Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. *75* (11) 1481–1489 (2010)
JSCS–4070
UDC 577.112+577.175.72:539.2:543.544
Original scientific paper

An investigation of the different molecular forms of IGFBP-1 using immobilised metal-, immuno- and lectin-affinity chromatography

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(Received 30 March, revised 2 July 2010)

Abstract: The insulin-like growth factor-binding protein 1 (IGFBP-1) is a member of a family of six homologous proteins that regulate the action of the insulin-like growth factors. IGFBP-1 is a 25 kDa protein that, in addition to its native form, may exist in several phosphoforms (30 kDa), which are predominant in the circulation of humans. Phosphorylation of IGFBP-1 is a post-translational modification that has a great influence on the action of IGF-I. IGFBP-1 forms multimers and complexes with α2-macroglobulin (α2M). Polymerisation of IGFBP-1 was also reported. In order to analyse and separate these IGFBP-1 molecular species, affinity chromatography methods were used in this study. The results demonstrated that most of the IGFBP-1 circulates in complexes with α2M, which can be isolated by affinity chromatography using immobilised anti-α2M antibodies. IGFBP-1/α2M complexes may be differentiated from IGFBP-1 dimer and multimers using lectin-affinity chromatography, since the latter do not interact with lectins. It seems that the complexes contain not only monomeric IGFBP-1, but also its multimers. The dimer and multimers are stable under reducing conditions, suggesting a covalent linkage between the units. Free IGFBP-1 monomer can be separated from multimers using Con A-affinity chromatography. The concentration of free IGFBP-1 is relatively low in the circulation.

Keywords: IGFBP-1; isoforms; complexes; affinity chromatography.

INTRODUCTION

Insulin-like growth factors (IGF-I and IGF-II) are polypeptides, which regulate cell growth, differentiation and metabolism.[1] In the circulation they are associated with IGF binding proteins (IGFBPs), a family that consists of six homo-

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doi: 10.2298/JSC100330090L

logous proteins that modulate the bioactivity of IGFs. IGFBPs can either inhibit or enhance the activity of IGFs or have IGF independent effects.[2] Only the free (unbound) form of IGF is considered to be biologically active.[3]

IGFBP-1 is synthesised in many cell types, including hepatocytes, ovarian granulose cells, decidualised endometrium[2] and cells of connective tissue.[4] It is the predominant binding protein in amniotic fluid. In healthy individuals, the concentration of circulating IGFBP-1 fluctuates tenfold or more within a few hours as a response to changes in insulin concentration.[3] The concentrations of insulin and IGFBP-1 alter in opposite directions.

IGFBP-1 is a 25 kDa protein that may exist in several phosphoforms of approximately 30 kDa.[5] Up to five IGFBP-1 forms, varying in the degree of phosphorylation, have been identified.[6,7] Numerous studies have indicated that human IGFBP-1 may both potentiate or inhibit IGF-I stimulated biological activity.[8,9] The differences in IGFBP-1 actions are due to differential phosphorylation of IGFBP-1 that alters its affinity for IGF-I.[10] Potential phosphorylation sites of human IGFBP-1 are Ser101, Ser119 and Ser169.[9] Highly phosphorylated IGFBP-1 is the predominant isoform of IGFBP-1 in the circulation of a healthy adult, having affinity for IGF-I that is six- to eightfold higher than that of non-phosphorylated IGFBP-1.[11] Since the non-phosphorylated form binds IGF-I less tightly, enhancing its actions,[9,11] a delicate equilibrium between phosphorylated and non-phosphorylated isoforms is responsible for the regulation of IGF-I activity.

In the circulation, IGFBP-1 associates with the glycoprotein α_2-macroglobulin (α_2M, ≈700 kDa), which protects IGFBP-1 from proteolysis.[6] Thus, the IGFBP-1/α_2M complex may have an important role in controlling IGF-I action by regulating the amount of free IGFBP-1. In tissues, IGFBP-1 was found to form multimers, the role of which in the action of IGF remains to be determined.[12]

The aim of this study was to investigate IGFBP-1 isoforms, multimers and complexes in the circulation of healthy persons, using different affinity chromatography matrices. The affinity chromatography methods were based on the following interactions: metal ion–phosphoprotein, antigen–antibody and lectin–glycoprotein.

