Journal of Chromatography B, 879 (2011) 1704–1706

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Analysis of hSCOMT adsorption in bioaffinity chromatography with immobilized amino acids: The influence of pH and ionic strength

S.R. Costa [a], M.J. Bonifácio [b], J.A. Queiroz [a], L.A. Passarinha [a,*]

[a] CICS – Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, 6201-001 Covilhã, Portugal
[b] BIAL – Departamento de Investigação e Desenvolvimento, São Mamede do Coronado, Portugal

ARTICLE INFO

Article history:
Received 16 November 2010
Accepted 29 March 2011
Available online 12 April 2011

Keywords:
Amino acids immobilized ligands
Human soluble
catechol-*O*-methyltransferase
Bioaffinity interactions
Mixed-mode
Proteins

ABSTRACT

In the last years, chromatographic supports with amino acids as immobilized ligands (AAILs) were been used successfully for isolation of several biomolecules, such as proteins. In this context and based on specific properties of human soluble cathecol-*O*-methyltransferase (hSCOMT), we screened and analyzed the effect of experimental conditions, such as pH and ionic strength manipulation for hSCOMT adsorption, over six different AAIL commercial supports. Typically, the proteins adsorption on AAIL chromatographic supports is around their p*I*. While hSCOMT isoelectric point is around 5.5, this parameter leads us to design new adsorption strategies with several acid buffers for the chromatographic process. In terms of the ionic strength manipulation strategy, the results suggest that the AAILs–hSCOMT interaction is strongly affected by the intrinsic hSCOMT hydrophobic domains. On the other hand, the interaction mechanism of hSCOMT on amino acid resins appears to be highly dependent on the binding pH. Consequently the retention mechanism of the target enzyme on the AAILs can be as either in typical hydrophobic or ionic chromatographic supports, so long as selecting various mobile phases and separation conditions. In spite of these mixed-mode interactions and operation strategies, the elution of interferent's proteins from recombinant host can be achieved only with suitable adjusts in pH mobile phase set point. This lead to a new approach in biochromatographic COMT retention, while possess a higher specificity than other chromatographic methods reported in literature.

1. Introduction

The amino acids as immobilized ligands (AAILs) were introduced, in 1989 by Vijayalakshmi and coworkers, with the designation of "pseudo-biospecific affinity ligands". Nowadays, these chromatographic supports represent promising alternatives to traditional ligands in affinity chromatography, due to their resistance to harsh chemicals, high temperatures and low cost [1]. Indeed, these chromatographic supports have been extensively used in separation of several molecules such as oligouronides [2,3], oligonucleotides [4], pDNA [5,6], RNA [7] and proteins [8–11]. Typically, AAILs act as electron acceptors of NH, SH or OH groups from protein amino acid residues such as histidine, cysteine, tryptophan, and serine. Indeed, the global interaction between the target molecule and AAILs is a reflection of the several molecular interactions involved, namely, electrostatic, hydrogen bonding, hydrophobic and Van der Waals interactions [12]. The feasibility of these mixed-mode interactions, as described for other commercial supports [13] will depend on several conditions such as buffer composition, pH, temperature, ionic strength and support matrix. Nevertheless, the degrees of interactions can depend more specifically on the protein surface amino acids accessibility [14]. Also, the three-dimensional structure of the target protein can be a relevant factor in these systems, affecting the rate and extent of the interactions. In general, the native conformation is directly affected by environmental conditions, such as pH and ionic strength variation, used in order to promote interaction onto the AAIL supports [12]. To our best knowledge there are not yet known structural evidences or experimental data to preview the behavior of mammalian cathecol-*O*-methyltrasferase (COMT) on AAIL chromatographic matrices.

