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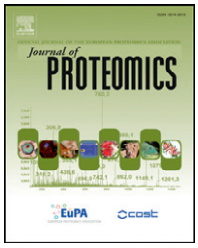

Technical note

Development of simple and rapid elution methods for proteins from various affinity beads for their direct MALDI-TOF downstream application☆

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ABSTRACT

Commercially available desalting techniques, necessary for downstream MALDI-TOF analysis of proteins, are often costly or time consuming for large-scale analysis. Here, we present techniques to elute proteins from various affinity resins, free from salt and ready for MALDI mass spectrometry. We showed that 0.1% TFA in 50% acetonitrile or 40% ethanol can be used as salt-free eluents for His-tagged proteins from variety of polyhistidine-affinity resins, while washing of resin beads twice with double-distilled water prior to the elution effectively desalted and recovered wide-range-molecular size proteins than commercially available desalting devices. Modified desalting and elution techniques were also applied for Flag- and Myc-tag affinity resins. The technique was further applied in co-precipitation assay, where the maximum recovery of wide-range molecular size proteins is crucial. Further, results showed that simple washing of the beads with double distilled water followed by elution with acetonitrile effectively desalted and recovered 150 kDa factor H protein of the sheep and its binding partner ~30 kDa BbCRASP-1 in co-precipitation assay. In summary, simple modifications in the desalting and elution strategy save time, labor and cost of the protein preparation for MALDI mass spectrometry; and large-scale protein purifications or co-precipitations can be performed with ease.
This article is part of a Special Issue entitled: (SI: Farm animal proteomics).

Immobilized Metal Affinity Chromatography (IMAC) is one of the most commonly used chromatography methods for purification of recombinant proteins fused with metal affinity polyhistidine tag. Nevertheless, other tags (like Flag, Myc, GST) and affinity beads coupled with monoclonal antibodies raised against Flag, Myc or GST peptides are also becoming a tool for purification of recombinant tagged proteins, co-immunoprecipitations and study of protein–protein interactions. IMAC is based on interaction between specific amino acid side chains (most commonly hexa-histidine) in proteins and transitional metal ions (Cu, Ni, Zn, Co and Fe), which are immobilized on a matrix. Some of the most common

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metalchelator systems for IMAC are the tridentate ligand IDA, Ni^{2+} bound to tetradentate ligand NTA (e.g. Ni-NTA from Qiagen) and Co^{2+} bound to tetradentate ligand CM-Asp (e.g. Talon™ from Clontech). In general, IMAC system provides higher sensitivity while, Flag or Myc tag based systems are good choice for more specificity and thus low background proteins in the purification, co-immunoprecipitation and protein–protein interaction assays [1–4].

SDS-PAGE and/or western blotting are the commonly used techniques for identification of the candidate proteins from above said assays, however gel based identification is laborious and time consuming. MALDI-TOF mass spectrometry provides rapid, sensitive and robust alternative for identification of proteins. One of the limiting factors of the MALDI mass spectrometry is high salt concentration in the sample [5], which necessitates further desalting of proteins either with molecular weight cut-off (MWCO) desalting columns or reversed phase media (RPM) tips like ZipTip (Millipore). Both desalting techniques add more cost and experimental steps. Moreover, such desalting procedures become cumbersome when large number of samples has to be processed.

To overcome this obstacle a simple desalting step was incorporated in the purification protocols of proteins tagged with His, Flag and Myc, moreover salt free elution solvents (acetonitrile and ethanol) were tested for elution of tagged proteins from affinity beads for their direct analysis on MALDI-TOF. These modifications were then successfully applied in co-precipitation assay to study interaction between ovine complement factor H protein (fH) of sheep and fH binding ligand of *Borrelia*.

