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Contributions of commercial sorbents to the selectivity in immobilized metal affinity chromatography with Cu(II)

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Abstract

Immobilized copper(II) affinity chromatography [Cu(II)-immobilized metal affinity chromatography (IMAC)] has been used in proteomics to simplify sample mixtures by selecting histidine-containing peptides from proteolytic digests. This paper examines the specificity of four different support materials with an iminodiacetic acid (IDA) stationary phase in the selection of only histidine-containing peptides in the single step capture-release mode. Three of the sorbents examined were commercially available: HiTrap Chelating HP (agarose), TSK Chelate-5PW, and Poros 20MC. IDA was also immobilized on CIM discs (monolithic glycidylmethacrylate-ethylene dimethacrylate). Tryptic digests of transferrin and β-galactosidase were used as model samples to evaluate these sorbents. It was found that among the examined matrices, the TSK Chelate-5PW sorbent bound histidine-containing peptides the strongest, while Poros matrix was found to have a high degree of non-specific bindings. Agarose-based columns showed relatively high selectivity and specificity.

Keywords: Immobilized metal affinity chromatography; Proteomics; Stationary, LC; Peptides; Copper

1. Introduction

Proteomics is a major field in separation science. The selection of histidine-containing peptides is an important aspect of proteomics in sample simplification and database search. Immobilized metal affinity chromatography (IMAC) has been reported to select both proteins and peptides containing histidine residues from complex biological extracts [1–5]. By attaching six histidine residues tag, Ni(II)-IMAC selection is very useful in purification of the recombinant proteins in biochemistry. However, the issue in proteomics is whether IMAC columns can capture only histidine-containing peptides. When it can be assumed that all the peptides in a mixture being examined by LC-MS contain histidine residues, peptide identification is greatly simplified. Based on a recent report there is reason to believe that Cu(II)-IMAC columns come close to selecting only peptides that contain histidine [6]. Moreover, this report shows that selectivity is strongly impacted by mobile phase composition. At high salt concentration, electrostatic interactions are reduced along with non-specific bindings. Elution can be achieved with either strong displacer such as imidazole, or protonation by decreasing elution buffer pH. When an imidazole elution is applied, peptides with increasing numbers of histidine residues, which bound to the stationary phase strongly, are preferentially eluted. Finally, derivatization the N-terminal amino group by such reagents as *N*-acetoxysuccinamide (NAS), succinic anhydride, or quaternary amines reduces the number of histidine-containing peptides retained onto the Cu(II)-IMAC column. For example, the number of peptides selected from a tryptic digest of the yeast proteome decreased 10-fold when the primary amine groups in peptides were acetylated with *N*-acetoxysuccinamide. This report suggests that acylation of N-terminal amine groups in peptides has a far stronger impact on peptides that contain a single histidine than those having multiple histidines, especially with histidine residues that are close together in the sequence.

It is well known that sorbent matrix effects have a major impact on selectivity in liquid chromatography [7] and that the chemical nature of a support [8], chelating ligands

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[9–12], and stationary phase density [13] can influence the interaction between a sorbent matrix and target peptides. The type of chelating ligand and support medium have been reported to influence selectivity and specificity of peptide selection in Cu(II)-IMAC [14], although iminodiacetic acid (IDA) is by far the most widely used stationary phase in IMAC. Typical matrices for IMAC can be soft-gel agarose, inorganic sorbents, and synthetic polymer-based particles [15]. However, these supports have not been evaluated for use in the capture-release mode of histidine-containing peptide selection in a protein tryptic digest mixtures. Recognizing that supports could impact the capture efficiency of histidine-containing peptides, this study was initiated to examine the influence of most commercially available soft-gel, hard-gel, and porous-based Cu(II)-IMAC sorbent matrices on peptide selectivity and specificity with tryptic digests of model proteins. Four sorbent matrices were examined based on differences in their chemical and physical properties such as mechanical strength, the potential of hydrophobicity contributed by the support matrix.

2. Experimental

2.1. Materials

β-Galactosidase and imidazole were purchased from Fluka (Milwaukee, WI, USA). Transferrin (human), TPCK-treated trypsin (bovine), copper(II) sulfate pentahydrate, urea, dithiothreitol (DTT), iodoacetic acid (IAA), *N*-(2-hydrozyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), *N*-tosyl-L-lysylchloromethyl ketone (TLCK), acetic acid, ethylenediaminetetraacetic acid (EDTA) and calcium chloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). Trifluoroacetic acid (Sequenal grade) was acquired from Pierce (Rockford, IL, USA). Sequencing grade modified trypsin was from Promega (Madison, WI, USA). HPLC grade acetonitrile (ACN), sodium acetate, sodium chloride, sodium phosphate dibasic heptahydrate, sodium phosphate monobasic and phosphoric acid were obtained from Mallinckrodt Baker (Paris, KY, USA).