The COMT enzyme catalyzes the *O*-methylation in catecholamines and other catechols and is a significant target in pharmacological studies due to its role not only in normal brain function but also its possible involvement in some human disorders [15]. Specifically, the protein is composed of a seven-stranded β-sheet core, wedged between two sets of α-helices. Its active site consists on the *S*-adenosyl-L-methionine binding domain [16] and also few amino acids extremely relevant for binding sub-

Abbreviations: AAILs, amino acids as immobilized ligands; AS, ammonium sulphate; hSCOMT, human soluble catechol-*O*-methyltransferase; SC, sodium chloride.

* Corresponding author at: Av. Infante D. Henrique, 6200-506 Covilhã, Portugal. Tel.: +351 275 329 002/3; fax: +351 275 329 099.
E-mail addresses: lpassarinha@fcsaude.ubi.pt, luis_passarinha@hotmail.com (L.A. Passarinha).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.03.056

strate, water, and Mg^{2+}. For instance, amino acids residues such as Lys144 that accepts the proton from the hydroxyl group, and the "gatekeeper" residues Trp38, Trp143, and Pro174 that form the hydrophobic "walls" which define the hSCOMT substrate selectivity [17]. In this context, the chromatographic strategies applied for highly sensitive proteins, such as hSCOMT, should be careful designed since it can compromise the native intrinsic kinetic properties of the target protein.

In this work, recombinant hSCOMT obtained from *Escherichia coli* (*E. coli*) lysates was chosen as a model enzyme, in order to study the incorporation of AAIL chromatographic supports in a hSCOMT isolation process and explore the selection of experimental conditions, such as pH and ionic strength. This strategy described for the first time for hSCOMT enzyme, could be particularly promising while combines a natural biological interaction, improving the selectivity onto the support and the purity achieved in the target protein fractions.

2. Materials and methods

2.1. Recombinant hSCOMT expression

Plasmid pET101/D-hSCOMT (Invitrogen, Carlsbad, USA) was used as the expression construct. Commercial *E. coli* BL21-star (Invitrogene, Carlsbad, USA) was used as the recombinant strain for hSCOMT over expression under the control of the IPTG (Sigma, Missouri, USA) inducible promoter and employing carbanecillin disodium salt (Sigma, Missouri, USA) supplementation as a selection marker. The fermentation conditions were previously described by our group [18].

2.2. Recombinant hSCOMT recuperation

Cells were resuspended in a standard buffer and lysis by consecutive freeze–thaw cycles [18]. After bacterial lysis, a supernatant sample was obtained and injected directly into the AAIL chromatographic supports.

2.3. Protein quantification

Protein contents in samples were measured by the Bio-Rad protein assay reagent (Bio-Rad, Carlsbad, USA), with bovine serum albumin as the standard and calibration control samples (1.2–10.0 μg/mL), according to manufacturer's indications.

2.4. AAIL preparative chromatography

The AAIL media (L-arginine, L-methionine, L-histidine, L-aspartate, L-glutamine and L-leucine obtained from Sigma, Missouri, USA) were packed according to company guidelines (5 mL of gel volume), into a Econo-Pac® disposable Chromatography Columns (Bio-Rad, Carlsbad, USA). Aliquots (500 μL with an estimated protein concentration of about 11.5 mg/mL) of recombinant hSCOMT-containing supernatant were loaded onto the columns with two distinct strategies. In adsorption using pH manipulation, columns were initially equilibrated with Tris (Sigma, Missouri, USA)–HCl buffer (10 mM) at different pH values, and isocratic elution was performed with the same pH value. After elution of unretained species, the bound proteins were eluted in a stepwise gradient with sodium chloride (SC) (Fluka, Buchs, Switzerland) 1 M in 10 mM Tris–HCl buffer, pH 7.8. The same procedure was performed in adsorption using salt manipulation, where the binding buffer was 1.5/2 M ammonium sulphate (AS) (Sigma, Missouri, USA) in Tris–HCl 10 mM, pH 7.8 and elution buffer was Tris–HCl 10 mM, pH 7.8. In all chromatographic experiments, the optical density was continuously monitored at 280 nm while 1 mL fractions were collected and evaluated for hSCOMT detection. All assays were performed at room temperature.