Four His-tagged proteins of different sizes (11 kDa fragment of bovine C4BP protein, 31 kDa bovine CD40, 54.3 kDa ovine vitronectin and 150 kDa ovine fH protein) and three different resins (Talon, Clontech USA; Ni-NTA, Quiagen USA; and Magnetic Bead based IMAC [MB-IMAC], Bruker Germany) were used to assess modified desalting and elution solvents in IMAC. As a control, IMACs were first performed according to

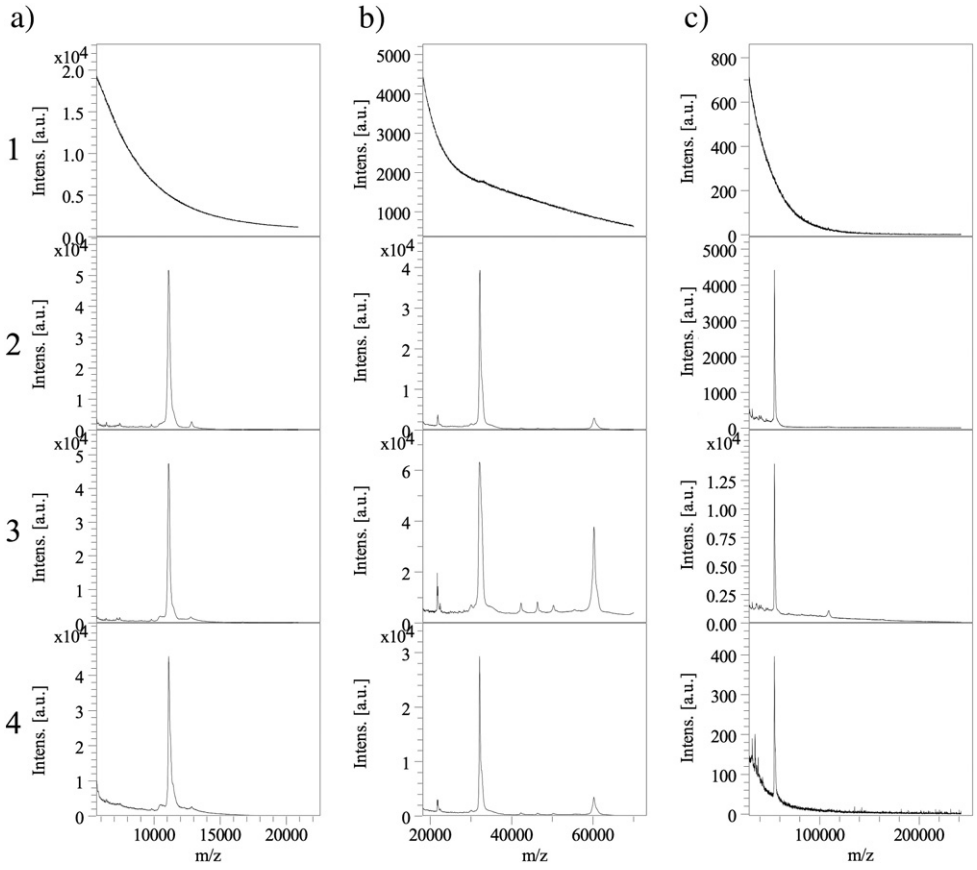

Fig. 1 – Immobilized metal affinity chromatography (Talon beads). Results of IMAC performed for purification of three His-tagged proteins [11 kDa truncated bovine C4BP (column a), 31 kDa bovine CD40 (column b) and 54 kDa ovine vitronectin (column c)] overexpressed in *E. coli*, are presented. Classical IMAC, according to manufacturer's instructions was performed and eluted proteins were analyzed on MALDI-TOF without prior desalting (row 1) or desalting with $ZipTip_{C4}$ (row 2). Desalting of proteins only with double distilled water followed by elution with TA50 (row 3) or 40% ethanol (row 4) gave promising results. Note that desalting and elution efficiencies of both TA50 and 40% ethanol were equal to commercially available ZipTips for smaller molecular weight proteins (columns a and b, note peak intensities ~3 to 5×10^4 in rows 2, 3 and 4); however for larger protein molecules recovery with ZipTips and 40% ethanol was repeatedly poor than TA50 (column C, note peak intensity ~1.3×10^4 for TA50 elution [row 3] vs. ~4500 and ~400 for ZipTips and ethanol, respectively [rows 2 and 4]).