EXPERIMENTAL

Samples

Blood samples were obtained from healthy adult volunteers (n = 10). Venous blood was collected after 12 h fasting. The serum was separated by centrifugation within 45 min and kept frozen at –20 °C until used. The study was approved by the local ethics committee of the Institute for the Application of Nuclear Energy.

Immobilised metal-affinity chromatography (IMAC)

Iminodiacetic acid-agarose (IDA) was purchased from Sigma-Aldrich (Steinheim, Germany) and 1 mL of gel was packed into a column. After the column had been washed with distilled water, 1.0 mL of 0.10 mol L^{-1} ferric chloride solution was applied in order to saturate

the column with ferric ions. Unbound metal ions were washed out with 5 ml of distilled water and 5 ml of 0.1 % acetic acid. The column was equilibrated with dilution buffer: 0.05 mol L^{-1} MES (2-(*N*-morpholino)ethanesulphonic acid)/0.5 mol L^{-1} NaCl buffer pH 5.5.[13] Serum samples (100 μL diluted in 900 μL of dilution buffer) were circulated through the column for 1 h to ensure maximal binding. The unbound material was washed out with 5 mL of MES buffer. Elution of the bound molecules was performed using 8 mL of 0.2 mol L^{-1} Na-phosphate buffer pH 8.0. The column was regenerated using 10 mL of 0.1 mol L^{-1} Na-borate/1.0 mol L^{-1} NaCl buffer pH 10.0 and 10 mL of distilled water, followed by saturation with ferric chloride. The collected fractions (1.0 mL each) were kept at –20 °C until electrophoresis and immunoblotting were performed.

Immunoaffinity chromatography (IgY-C)

An IgY-12 column (1.2 mL of microbeads) was purchased from Beckman Coulter (Fullerton, USA, Proteomelab IgY-12 High Capacity Proteome Partitioning kit). The matrix with immobilised antibodies enables the binding and removal of albumin, IgG, IgA, IgM, α1-antitrypsin, haptoglobin, transferrin, orosomucoid, α_2M, HDL (apo A-I and apo A-II) and fibrinogen. The IgY column was loaded with 20 μL of serum diluted with 480 μL of dilution buffer: 10 mmol L^{-1} Tris (tris(hydroxymethyl)aminomethane)–HCl/1 mol L^{-1} NaCl buffer pH 7.4, and incubated for 15 min at room temperature using a rotator.[14] Unbound proteins were separated by centrifugation for 30 s at 2000 × g and the column was washed three times with dilution buffer. Bound proteins were eluted with 500 μL of elution buffer: 0.1 mol L^{-1} glycine–HCl buffer pH 2.5, for 3 min at room temperature using a rotator. They were separated from the gel by centrifugation and immediately neutralised with 50 μL of 1 mol L^{-1} Tris–HCl buffer pH 8.0. The column was washed three times with the elution buffer, neutralised with 600 μL of 2 mol L^{-1} Tris–HCl buffer pH 8.0 and washed with a dilution buffer prior to the next chromatographic cycle. The collected fractions (1 mL each) were kept at –20 °C until electrophoresis and immunoblotting were performed.

