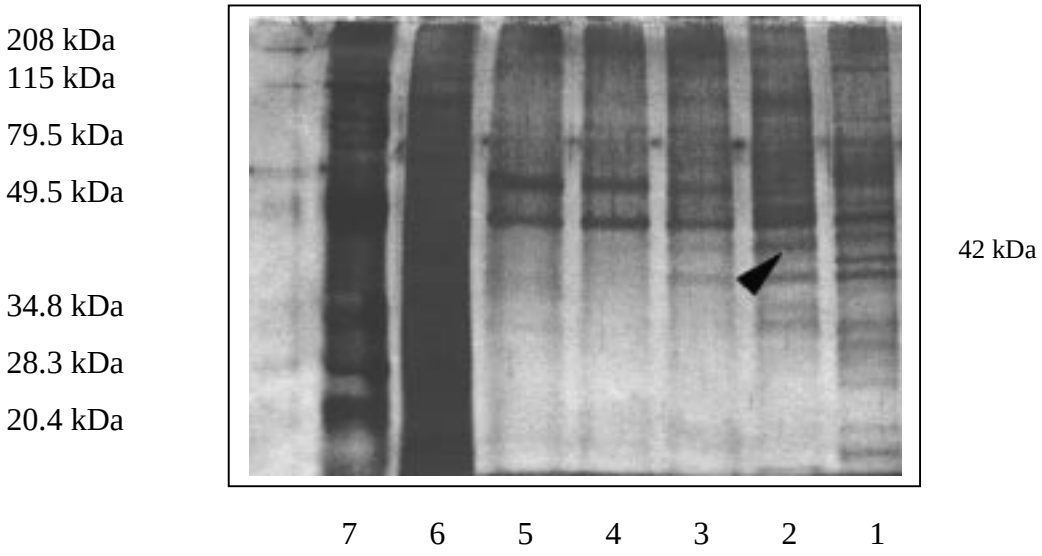

Figure 3: SDS-polyacrylamide analysis of fractions obtained from CIM® DEAE chromatography. Lane 1: 2.19 μg of fraction 1, lane 2: 2.51 μg of fraction 2, lane 3: 1.96 μg of fraction 3, lane 4: 1.86 μg of fraction 4, lane 5: 2.27 μg of fraction 5, lane 6: 10 μg of membrane extract, lane 7: 6.25 μg of standard

Comparison between CIM® DEAE and DEAE-Sephacel methods[4] showed the same effectiveness in purification steps up to 10 fold. However, in the case of CIM® DEAE column the analysis time was 10-100 time shorter. A difference in absolute specific activity, which is almost one order of magnitude lower in case of porcine brain plasma membranes, can be attributed to different source material or/and in different plasma membrane preparation protocols (Table 2).

Table 2: CIM® DEAE (A) and conventional DEAE-Sephacel purification (B) of the proteins that binds GTPγS in membranes from bovine brain (A) and porcine brain (B). Data for conventional DEAE-Sephacel purification are taken from literature.[4]
A)

STEP	PROTEIN CONC.	PROTEIN	GTPγS BINDING	SPECIFIC ACTIVITY	PURIFICATION
	mg/ml	*μg*	*pmol*	*nmol/mg*	*fold*

Membranes	7.5	66.1	3.5	0.05	1.0
Extract	2.7	23.7	2.4	0.10	2.0
DEAE CIM	0.3	0.7	0.4	0.56	11.2

B)

STEP	PROTEIN KONC.	PROTEIN	GTPγS BINDING	SPECIFIC ACTIVIY	PURIFICATION
	mg/ml	*mg*	*nmol*	*nmol/mg*	*fold*
Membranes	6.0	12.00	4.40	0.37	1.0
Extract	2.3	3.80	2.60	0.70	1.9
DEAE-Sephacel	1.1	0.28	1.07	3.80	10.0

It is known that melittin, component of bee venom, can activate GTPase activity of rat brain cortical membranes,[12] stimulate G_i and G_{11} and inhibit G_s activities of synaptic membranes of rat brain cerebral cortex.[13] We used melittin as a source for preparation CIM® MELITTIN affinity column. When we tried to elute bound protein with the decrease of pH value as normally recommended for affinity elution, the mobile phase become milky white due to the presence of a detergent. Since we couldn't avoid detergent we applied elution with 2M NaCl instead of pH change, having in mind that the analysis might show false results. Nevertheless, affinity separation revealed one peak much higher the baseline signal (Fig. 4). To verify if the peak is due to the non-specific binding to the matrix we repeated similar experiment, this time however with the CIM® epoxy column instead of CIM® MELITTIN column. Although there was some protein binding with the matrix, but the peak was significantly lower than in the case of affinity column (Fig. 5). Analysis of GTPγS-binding proteins after CIM® MELITTIN affinity column chromatography revealed an insignificant increase in GTPγS binding activity. According to our results purification of heterotrimeric G-proteins using melittin affinity column has not been successful. The reason could be a low concentration of melittin incubated with CIM® disk or a very low yield of protein eluted from disk. CIM® column affinity chromatography using different amphipathic peptides, such are melittin and mastoparan, or different types of immunoglobulins, is still under further investigation.