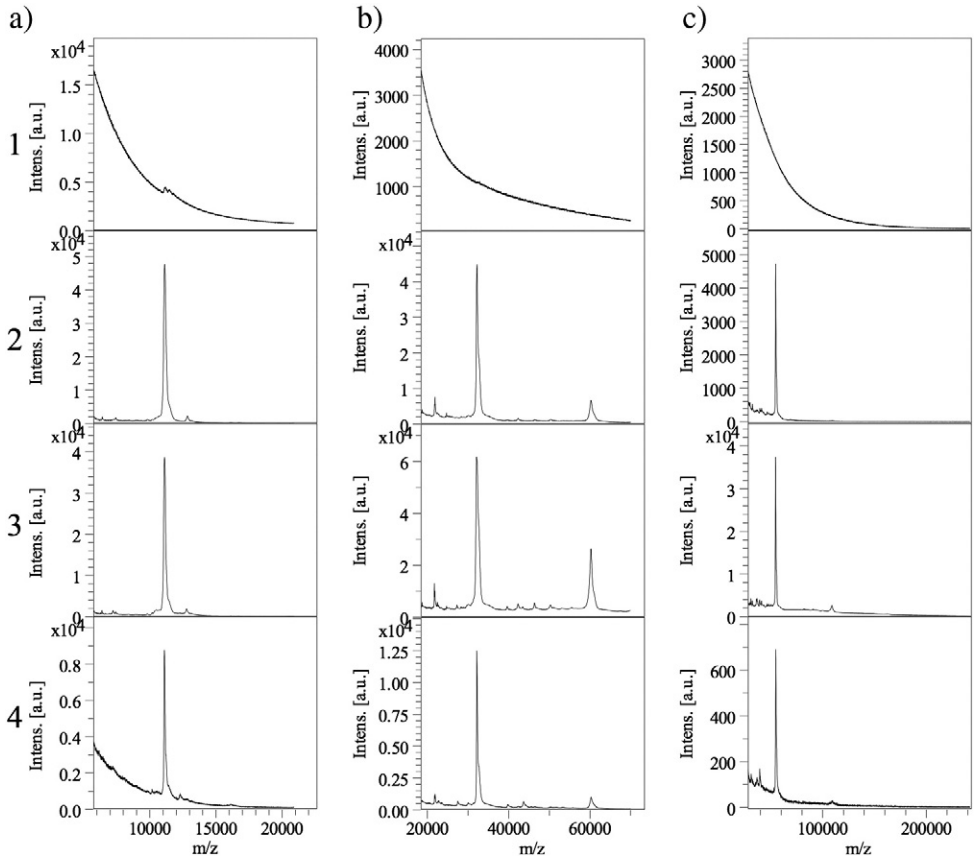

Fig. 2 – Ni-NTA based IMAC. Column a—C4BP; column b—CD40 and column c—vitronectin. Proteins in elutes obtained from classical IMAC without prior desalting (row 1) or desalting with ZipTipC4 (row 2). Modified IMAC with TA50 elution (row 3) and 40% elution (row 4). Consistent lower recovery of vitronectin, also reported in Fig. 1, was found in case of ZipTip or 40% ethanol (note peak intensity ~3.8x10^4 for TA50 elution [row 3] vs. ~4900 and ~700 for ZipTips and ethanol, respectively [rows 2 and 4]).