Lectin-affinity chromatography (LAC)

Eleven agarose-immobilised lectins purchased from Vector Laboratories (Burlingame, CA, USA) were packed into columns: Con A (lectin from *Canavalia ensiformis*), SNA (*Sambucus nigra* agglutinin), RCA-I (*Ricinus communis* agglutinin I), PHA-E (*Phaseolus vulgaris* erythroagglutinin), PHA-L (*Phaseolus vulgaris* leukoagglutinin), WGA (wheat germ agglutinin), succinylated WGA, ECL (*Erythrina cristagalli* lectin), UEA (*Ulex europaeus* agglutinin), LCA (*Lens culinaris* agglutinin) and MAL (lectin from *Maackia amurensis*). All columns except Con A- and LCA-agarose were equilibrated in 0.01 mol L^{-1} HEPES buffer, containing 0.15 mol L^{-1} NaCl, 0.8 g L^{-1} NaN_3, pH 7.5 (HBS). The Con A-agarose was equilibrated in 0.02 mol L^{-1} HEPES buffer containing 0.5 mol L^{-1} NaCl, 0.8 g L^{-1} NaN_3, 1.0 mmol L^{-1} $CaCl_2$, $MgCl_2$ and $MnCl_2$, pH 7.5, whereas the LCA-agarose was equilibrated in 0.01 mol L^{-1} HBS containing 0.8 g L^{-1} NaN_3, 0.1 mmol L^{-1} $CaCl_2$ and 0.01 mmol L^{-1} $MnCl_2$, pH 7.5. The HBS used for ECL-, SNA-, UEA- and MAL-agarose equilibration contained 0.1 mmol L^{-1} $CaCl_2$, whereas the HBS used for the equilibration of PHA-E and PHA-L-agarose contained 0.1 mmol L^{-1} $CaCl_2$ and 0.01 mmol L^{-1} $MnCl_2$. The pH of the HBS for the PHA-E-agarose equilibration was 8.0. Serum samples (100 μL diluted with 900 μL of the corresponding HBS) were circulated through the columns for 1 h. The unbound material was washed out with 20 mL of the appropriate HBS. The elution of the bound glycoproteins was generally performed in two consecutive steps: 1) neutral elution using hapten sugar dissolved in 15 mL of the appropriate HBS, pH 7.5 and 2) acidic elution with 7 mL of hapten sugar solution in 0.1 M

acetic acid, pH 3.0. The PHA-E and PHA-L-agarose columns were eluted in one-step only, using 10 mL of a 0.1 mol L^{-1} acetic acid solution. The hapten sugars used for specific elutions were: 0.5 mol L^{-1} lactose for MAL- and SNA-agarose, a mixture of 0.2 mol L^{-1} methyl-α-glucopyranoside and 0.2 mol L^{-1} methyl-α-mannopyranoside for Con A- and LCA-agarose, 0.2 mol L^{-1} lactose for the RCA I- and ECL-agarose, 0.5 mol L^{-1} *N*-acetyl-glucosamine for the WGA- and succinylated WGA-agarose and 0.1 mol L^{-1} fructose for the UEA-agarose.[15] The most concentrated protein fractions (1 ml each) were collected and the acidic fractions were immediately neutralised using 150 μL of 2 mol L^{-1} Tris–HCl buffer pH 8.9. The fractions were dialysed first against distilled water for 3 h at room temperature and then against 0.15 mol L^{-1} NaCl overnight at 4 °C. After being concentrated to approximately 1.0 ml on Microcon centrifugal filter device (Millipore, Billerica, MA, USA) with a 10 kDa cut-off membrane, the samples were kept frozen at –20 °C until electrophoresis and immunoblotting.

Electrophoresis and Western immunoblotting (WIB)

The most concentrated protein fractions obtained after affinity chromatography were subjected to: native polyacrylamide gel electrophoresis (nPAGE), denaturing non-reducing sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and denaturing reducing SDS-PAGE with 10 % 2-mercaptoethanol (v/v) in the sample buffer (rSDS-PAGE) using 12 % gels.[16]