C_{18} columns (1 mm × 250 mm) were purchased from Vydac (Hesperia, CA, USA). HiTrap Chelating HP affinity columns (0.7 cm × 2.5 cm) were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). The TSK Chelate-5PW column (7.5 mm × 7.5 mm) was purchased from TosoHaas (Montgomeryville, PA, USA). Poros 20MC (polystyrene-divinylbenzene) was obtained from PerSeptive Biosystems. The CIM disc (monolithic glycidylmethacrylate-ethylene dimethacrylate) column was acquired from BiaSeparations (Ljublyana, Slovenia). IDA was immobilized onto CIM disc media following procedures described in the literature [16,17].

Deionized water was produced with a Milli-Q Gradient A10 system from Millipore (Bedford, MA, USA).

2.2. Proteolysis of proteins

Protein samples (3–5 mg/ml) were dissolved in 50 mM HEPES buffer (pH 7.8) containing 20 mM $CaCl_2$ and 6 M urea. A 20-fold molar excess of DTT was added to the protein solution, which was then incubated for 2 h at 37 °C. Next, 40-fold molar excess of iodoacetic acid was added and incubated in darkness on ice for another 2 h with subsequent addition of 20-fold molar excess of cysteine. The mixture was incubated for 15 min. The protein solution was then diluted 4 times before adding trypsin (at 1/50 the total amount of protein). Proteolysis was achieved by incubation overnight at 37 °C. The reaction was terminated by adding TLCK in a molar excess (i.e. at a ratio of 1.5:1) over that of trypsin or by freezing the peptide mixture in liquid nitrogen.

2.3. Derivatization of peptides

Protocols for the synthesis of the *N*-acetoxysuccinamide and its use in the derivatization of peptides were obtained from the literature [18,19]. Briefly, a 50-fold molar excess of NAS was added to a tryptic digest and the reaction was allowed to proceed for 2 h at room temperature (pH 7–8). After the reaction was finished, *N*-hydroxylamine was added to hydrolyze esters by adjusting the pH of the peptide solution to pH 11–12. The reaction was allowed to proceed for 10 min before the pH was adjusted back to 7–8 with glacial acetic acid.

2.4. Cu(II)-IMAC selection

Cu(II)-IMAC selections were performed on a BioCAD 20 Micro-Analytical Workstation (Applied Biosystems, Framingham, MA, USA). The flow rate was set at 1 ml/min, and detection was performed at 280 nm. The column was first equilibrated with five column volumes of 50 mM EDTA (pH 8.0, 0.5 M NaCl) and then with five column volumes of binding buffer containing 20 mM phosphate (pH 7.0) and 0.5 M NaCl. Following sample (pH 7–8) injection, the column was washed again with three column volumes of binding buffer and the selected peptides were eluted with 0.1 M NaAc (pH 4.0, 0.5 M NaCl). For strongly bound histidine-containing peptides, which cannot be eluted by sodium acetate solution, additional elution step with 50 mM imidazole at pH 7.0 was applied. In between the two elution steps, Cu(II)-IMAC column was re-equilibrated with 20 mM phosphate buffer at pH 7.0 [6]. No further strong elution conditions were tested due to the possibility of causing severe Cu(II) leaching from the column. Elution fractions were manually collected and analyzed by LC-MS.

2.5. LC-MS analysis

Peptide mixtures collected from Cu(II)-IMAC elution were separated on a Vydac C_{18} column (1 mm × 250 mm) using an Integral Micro-Analytical Workstation (Applied

Biosystems) at 50 μl/min. The column was equilibrated with solvent A (0.01% TFA in deionized water) and peptides were eluted with increasing concentration of solvent B (95% ACN/0.01% TFA in deionized water). The column was directly connected to the QSTAR workstation (Applied Biosystems) through an electrospray interface. Spectra were obtained in the positive TOF mode at a sampling rate of one spectrum per second. Peptide identification was achieved by matching the observed *m/z* values to the theoretical *m/z* of the trypsin digested peptides in the SWISS-PROT database [20].