the manufacturer's instructions followed by desalting with RPM tips. In short, 50 μl of each resin was washed (equilibrated) twice with denaturating wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 8 M urea, 10 mM imidazole, 20 mM 2-mercapto-ethanol). To the resin 100 μl of cleared whole cell lysate of *E. coli* containing overexpressed His-tagged protein was added and binding of tagged protein to resin was allowed for 15 min at room temperature. Resins were then centrifuged at 10,000×*g* for 1 min, washed four times with wash buffer and proteins were eluted by imidazole denaturing elution buffer (45 mM sodium phosphate, 7.2 M urea, 270 mM NaCl, 150 mM imidazole). In case of MB-IMAC resin, denaturing imidazole wash and elution buffers were replaced by buffers provided in the kit. For elution, 50 μl of elution buffer was added to resin, mixed vigorously, centrifuged at 10,000×*g* for 2 min and supernatant containing tagged proteins was collected. This elution step was repeated one more time. All elutions were desalted with ZipTip$_{C4}$ pipette tips (Millipore, MA, USA) according to manufacturer's instructions.

Two modifications were incorporated in the classical IMAC described above. The first: after the binding of proteins on beads and consecutive washing with wash buffer, resins were washed two more times with double distilled water (500 μl, pH 7); the second: for elution of proteins from beads the imidazole denaturing elution buffer and commercially available elution buffer from the MB-IMAC kit were replaced either with TA50 (50:50 v/v of acetonitrile: TFA 0.1% in water) or 40% ethanol. No further desalting step was applied for His-tagged proteins eluted with modified IMAC before MALDI mass spectrometry analysis.

For MALDI-TOF analysis 1 μl of elute (desalted with ZipTip from classical IMAC or without desalting from modified IMAC) was mixed with the 1 μl of sDHB matrix (Bruker-Daltonics, Germany) in TA50, transferred onto a ground steel target and allowed to air dry. Measurements were performed on a Microflex mass spectrometer (Bruker-Daltonics). Mass spectra were recorded in the linear, positive mode at a laser frequency of 60 Hz (250 shots total). The spectra were calibrated using the protein Standard I (5.000 to 20.0000 kDa range) and II (20.000 to 70.0000 kDa range) from Bruker-Daltonics.

Results from the standard IMAC and IMAC with modifications (Figs. 1–4) clearly show that washing of affinity beads