Immunoblotting was performed using goat polyclonal anti-IGFBP-1 antiserum (DSL, Webster, USA), followed by an anti-goat IgG antibody coupled to horseradish peroxidase (Biosource, Camarillo, CA, USA) and a rabbit polyclonal anti-α2M antibody (IgG fraction, AbD Serotec, Oxford, UK), followed by an anti-rabbit IgG antibody coupled to horseradish peroxidase (Pierce Biotechnology, Rockford, IL, USA). The immunoreactive proteins were visualised using an enhanced chemiluminescence (ECL) reagent kit (Pierce, Minneapolis, MN, USA).[16] Molecular mass markers were from BioRad Laboratories (Hertfordshire, UK) and the cytosol from human placental cells was used as a source of a physiological marker of IGFBP-1.[17]

RESULTS AND DISCUSSION

Several post-translational modifications are known to modify the affinity of IGFBPs for IGFs, including phosphorylation,[7,18] proteolysis[19] and polymerisation.[20] The mechanisms accounting for these changes remain to be elucidated.[12] Phosphorylation of human IGFBP-1 leads to inhibition of IGF-I action.[9,11] The non-phosphorylated isoform forms multimers more rapidly and to a greater extent compared to the phosphorylated isoform.[12] When complexed to α_2M, IGFBP-1 is protected from proteolysis, but still able to bind IGFs and, thus may be expected to influence the IGF action as well.[6]

In order to assess the importance of various molecular forms of IGFBP-1, it is crucial to develop analytical tools for their investigation. To fulfil this goal, affinity chromatography methods based on three types of molecular recognition, *i.e.*, IMAC, immunoaffinity chromatography and LAC, were employed. Electrophoretic separation followed by immunoblotting of the fractionated proteins was used to identify the different molecular forms of IGFBP-1. Fractions of all serum samples were subjected to SDS-PAGE, rSDS-PAGE and nPAGE. Since all

samples showed the same pattern of IGFBP-1 distribution upon immunoblotting, only the representative results are shown in the figures.

SDS-PAGE revealed that almost all immunoreactive IGFBP-1 forms bound to the IDA column (Fig. 1a). Anti-IGFBP-1 antibodies recognised high molecular mass species as a very wide protein band (or several bands that could not be clearly distinguished) at ≈100 kDa, a protein at ≈60 kDa together with proteins at the start. IGFBP-1 monomer (≈30 kDa) was hardly visible. Matrices with immobilised IDA and chelated ions are widely used for the isolation of phosphoproteins.[13] Phosphate groups are electron-donors and, hence, can serve as ligands for coordination of metal ions. Ions that have the greatest affinity for binding to phosphoproteins are Fe^{3+}, Ga^{3+} and Ni^{2+}, Fe^{3+} being the most widely used metal ion in IMAC.[13,21,22] According to the presented results, different molecular species of IGFBP-1 could not be separated using IMAC, since all of them bound to the matrix. rSDS-PAGE was performed in order to investigate the stability of IGFBP-1 complexes in the presence of 2-mercaptoethanol. Almost all high molecular mass complexes, both in the bound and unbound fractions, dissociated, which resulted in the appearance of proteins of predominantly ≈60 and ≈30 kDa (Fig. 1b). This result implicates the existence of disulphide bonds that maintain molecules within the complex. It also suggests that the ≈ 60 kDa form is stable and resists dissociation into IGFBP-1 monomers. nPAGE was performed aiming to differentiate between the phosphoforms of IGFBP-1 monomer. Unfortunately, all isoforms appeared clustered in the middle of the gel and they could not be clearly separated from one another (Fig. 1c). Only proteins at the start were identified by immunoblotting with anti-α_2M antibodies (Fig. 1d), suggesting that these species of very high molecular mass were IGFBP-1/α_2M complexes. Cytosol