3. Results and discussion

Four IDA modified matrices were chosen for this study: HiTrap Chelating HP (agarose), TSK Chelate-5PW, Poros 20MC, and CIM disc (monolithic glycidylmethacrylate-ethylene dimethacrylate). Although the agarose, consisting of alternating residues of D-galactose and 3-anhydrogalactose, is widely used as a purification medium for proteins, it is seldom used as an analytical matrix for peptides. This sorbent has low mechanical strength. Therefore, it was not possible to execute the direct transfer of analytes from agarose-based IMAC columns to the C_{18} reversed phase column. Instead fractions from Cu(II)-IMAC column were manually collected and transferred to the reversed phase column.

Polymer-based supports made of polystyrene or polymethacrylate approach silica in requisite mechanical strength for high performance operation and are of excellent chemical stability. The TSK Chelate-5PW column is a porous spherical gel synthesized from methacrylate-based monomers. It can withstand high pressures but the surface environment within which the chelating stationary phase is immobilized is mildly hydrophilic and could potentially display some hydrophobic tendencies under certain conditions. The monolithic support from BiaSeparations is very similar to the TSK Chelate-5PW columns in chemical properties although it varies substantially in pore structure. The advantage of CIM disc columns is that they allow very high flow rates (up to 10 ml/min) without loss of efficiency and capacity. The polystyrene/divinylbenzene based Poros 20MC is even more stable and rigid than the other two organic resins and tolerates very high flow rates. However, it is potentially more hydrophobic and of lower loading capacity. A summary of these supports is presented in Table 1.

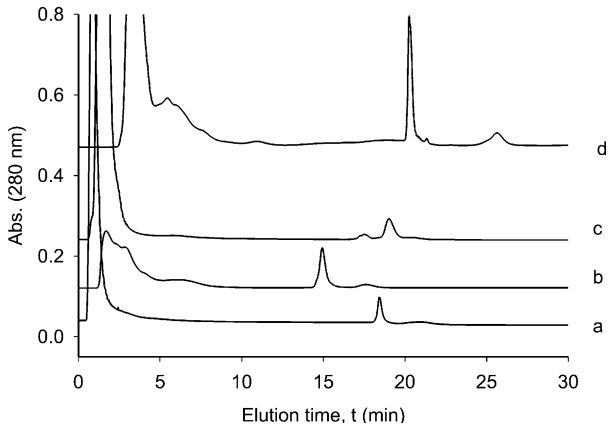

Fig. 1. Cu(II)-IMAC elution profiles of histidine-containing peptides from transferrin on different matrices using 0.1 M NaAc elution condition (pH 4.0): (a) CIM disc column; (b) HiTrap Chelating column; (c) Poros column; (d) TSK Chelate-5PW column.

Selectivity and specificity of the different affinity columns were investigated with trypsin digested transferrin. Cu(II)-IMAC elution profiles from each of the columns are shown in Fig. 1. The elution peak area observed for histidine-containing peptide selection from the TSK Chelate-5PW column was relatively big. This suggested that this column retained more peptides, as was later confirmed in the data analysis (Table 2). However, a small number of these peptides were non-specifically bound and did not contain histidine (data not shown). The number of histidine-containing peptides selected by the HiTrap Chelating and TSK Chelate-5PW media was similar. Poros retained the smallest number of histidine-containing peptides from transferrin, while the CIM disc matrix selected an intermediate number (Table 2). Two structurally similar histidine-containing peptides, WCALS**HH**ER and CALS**HH**EK, were observed to elute from all the columns except the TSK Chelate-5PW column when 0.1 M NaAc (0.5 M NaCl) was applied. As was noticed in a previous study [6], an imidazole elution tends to elute peptides that were strongly bound onto the column. Therefore, retained peptides on the TSK Chelate-5PW column were first washed with 0.1 M NaAc (pH 4.0) followed by re-equilibrating with 20 mM phosphate binding buffer (pH 7.0), finally, 50 mM imidazole (pH 7.0) buffer was used to elute strongly bound peptides (WCALS**HH**ER and CALS**HH**EK) as confirmed by LC-MS. These results suggested that the TSK Chelate-5PW column is more

Table 1
Selected sorbents[a]

Company	Product	Matrix	Particle diameter
Amersham Pharmacia	HiTrap Chelating	Highly cross-linked spherical agarose	34 μm
Perseptive Biosystems	Poros 20MC	Polystyrene/divinylbenzene	20 μm
TosoHaas	TSKgel Chelate-5PW	Methacrylate-based polymer	10 μm
BiaSeparations	CIM disc	Monolithic glycidylmethacrylate-ethylene dimethacrylate	Through-pores: 1500 nm, mesopores: 100 nm, disc volume 0.34 ml

[a] All sorbents were immobilized with iminodiacetic acid group.