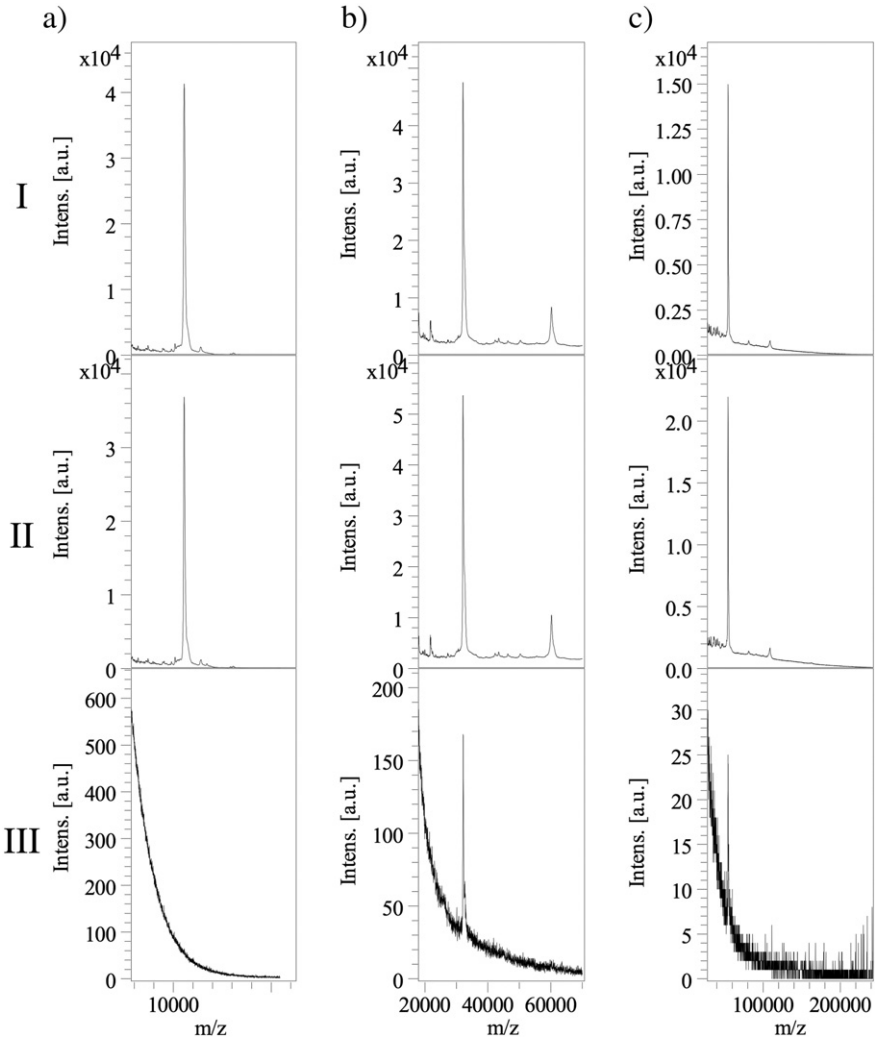

Fig. 3 – Magnetic beads immobilized metal affinity chromatography (MB-IMAC). Column a—C4BP; column b—CD40 and column c—vitronectin. Note that elution buffer provided in the kit (MB-IMAC, Bruker) was fully compatible with downstream MALDI assay and no desalting was necessary (row I). Composition of this elution buffer is undisclosed and under Bruker's trademark. TA50 (row II) was the best alternative for commercial elution buffer, however 40% ethanol was not suitable at all (row III). Note that recovery of vitronectin was equally good in both commercial elution buffer (peak intensity ~1.5×10^4, row 1) and TA50 (peak intensity ~2.2×10^4, row 2). The only disadvantage of this commercial MB-IMAC is that it is not suitable for large-scale protein purification.

with double distilled water (pH 7) ensures effective removal of the salts. Moreover, the peak intensities obtained in the modified protocol indicate that the washing with water causes minimum, if at all, loss of the tagged proteins from affinity beads. It is important to note that pH of water used in washing step is crucial as most of the IMAC resins bind/retain His-tagged proteins at neutral or slightly basic pH. Whereas, at acidic pH, affinity between metal ions and polyhistidine tags decreases substantially, that leads to the detachment of His-tagged proteins from beads. Thus the use of high quality double distilled water with pH 7 (adjusted with NaOH) is crucial in the washing step.

Acetonitrile and ethanol are the commonly used MALDI-TOF compatible organic solvents. Furthermore, use of 50% acetonitrile and 40% ethanol with IMAC affinity beads do not cause increase in the noise during MALDI-TOF analysis. In terms of noise vs. signal ratio 50% acetonitrile with 0.1% TFA was found better eluent than 40% ethanol for Talon and Ni-NTA affinity beads, whereas, 40% ethanol was not suitable for MB-IMAC beads (Figs. 1–4).

Desalting columns or tips with RPM (like ZipTips) are used commonly for desalting and concentration of the proteins eluted with standard IMAC protocols. The major disadvantage of RPM tips, often observed, is low retention of high molecular weight proteins. For example, RPC tips with C4 can be used for proteins up to 100 kDa size however with poor protein recovery. Here we showed an excellent desalting and elution of higher molecular weight protein (150 kDa fH) with