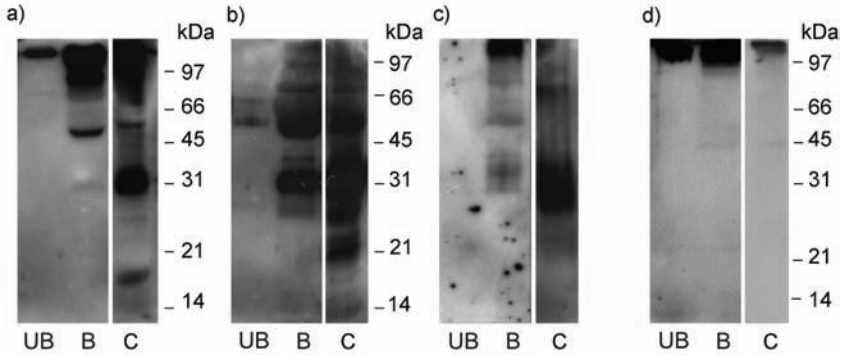

Fig. 1. Immunoblotting of the unbound (UB) and bound (B) fractions obtained after IMAC with anti-IGFBP-1 antibodies after a) SDS-PAGE, b) rSDS-PAGE c) nPAGE, as well as with anti-α_2M antibodies after d) SDS-PAGE. IGFBP-1 from placental cytosol (C) was used as a physiological marker. The positions of the molecular mass markers are indicated on the right side.

gave a different protein profile than that of serum, mostly in terms of the intensity of the bands, as it contains a different proportion of IGFBP-1 molecular forms than serum. The positions of some protein bands in the two types of samples varied slightly due to a difference in sample milieu (the number and abundance of other proteins).

After IgY-C, proteins in the bound fraction were identified as at least three bands of high molecular mass (Fig. 2a), one band at ≈60 kDa and a weak band at ≈30 kDa. In the unbound fraction, high molecular mass bands appeared together with monomer IGFBP-1. In rSDS-PAGE (Fig. 2b) high molecular mass species dissociated giving predominantly a ≈60 kDa protein band in the bound fraction and a ≈30 kDa protein band in the unbound fraction. nPAGE (Fig. 2c), again, proved itself not to be useful for the study of the isoform pattern of IGFBP-1. Immunoblotting with anti-α_2M antibodies (Fig. 2d) confirmed that only proteins at the start of the gel were IGFBP-1/α_2M complexes.

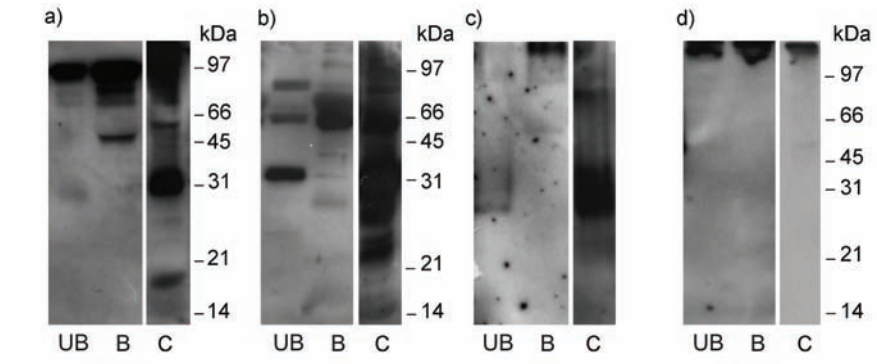

Fig. 2. Immunoblotting of unbound (UB) and bound (B) fractions obtained after IgY-C with anti-IGFBP-1 antibodies after a) SDS-PAGE, b) rSDS-PAGE c) nPAGE, as well as with anti-α_2M antibodies after d) SDS-PAGE. IGFBP-1 from placental cytosol (C) was used as a physiological marker. The positions of the molecular mass markers are indicated on the right side.