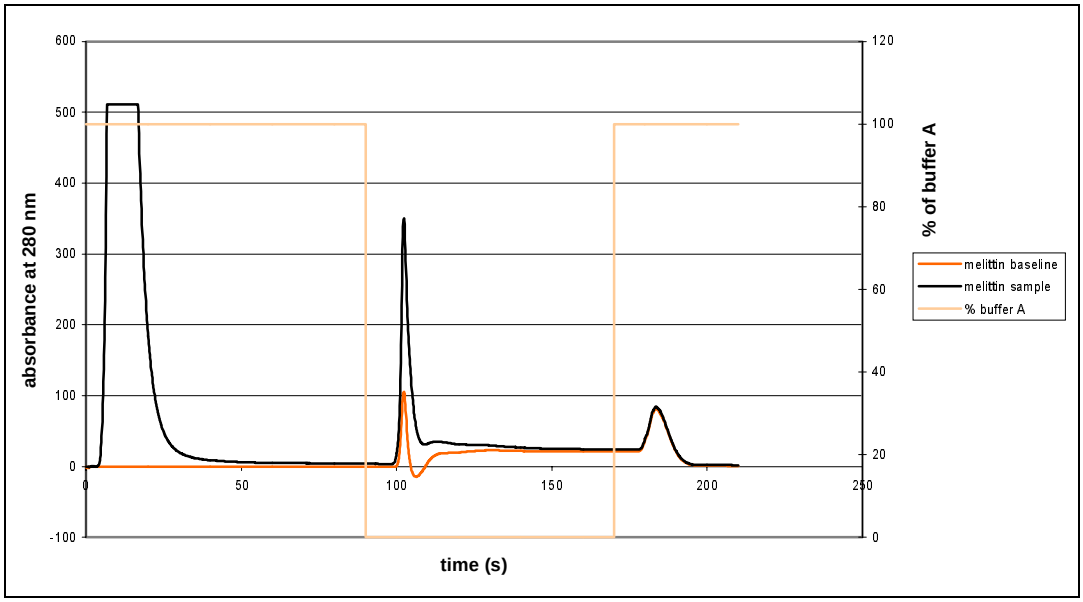

Figure 4: Separation of GTPγS-binding plasma membrane proteins by HPLC on CIM MELITTIN affinity disk. Buffer A: TED/1% Sodium cholate, pH = 8; buffer B: buffer A + 2 M NaCl; gradient: 100% A in 90 s, 100% B in 80 s, 100% A for 40 s; flow rate: 4 min/ml. Detection: UV-VIS at 280 nm.