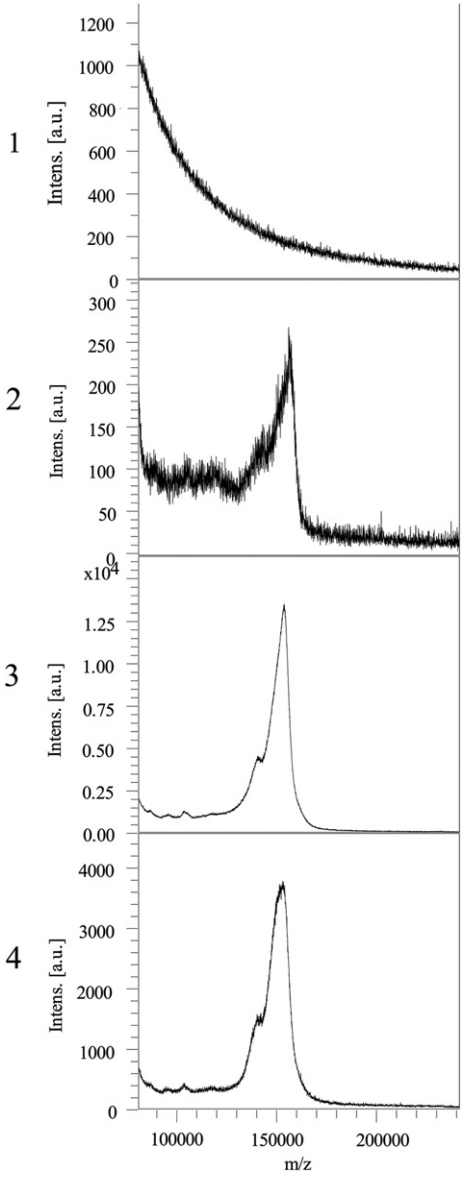

Fig. 4 – Purification of higher molecular weight protein (ovine factor H) with IMAC. Comparative recovery of higher molecular weight protein (for example 150 kDa fH) by RPM column — ZipTip$_{C4}$, TA50 and 40% ethanol is presented. MALDI-TOF analysis of sample eluted from classical IMAC with imidazole elution buffer without desalting (row 1) and after desalting with ZipTip$_{C4}$ (Row 2). MALDI-TOF analysis of sample eluted from modified IMAC with TA50 as eluent (row 3) and 40% ethanol (row 4). Extremely higher recovery of high-molecular weight protein fH with TA50 than 40% ethanol and ZipTip was observed (note peak intensity ~1.3 × 10^4 for TA50 elution [row 3] vs. ~270 and ~3900 for ZipTips and ethanol, respectively [rows 2 and 4]).

significantly higher recovery than commercially available C4 RPM tips (Fig. 4).

Anti Flag or Myc antibodies coupled to agarose beads are prone to degradation when inadequate elution buffers are used. This degradation causes to two major background peaks in MALDI-TOF analysis, the higher molecular weight peak ~50 kDa corresponding to heavy chain and ~26 kDa peak of light chain of the IgG, which complicates the identification of the protein of interest (Fig. 5). Use of 40% ethanol as an eluent did not cause degradation of both Myc and Flag monoclonal antibodies, however failed to elute Myc- or Flag-tagged proteins effectively (results not presented). Use of TA50 showed evident degradation of the antibody resulting into the high background peaks of heavy and light chains (Fig. 5). Previously, it was shown that increase in the percentage of acetonitrile makes the antibody unstable and reduces its activity by nearly 500 times than that in aqueous medium. This reduction in the activity is due to the irreversible conformation changes in the antibody [6]. Authors further reported that acetonitrile at concentration 40% or below in water did not change the conformation of antibody irreversibly [6]. To avoid degradation of antibodies different concentrations of acetonitrile were assessed. In short, 20 μl of affinity beads coupled with Flag- or Myc-tagged monoclonal antibodies were washed twice with TBS followed by two washing with double distilled water (pH 7.2). Affinity beads were then incubated with 0.1% TFA containing decreasing concentrations of acetonitrile (50% up to 10%), centrifuged at 10,000×*g* for 1 min and supernatant was analyzed by MALDI-TOF. Effect of acetonitrile on IgG degradation is presented in Fig. 5. Finally, TA10 was used as most suitable eluent for Flag and Myc-tagged proteins. For this purpose, Flag- (~45 kDa) or Myc-tagged (~42 kDa) truncated forms of ovine toll-like receptor 1 protein were used. 50 μl of agarose beads coupled with monoclonal-anti-Flag or anti-Myc antibodies (both Sigma, Slovakia) was washed twice with TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.4). Beads were then incubated at 4 °C for overnight with cleared whole cell lysate of *S. cerevisiae* containing overexpressed Flag or Myc-tagged proteins. Unbound proteins from agarose beads were removed by washing with TBS buffer for two times, while desalting was performed by washing the beads two times with double distilled water (pH 7.2). Proteins were eluted from agarose beads with TA10 or 0.1 M glycine HCl (pH 3.5). For proteins eluted with glycine HCl, desalting with ZipTip$_{C4}$ (Millipore) was performed as per manufacturer's instructions, whereas no desalting of the proteins eluted with acetonitrile or ethanol was performed before MALDI-TOF analysis. Results depicted in Fig. 5 shows that desalting of Flag- and Myc-tagged proteins with water and TA10, as eluent, effectively elutes tagged proteins without background noise.