The IgY matrix contains immobilised antibodies against α_2M and, according to the intensity of the stained protein band, the majority of IGFBP-1 in circulation is engaged in complex formation with α_2M. The presence of other protein bands in the bound fraction after SDS-PAGE was most likely due to the dissociation of complexes either during the elution of the sample (acidic buffer) and/or during sample preparation for electrophoresis. Since anti-α_2M antibodies recognised only proteins positioned at the start, the immunoreactive bands of ≈100 and ≈60 kDa do not contain fragments of α_2M. These proteins are likely to be multimers of IGFBP-1. The immunoreactive band of ≈60 kDa most likely represents dimeric IGFBP-1, the formation of which may be catalysed by a transglutaminase.[12] To date, dimers were identified only in tissues consisting predominantly

of the non-phosphorylated IGFBP-1 form. IGFBP-1 forms having a mass greater than 60 kDa are most likely higher multimers, resisting the reduction by 2-mercaptoethanol. Multimers of ≈100 kDa were also found in tissues.[12]

Another way to differentiate IGFBP-1 complexes with α_2M from IGFBP-1 multimers is to employ lectins. Lectins have become very important analytical tool in glycoproteomics.[23] In the present study, complexes of IGFBP-1 with glycoprotein α_2M were expected to interact with certain lectins, whereas IGFBP-1 multimers were not, since IGFBP-1 is not glycosylated. The reactivity was tested with eleven immobilised lectins covering a wide range of saccharide specificity. IGFBP-1 immunoreactive forms were detected in pH 7.5 eluates from: Con A-, SNA-, RCA-I- and WGA-agarose as well as in pH 3.0 eluates from Con A- and PHA-E-agarose (Fig. 3a). As expected, anti-IGFBP-1 antibodies recognised only the high molecular mass species (complexes with α_2M).

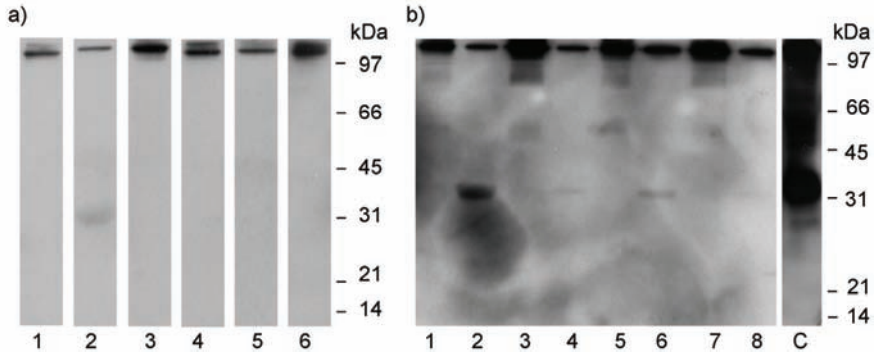

Fig. 3. Immunoblotting with anti-IGFBP-1 antibodies of the bound fractions obtained after LAC and SDS-PAGE. a) The presented samples were eluted from: Con A-agarose (pH 7.5 and 3.0; lanes 1 and 2), SNA-agarose (pH 7.5; lane 3), RCA-I-agarose (pH 7.5; lane 4), PHA-E-agarose (pH 3.0; lane 5) and WGA-agarose (pH 7.5; lane 6); b) The presented samples were eluted from Con A-agarose: in the presence of Ca^{2+}, Mn^{2+} and Mg^{2+} (pH 7.5 and 3.0; lanes 1 and 2), in the presence of Ca^{2+} and Mg^{2+} (pH 7.5 and 3.0; lanes 3 and 4), in the presence of Ca^{2+} and Mn^{2+} (pH 7.5 and 3.0; lanes 5 and 6) and in the presence of Mn^{2+} and Mg^{2+} (pH 7.5 and 3.0; lanes 7 and 8). IGFBP-1 from placental cytosol (C) was used as a physiological marker. The positions of the molecular mass markers are indicated on the right side.