2.5. hSCOMT specific activity assays

The experiments of activity were designed to evaluate the methylation efficiency of recombinant hSCOMT, in a HPLC system with electrochemical detection (Waters, Milford, MA, USA), by measuring the amount of metanephrine using epinephrine (Sigma, Missouri, USA) as substrate, as previously described [19].

3. Results and discussion

In general, the interaction between AAILs and proteins are predominantly due to different types of intermolecular forces. Specifically, the pH and the ionic strength of the mobile phase can have a relevant effect on the establishment of appropriate adsorption/elution equilibriums, for a target protein isolation process from complex bacterial lysate extracts. Indeed, the manipulation of these operatory parameters is the most common strategy that can be found in bioaffinity chromatography. Specific experiments were carried out in order to study the behavior of hSCOMT in several stock buffer conditions that attempt to exemplify several adsorption patterns performed in AAIL chromatographic step. Therefore, firstly we focus on the ionic strength parameter, testing the effects of several AS concentrations, normally applied for hSCOMT adsorption in traditional HIC chromatographic media [18]. Secondly, we center the study in pH manipulation at adsorption mobile phases formulation, in order to understand the hSCOMT adsorption behavior in AAIL chromatographic supports, for a suitable integration on a hSCOMT isolation process.

Normally in salt mediated separations, the salts modulate affinity of proteins for particular columns and nonspecific protein–protein or protein–surface interactions, depending on the type and concentration, in both specific and nonspecific approaches [20]. Typically, the protein adsorption is carried out by moderate to high concentrations of anti-chaotropic salts, and the commonly used is AS [21]. In spite of the salt concentration strongly influences the selectivity in protein adsorption; high salt levels can lead to hSCOMT denaturation and aggregation with partially or total inactivation, as previously described by our group [22]. Nevertheless, the enzyme deactivation and/or modification can be reduced by shortening process time and by applying a lower ionic strength in the bioseparation process [18]. Therefore, we studied the applicability of AS in adsorption buffers on AAIL chromatographic supports, for the direct capture of hSCOMT from cell culture supernatants. The experiments were carried out through increasing AS concentrations from 0.5 to 2 M in 10 mM Tris–HCl, pH 7.8. The results showed that in the six AAIL chromatographic supports tested, a complete binding of hSCOMT was achieved above 1.25 M of AS concentration (data not shown). Specifically, in all chromatographic supports the concentration of SA for hSCOMT-adsorption was similar. The AAILs–hSCOMT interaction appears to be independently of the chromatographic support used but emerge to be dependent of the native hSCOMT specific hydrophobic characteristics, suggesting a higher selectivity adsorption phenomenon when the ionic strength was manipulated.

The specific protein adsorption on the AAILs occurred mainly at pH values around its isoelectric point [14]. So ideally, the adsorption pH for hSCOMT can be selected early based on the simple knowledge of its p*I* value (5.5). Over the last years, this approach has been described for several proteins for instance yeast carboxypeptidase (pH 3–4) and goat chymosin (pH 5.5), revealing that acidic pH can be applied successfully in several purification strategies without

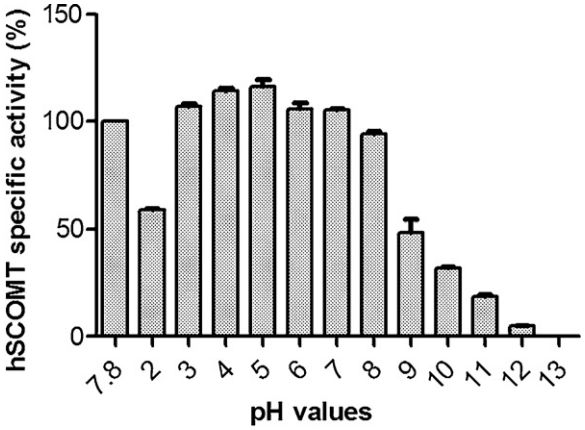

Fig. 1. The hSCOMT specific activity performed at pH ranges from 2 to 13 in 10 mM Tris–HCl buffer. The positive control of specific activity data achieved at pH 7.8 in the same buffer. The experiments were performed at 4 °C during 12 h and repeated independently three times.