Table 2
Sorbent effects on recovery of histidine-containing peptides from trypsin digested transferrin

Peptide mass	Sequence	HiTrap Chelating	CIM disc	Poros	TSK Chelate-5PW
1196.53	WCALS**HH**ER	+	+	+	*
1010.44	CALS**HH**EK	+	+	+	*
2550.27	KPVDEYKDC**H**LAQWPS**H**TVVAR	+	+	+	+
2233.12	DGAGDVAFVK**H**STIFENLANK	+	+	+	+
2070.03	EDLIWELLNQAQE**H**FGK	+	+	*	+
1587.76	KPVEEYANC**H**LAR	+	+	+	+
1413.71	ELLNQAQE**H**FGK	+	+	+	+
1276.63	EFQLFSSP**H**GK	+	+	+	+
1273.65	**H**STIFENLANK	+	+	+	+
964.53	APN**H**AVVTR	+	+	+	+
874.44	DSA**H**GFLK	+	+	+	+
1166.59	**H**QTVPQNTGGK	+	+		+
987.46	DKEACV**H**K	+	+		+
1690.83	DC**H**LAQVPS**H**TVVAR	+	+		+
1016.49	KSC**H**TGLGR	+	+		+
1318.57	WCAVSE**H**EATK	+			+
1209.51	CQSFRD**H**MK	+			+
744.33	EACV**H**K	+			+
700.32	LN**H**CR			+	+

Peptides eluted with 0.1 M sodium acetate (pH 4.0) alone (+); peptides observed when 50 mM imidazole (pH 7.0) was applied following 0.1 M sodium acetate (pH 4.0) elution (*).

retentive for some peptides than the other columns and that 50 mM imidazole (pH 7.0) should always be used in the elution of this column to assure that all peptides retained can be eluted.

A similar study was performed with more complicated peptide mixture obtained from tryptic digested β-galactosidase using TSK Chelate-5PW, Poros and HiTrap Chelating columns. Considering the presence of strongly bound histidine-containing peptides that are difficult to be eluted, a combination of sodium acetate (pH 4.0) and 50 mM imidazole (pH 7.0) elution was applied in order to elute all retained histidine-containing peptides from a column. As in the case for transferrin, Cu(II)-IMAC elution profiles of β-galactosidase were also matrix dependent (Fig. 2). Each of the matrices selected a different population of histidine-containing peptides (Table 3). The data analysis showed that the relatively hydrophobic media, TSK Chelate-5PW and Poros, tend to retain more histidine-containing peptides as compared to those from HiTrap Chelating. For example, peptide with up to four histidine residues **H**E**HH**PL**H**GQVMDEQTMVQDILLMK was observed using TSK Chelate-5PW and Poros columns, but not with HiTrap Chelating column. During imidazole elution, seven and nine histidine-containing peptides were eluted from the TSK Chelate-5PW and Poros media, respectively, while only one was eluted from HiTrap Chelating sorbent. Of the peptides eluted from the TSK Chelate-5PW column with strong displacer, imidazole, six of the seven histidine-containing peptides could only be eluted by imidazole, whereas two of the nine histidine-containing peptides were newly released from the Poros columns. Again it showed that the TSK Chelate-5PW tends to retain histidine-containing peptides more strongly. It should also be pointed out that there were some non-specific bindings observed when Poros and TSK Chelate-5PW columns were used due to the relative stronger interactions between these matrices and peptides. However, agarose base sorbent showed high specificity and selectivity as studied previously [6].

Quantification is another important issue in proteomic studies. Acylation of peptides is often used to achieve this goal [21]. It is thus important to understand the effect of N-terminus derivatization of the peptides on the interaction with the stationary phases during a Cu(II)-IMAC selection. Upon *N*-acetoxysuccinamide derivatization of tryptic digested transferrin, the number of histidine-containing peptides selected was drastically changed with different