Despite the fact that IGFBP-1 is not glycosylated at all, it bound to Con A and was eluted with the pH 3.0 buffer (Fig. 3a, lane 2). In order to investigate further the nature of this interaction, a series of experiments were performed by varying the presence of ions required for Con A activation (Ca^{2+}, Mn^{2+} and Mg^{2+}). It was demonstrated that all three ions are necessary for the interaction between IGFBP-1 and Con A (Fig. 3b). The absence of Mg^{2+} and Mn^{2+} reduced the binding of IGFBP-1 to some extent, while the absence of Ca^{2+} inhibited the interact-

tion completely. Lectin Con A is a metalloprotein, the 3D structure of which can adopt two conformational states, a “locked” form and an “unlocked” form.[24] The unlocked form of Con A weakly binds metal ions and a saccharide ligand, while the locked form binds two metal ions per monomer, enabling its full binding capacity for the saccharide. Many metastable complexes can be formed as well. Binary, ternary and quaternary complexes having both Con A conformers were identified.[25] Although Con A preferably binds methyl α-D-mannopyranoside and α-D-mannosyl groups, the synthesised ligand peptides, which structurally imitate these saccharides, can also bind to this lectin.[26] The specificity of Con A is not limited to saccharide units, but sugar-mimics may be identified by Con A binding sites as well. Therefore, the binding of IGFBP-1 monomer to Con A-agarose could be explained in the light of the appropriate conformational recognition. Metal ions, especially Ca^{2+}, are crucial for the interaction to occur.

CONCLUSIONS

The results obtained in this study demonstrated that most of the IGFBP-1 circulates associated with α_2M. These complexes can be isolated by affinity chromatography with immobilised anti-α_2M antibodies. IGFBP-1/α_2M complexes may be differentiated from IGFBP-1 dimer and multimers using lectin-affinity chromatography, since the latter do not interact with lectins. It seems that the complexes contain not only monomeric IGFBP-1, but also its multimers. Dimer and multimers are stable under reducing conditions, suggesting covalent linkage between the units. Free IGFBP-1 monomer can be separated from the multimers using Con A-affinity chromatography. The concentration of free IGFBP-1 in circulation is relatively low.

Acknowledgement. This work was supported by the Ministry of Sciences and Technological Development of Serbia, Grant No. 143019.

ИЗВОД

ИСПИТИВАЊЕ РАЗЛИЧИТИХ IGFBP-1 МОЛЕКУЛСКИХ ФОРМИ ПРИМЕНОМ АФИНИТЕТНЕ ХРОМАТОГРАФИЈЕ СА ИМОБИЛИЗОВАНИМ МЕТАЛНИМ ЈОНОМ, АНТИТЕЛИМА И ЛЕКТИНИМА

ДРАГАНА ЛАГУНЏИН, РОМАНА МАСНИКОСА, ГОРАН МИЉУШ, ДРАГАНА РОБАЈАЦ и ОЛГИЦА НЕДИЋ

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Везујући протеин тип 1 за инсулину сличне факторе раста (IGFBP-1) је члан фамилије која садржи шест хомологих везујућих протеина, чија је улога у регулацији активности инсулину сличних фактора раста. IGFBP-1 је протеин од 25 kDa, који осим нативне, може постојати и у облику неколико фосфоформи (30 kDa), које доминирају у циркулацији људи. Фосфориловање IGFBP -1 је пост-транслациона модификација која има велики утицај на активност IGF-I. IGFBP-1 формира мултимере и комплексе са α_2-макроглобулином (α_2M). У циљу анализе и раздвајања IGFBP-1 молекулских врста, у овом раду су примењене методе афинитетне хроматографије. Резултати су показали да се већина IGFBP-1 у циркулацији на-

лази у комплексима са α_2M, који се могу изоловати применом афинитетне хроматографије са имобилизованим анти-α_2M антителима. IGFBP-1/α_2M комплекси се могу раздвојити од IGFBP-1 димера и мултимера применом афинитетне хроматографије са имобилизованим лектинима, пошто се олигомери не везују за лектине. Димерна форма и мултимери су стабилни при редукционим условима, што указује на ковалентну везу између јединица. Слободни IGFBP-1 мономер је присутан у релативно малој концентрацији у циркулацији и може се раздвојити од мултимера применом афинитетне хроматографије са имобилизованим лектином Con A.

(Примљено 30. марта, ревидирано 2. јула 2010)

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