changes in the native structure [14].Preliminary control experiments were conducted to analyze the effects of pH buffers ranging from 2 to 13 on hSCOMT stability (Fig. 1). The results of hSCOMT stability trials at several pH showed that the enzyme maintains its activity over pH ranges from 3 to 7. Also, we observed a decreasing for hSCOMT specific activity, at values outside the range mentioned. So concerning these stability results, pH set point selection for hSCOMT adsorption onto the AAIL chromatographic supports was fixed in a pH range between 3 and 7. Specifically, these experiments were carried out in order to study the behavior of the hSCOMT onto the six AAIL chromatographic supports at several pH conditions. As shown in Fig. 2, lysate extracts containing hSCOMT were loaded onto AAIL supports using 10 mM Tris–HCl with pH 7.8 (L-arginine), 4 (L-aspartate), 6.5 (L-glutamine), 5 (L-methionine and L-histidine supports) and 5.7 (L-leucine). Specifically, immunoreactivity screening of all chromatographic fractions showed that hSCOMT is totally adsorbed in the operation conditions established, and the protein is eluted by a simple stepwise gradient with 1 M of SC onto the mobile phase. In the six AAIL matrices, SDS-PAGE and Western blot (data not shown) screening of chromatographic peaks show that the target protein eluted in the second peaks with 1 M sodium chloride, with a considerable removal of host proteins. Overall, the purification degree achieved with pH manipulation, specifically in methionine ligand, is remarkably acceptable and higher than in routine hydrophobic resins.In all chromatographic trials performed (Fig. 2) was used an acidic pH to promote hSCOMT adsorption. However, in L-arginine support the adsorption can be performed not only with neutral pH (7.8; described as the model pH for activity enzymatic assays) but also with pH 4 (data not shown).

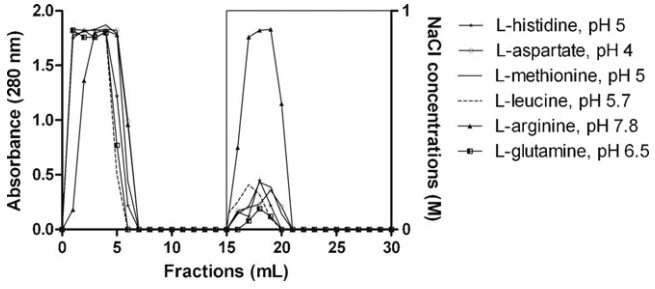

Fig. 2. Chromatographic profiles of an *E. coli* lysate extract with recombinant hSCOMT onto the six AAILs tested with a stepwise gradient from 0 to 100% buffer A (buffer A: 10 mM Tris–HCl, pH 7.8 (L-arginine), 4 (L-aspartate), 6.5 (L-glutamine), 5 (L-methionine and L-histidine), 5.7 (L-leucine); buffer B: SC 1 M in 10 mM Tris–HCl, pH 7.8).

Indeed, the differences observed in the enzyme adsorption process shows that retention depends on several factors such as hydrophobic, electrostatic and hydrogen bonding interactions, which can change with the surrounding pH, depending on the p*K* values from the different chemical groups involved. Therefore, these chromatographic experiments demonstrated that hSCOMT adsorption onto AAIL resins can be based on specific affinity properties achieved by a detailed strategy with a selective pH onto the mobile phase.