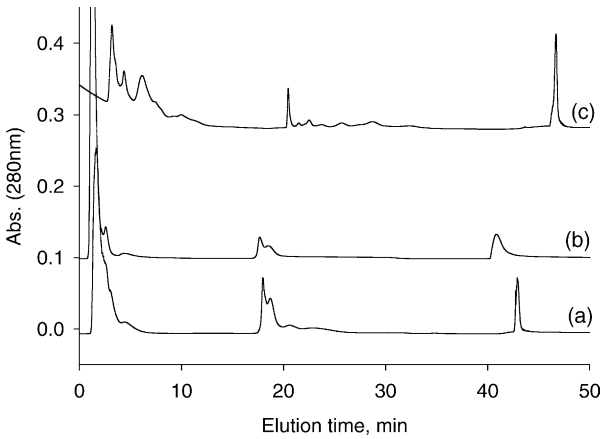

Fig. 2. Cu(II)-IMAC elution profiles of histidine-containing peptides from β-galactosidase on different matrices using 0.1 M NaAc (pH 4.0), followed by re-equilibrating with 20 mM phosphate binding buffer (pH 7.0) and eluted with displacer 50 mM imidazole (pH 7.0): (a) HiTrap Chelating column; (b) Poros column; (c) TSK Chelate-5PW column.

Table 3
Sorbent effects on recovery of histidine-containing peptides from trypsin digested β-galactosidase

Peptide mass	Sequence	HiTrap Chelating	TSK Chelate-5PW	Poros
2866.38	**H**E**HH**PL**H**GQVMDEQTMVQDILLMK		*	+, *
4324.02	**H**LL**H**AEEGTWLNIDGF**H**MGIGGDDSWSPSVSAEFQLSAGR		*	
3832.87	AAG**H**YQAEAALLQCTADTLADAVLITTA**H**AWQ**H**QGK	+, *	+, *	+, *
3015.46	DRN**H**PSVIIWSLGNESG**H**GAN**H**DALYR	+	*	*
2744.33	N**H**PSVIIWSLGNESG**H**GAN**H**DALYR	+	*	+, *
3423.74	**H**SDNELL**H**WMVALDGKPLASGEVPLDVAPQGK	+	*	+, *
2265.20	DVSLL**H**KPTTQISDF**H**VATR	+	+	+
2120.90	CS**H**YPN**H**PLWYTLCDR	+	*	+
3736.87	LAENLSVTLPAAS**H**AIP**H**LTTSEMDFCIELGNKR		+	+, *
3146.57	DVSLL**H**KPTTQISDF**H**VATRFNDDFSR		+	+
2311.17	TP**H**PALTEAK**H**QQQFFQFR	+	+	
3580.77	LAENLSVTLPAAS**H**AIP**H**LTTSEMDFCIELGNK		+	+
2956.55	MSGIFRDVSLL**H**KPTTQISDF**H**VATR		+	
3254.58	AAG**H**YQAEAALLQCTADTLADAVLITTA**H**AW			*
1797.82	SLGNESG**H**GAN**H**DALYR			+
3189.59	WLSLPGETRPLILCEYA**H**AMGNSLGGFAK	+	+	+, *
2474.19	AVVEL**H**TADGTLIEAEACDVGFR	+	+	+
2006.95	IIFDGVNSAF**H**LWCNGR	+	+	+, *
1299.62	ELNYGP**H**QWR	+	+	+
1265.62	**H**QQQFFQFR	+	+	+
1252.66	LAA**H**PPFASWR	+	+	+
1507.70	YSQQQLMETS**H**R	+	+	+
1425.66	Y**H**YQLVWCQK	+	+	+
1064.57	TP**H**PALTAK	+	+	+
2522.23	VVQPNATAWSEAG**H**ISAWQQWR	+		+
2466.19	IDGSGQMAITVDVEVASDTP**H**PAR		+	+
2408.13	YGLYVVDEANIET**H**GMVPMNR		+	+
3919.87	IDGSGQMAITVDVEVASDTP**H**PARIGLNCQLAQVAER			+
2522.22	VVQPNATAWSEAG**H**ISAWAQWR			+
2364.12	ELNYGP**H**QWRGDFQFNISR			+
1236.65	DRN**H**PSVIIW			+
1096.55	LAA**H**PPFASW			+
3317.68	KWLSLPGETRPLILCEYA**H**AMGNSLGGFAK		+	
2886.40	TYRIDGSGQMAITVDVEVASDTP**H**PAR		+	
1911.91	VVDEANIET**H**GMVPMNR		+	

Peptides eluted with 0.1 M sodium acetate (pH 4.0) alone (+); peptides observed when 50 mM imidazole (pH 7.0) was applied following 0.1 M sodium acetate (pH 4.0) elution (*).