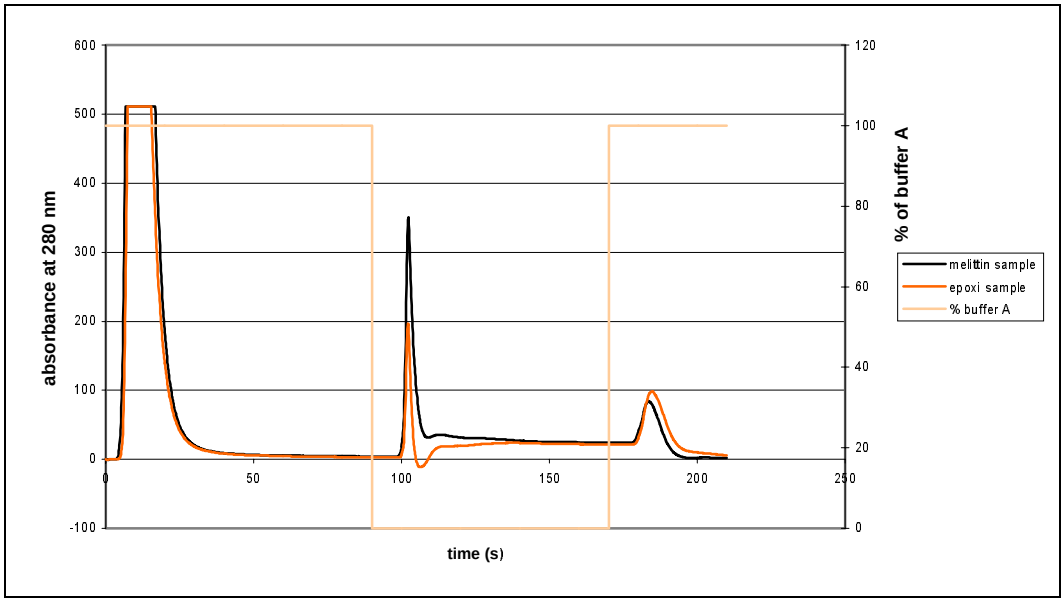

Figure 5: Separation of GTPγS-binding plasma membrane proteins by HPLC on CIM® MELITTIN affinity disk and CIM (epoxy) disk. Buffer A: TED/1% Sodium cholate, pH = 8; buffer B: buffer A + 2 M NaCl; gradient: 100% A in 90 s, 100% B in 80 s, 100% A for 40 s; flow rate: 4 min/ml. Detection: UV-VIS at 280 nm.

Conclusions

Our results suggest that membrane bound proteins might be isolated and partly purified by HPLC using CIM® DEAE supports. The analysis time is at least for one order of magnitude shorter in comparison with classical separation chromatography methods, which is great advantage in purification of highly unstable plasma membrane heterotrimeric G-proteins.

References and notes

1. G. Gilman, *Annu. Rev. Biochem.* **1987**, *56*, 615-649.
2. M. I. Simon, M. P. Strathmann, N. Gautman, *Science* **1991**, 25**2**, 802-808.
3. E. J. Neer, *Protein Sci.* **1994**, *3*, 3-14.
4. P. C. Sternweis, J. D. Robishaw, *J. Biol. Chem.* **1984**, *259*, 13806-13813.
5. T. Katada, M. Oinuma, M. Ui, *J. Biol. Chem.* **1986**, 261, 8182-8191.
6. H. Podgornik, A. Podgornik, A. Perdih, *Anal. Biochem.* **1999**, *272*, 43-47.
7. Štancar, M. Barut, A. Podgornik, P. Koselj, Dj. Josić, A. Buchacher, *LCGC Int*. **1998**, *11*(10), 660-669.
8. Bavec, A.Juréus, B. Cigić, Ü. Langel, M. Zorko, *Peptides* **1999**, *20*, 177-184.
9. U. K. Laemmli, *Nature* **1970**, *227*, 680-685.
10. F. R. McKenzie, *Basic techniques to study G-protein function* In: E. Milligan, ed. Signal Transduction, Oxford University Press, London, 1992, pp 31-56.
11. J.R. Hepler,T. Kozasa, A.V. Smrcka, M.I. Simon, S.G. Rhee, P.C. Sternweis, A.G. Gilman. *J. Biol. Chem.* **1993**, *268*(19):14367-14375.
12. Bavec, U. Soomets, Ü. Langel, M. Zorko, *Acta Chim. Slov.* **1998**, *45*(1), 27-54.
13. N. Fukushima, M. Kohno, T. Kato, S. Kawamoto, K. Okuda, Y. Misu, H. Ueda, *Peptides* **1998**, *19*(5), 811-819.

Povzetek

Plazemske membrane iz prašičjih možgan vežejo relativno visoko količino gvanozin 5'(3-*O*-tio) trifosfata (GTPγS). GTPγS je analog GTP, ki z razliko od GTP zelo slabo hidrolizira na GDP in fosfat. Separacija membranskih proteinov, ki so bili solubilizirani z 1% detergentom natrijevim holatom pri ionsko izmenjevalni kromatografiji na CIM® DEAE koloni, je pokazala dva vrhova, ki vsebujata GTPγS vezavne proteine. SDS-PAGE analiza je potrdila, da samo frakciji, ki sta bogati z GTPγS vezavnimi proteini, vsebujeta proteine z M_r 42 kDa, ki ustreza G_s, G_i ali G_{11} podenoti G_α G-proteinov. Primerjava obeh kromatografij CIM® DEAE in DEAE–Sephacel je pokazala, da se heterotrimerni membranski proteini lahko izolirajo z uporabo CIM® nosilcev, pri čemer je hitrost

separacije pri CIM® DEAE od 10-100-krat večja kot pri standardni DEAE – Sephacel koloni.