4. Conclusions

This is the first report of AAIL chromatographic supports screening data show a suitable approach of using bioaffinity supports for hSCOMT isolation. Overall, the experiments provided a new insight of pH and ionic strength strategies in hSCOMT–AAILs interaction mechanism. The experimental chromatographic results suggest that the characteristics of the support, native enzyme affinity properties and the pH of the eluent play an important role in the adsorption of hSCOMT in each resin. Specifically, in the case of adsorption based on ionic strength, the AAILs–hSCOMT interaction seems to be independently on the chromatographic support used but highly influenced by the hSCOMT specific hydrophobic characteristics. Indeed, the ionic, hydrophilic and hydrophobic constituents groups of immobilized amino acids allow a mixed-mode of HIC and IEC interactions with target protein. Therefore, in AAIL resins the careful selection of operation conditions is extremely relevant, while minimize or even eliminate the occurrence of host impurities retention. In conclusion, the described AAIL chromatographic matrices to hSCOMT partial purification can open a promising unexplored field regarding the remaining set of more unwieldy process such as, a typical hydrophobic or ionic chromatographic supports.

Acknowledgement

This research was partially supported by University of Beira Interior – Health Sciences Research Centre (CICS).

References

[1] M.A. Vijayalakshmi, Trends Biotechnol. 7 (1989) 71.
[2] C. Delattre, A.S. Kamalanathan, P. Michaud, M.A. Vijayalakshmi, J. Chromatogr. B 861 (2008) 181.
[3] C. Delattre, P. Michaud, K. Hamze, B. Courtois, J. Courtois, M.A. Vijayalakshmi, J. Chromatogr. A 1099 (2005) 121.
[4] A. Sousa, F. Sousa, D.M.F. Prazeres, J.A. Queiroz, Biomed. Chromatogr. 23 (2009) 745.
[5] A. Sousa, F. Sousa, J.A. Queiroz, J. Chromatogr. B 877 (2009) 3257.
[6] F. Sousa, T. Matos, D.M.F. Prazeres, J.A. Queiroz, Anal. Biochem. 374 (2008) 432.
[7] D.S. Jones, H.K. Lundgren, F.T. Jay, Nucleic Acids Res. 3 (1976) 1569.
[8] L. Summaria, F. Spitz, L. Arzadon, I.G. Boreisha, K.C. Robbins, J. Biol. Chem. 251 (1976) 3693.
[9] S. Kanoun, L. Amourache, S. Krishnan, M.A. Vijayalakshmi, J. Chromatogr. 376 (1986) 259.
[10] A. el-Kak, M.A. Vijayalakshmi, J. Chromatogr. 570 (1991) 29.
[11] K. Haupt, M.A. Vijayalakshmi, J. Chromatogr. 644 (1993) 289.
[12] O. Pitiot, M.A. Vijayalakshmi, in: M.A. Vijayalakshmi (Ed.), Biochromatography: Theory and Practice, Taylor & Francis, London, 2002, chapter 16, p. 172.
[13] P. Liu, H. Yang, X. Geng, J. Cromatogr. A 1216 (2009) 7497.
[14] A. el-Kak, S. Manjini, M.A. Vijayalakshmi, J. Chromatogr. 604 (1992) 29.
[15] J. Axelrod, S. Senoh, B. Witkop, J. Biol. Chem. 233 (1958) 697.
[16] J. Vidgren, M. Ovaska, in: P. Veerepandian (Ed.), Structure-Based Drug Design, Marcel Dekker, Inc, New York, 1997, chapter 14, p. 343.
[17] P.T. Mannisto, S. Kaakkola, Pharmacol. Rev. 51 (1999) 593.
[18] L.A. Passarinha, M.J. Bonifacio, P. Soares-da-Silva, J.A. Queiroz, J. Chromatogr. A 1177 (2008) 287.
[19] L.A. Passarinha, M.J. Bonifacio, J.A. Queiroz, Biomed. Chromatogr. 20 (2006) 937.
[20] K. Tsumoto, D. Ejima, A.M. Senczuk, Y. Kita, T. Arakawa, J. Pharm. Sci. 96 (2007) 1677.
[21] J.A. Queiroz, C.T. Tomaz, J.M. Cabral, J. Biotechnol. 87 (2001) 143.
[22] V.S. Nunes, M.J. Bonifacio, J.A. Queiroz, L.A. Passarinha, Biomed. Chromatogr. 24 (8) (2010) 858.