Table 4
Sorbent effects on recovery of histidine-containing peptides from *N*-acetoxysuccinamide derivatized transferrin peptides

Peptide mass	Sequence	HiTrap Chelating	CIM disc	Poros	TSK Chelate-5PW
1196.53	WCALS**HH**ER	+	+	+	
1010.44	CALS**HH**EK	+		+	
1690.83	DC**H**LAQVPS**H**TVVAR	+	+	+	+
2550.27	KPVDEYKDC**H**LAQWPS**H**TVVAR	+			+
1276.63	EFQLFSSP**H**GK			+	+
1273.65	**H**STIFENLANK			+	+
1587.76	KPVEEYANC**H**LAR				+
1413.71	ELLNQAQE**H**FGK				+
964.53	APN**H**AVVTR				+
2070.03	EDLIWELLNQAQE**H**FGK				+
1166.59	**H**QTVPQNTGGK				+
874.44	DSA**H**GFLK				+
1318.57	WCAVSE**H**EATK				+
1016.49	KSC**H**TGLGR or KSC**H**TAVGR				+
1209.51	CQSFRD**H**MK				+
700.32	LN**H**CR				+
540.29	L**H**DR				+
530.24	D**H**MK				+

Peptides eluted using 0.1 M sodium acetate (pH 4.0) (+).

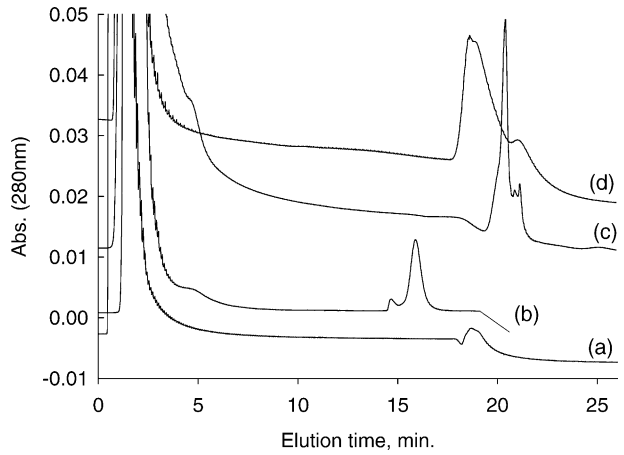

Fig. 3. Cu(II)-IMAC elution profiles of *N*-acetoxysuccinamide derivatized histidine-containing peptides from transferrin on different matrices using 0.1 M NaAc elution condition (pH 4.0): (a) CIM disc column; (b) HiTrap Chelating column; (c) TSK Chelate-5PW column; (d) Poros column.

sorbents (Table 4). This phenomenon was also observed in their corresponding Cu(II)-IMAC elution profiles (Fig. 3). Although the TSK Chelate-5PW media was still able to bind a large number of peptides, other matrices retained a substantially smaller number compared to the non-derivatized peptides. In fact, the monolith-based column was only able to bind two histidine-containing peptides; consequently, considerable information would be lost. It is not surprising to find a significant reduction of peptides retained on the Cu(II)-IMAC column because it is known that interaction of the peptide free amino-terminus with the Cu(II)-IDA matrix is important for peptide binding [22]. However, it is interesting to notice a large number of peptides retained on the TSK Chelate-5PW column. Two peptides, WCALSHHER and CALSHHEK, which were not observed by using 0.1 M sodium acetate elution buffer at pH 4.0, were eluted upon using 50 mM imidazole (pH 7.0). Once again it demonstrates the ability of TSK Chelate-5PW sorbent to bind strongly with histidine-containing peptides. Low molecular weight hydrophilic peptides, such as L**H**DR and D**H**MK, which were not previously observed, were retained on the TSK Chelate-5PW column upon N-terminus derivatization.

4. Conclusions

This work is targeted on the application of the commercially available columns for Cu(II)-IMAC selection in proteomics. Comparison of four different sorbents ranging from soft agarose gel to the mechanically stable porous media was evaluated in the capture-release mode of histidine-containing peptides. It is concluded that of these matrices the TSK Chelate-5PW media will be the most useful in proteomics because it interacts strongly with peptides that contain histidine and therefore selects the most histidine-containing peptides with an acceptable non-specific bindings from tryptic digests. The CIM disc and HiTrap Chelating columns are a close in utility, but for different reasons. HiTrap Chelating column selects a large number of histidine-containing peptides and in addition, showed the lowest non-specific binding. The disadvantage of the agarose is poor mechanical strength and difficulty of applying HiTrap Chelating columns in automated multidimensional separation systems. The CIM disc column was more like the TSK Chelate-5PW column but was less retentive. The Poros IMAC column was the least useful of the four matrices tested, showing substantial non-specific binding.

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