Effective desalting and maximum recovery of the proteins from affinity beads is essential in co-precipitation assays when novel protein–protein interactions are studied. When interacting proteins differ significantly in their molecular weights, the desalting and protein recovery of both candidates with single RPM tips becomes difficult. To overcome this problem, desalting of the protein complex on Talon beads was performed with double distilled water and proteins were eluted with TA50 as described above. In short, His-tagged 150 kDa fH protein of sheep was bound on Talon beads for 1 h at 4 °C in native wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, pH 7.0). After washing of unbound proteins with native wash buffer, beads were incubated in cleared whole cell lysate of *Borrelia burgdorferi* sensu stricto

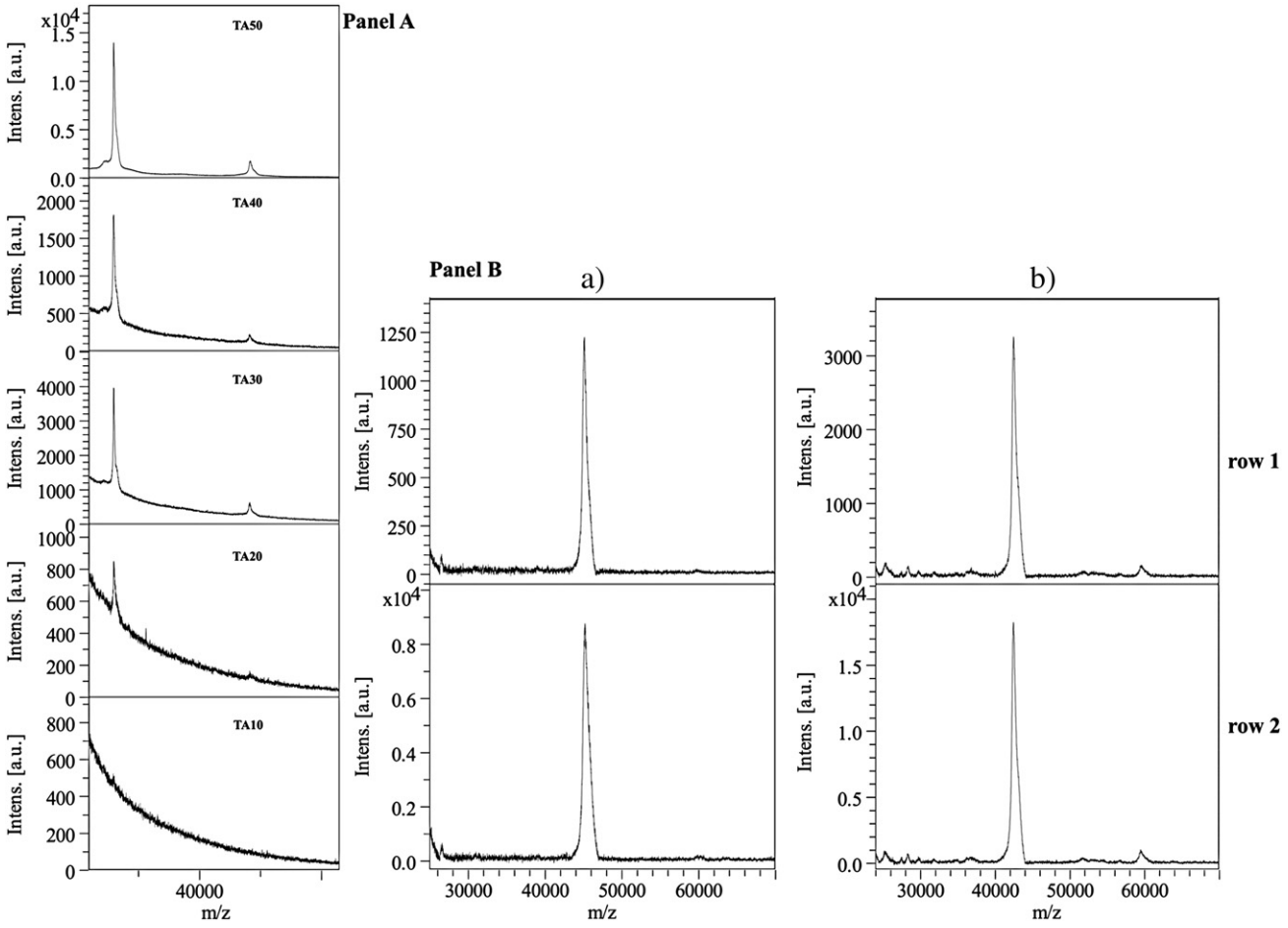

Fig. 5 – Use of modified wash and elution techniques for Flag- and Myc affinity beads. Panel a — standardization of the elution buffer for affinity beads coupled with monoclonal anti-Flag or anti-Myc antibodies. The decreasing concentrations of the acetonitrile in elution buffer were used to avoid degradation of the IgG molecule. Degradation of the IgG into light (peak at ~26 kDa) and heavy (peak at ~50 kDa) chains was the highest in TA50 and decreased gradually by decreasing concentration of acetonitrile up to 10% (TA10). Thus, TA10 was used as an optimal elution buffer for Flag- and Myc-tagged proteins. Panel b — Purification of ~45 kDa Flag-tagged (a) and ~42 kDa Myc-tagged (b) truncated ovine Toll like receptor-1 proteins. Row 1 presents elution of proteins with glycine-HCl after desalting with ZipTip$_{C4}$ and row 2 depicts results of modified purification technique, in which desalting was performed by washing of the beads with double distilled water and proteins were eluted with TA10.

(containing 1× protease inhibitor cocktail, Fermentas) for overnight at 4 °C with constant shaking. Non-interacting proteins of *Borrelia* were removed by washing two times with native wash buffer. Desalting of the proteins with double distilled water followed by elution of proteins with TA50 and their direct identification by MALDI-TOF was performed as described above. Results presented in Fig. 6 indicate that modified desalting and elution can be applied successfully in co-precipitation assay when protein candidates with wide-range of molecular masses are under study. Binding partner of 150 kDa ovine fH was identified as BbCRASP-1, one of the major fH binding proteins of *Borrelia*, in another study (details are not presented in this report).

In summary, overall results show that addition of two simple steps (washing of affinity beads with water and elution in salt free eluents) can shorten the time required for the identification of purified tagged proteins, circumvent need of costly desalting columns or tips, recover wide-rage molecular mass proteins from co-precipitations assays and ease the sample preparation for downstream MALDI-TOF assays for large-scale protein purifications or co-precipitation assays.

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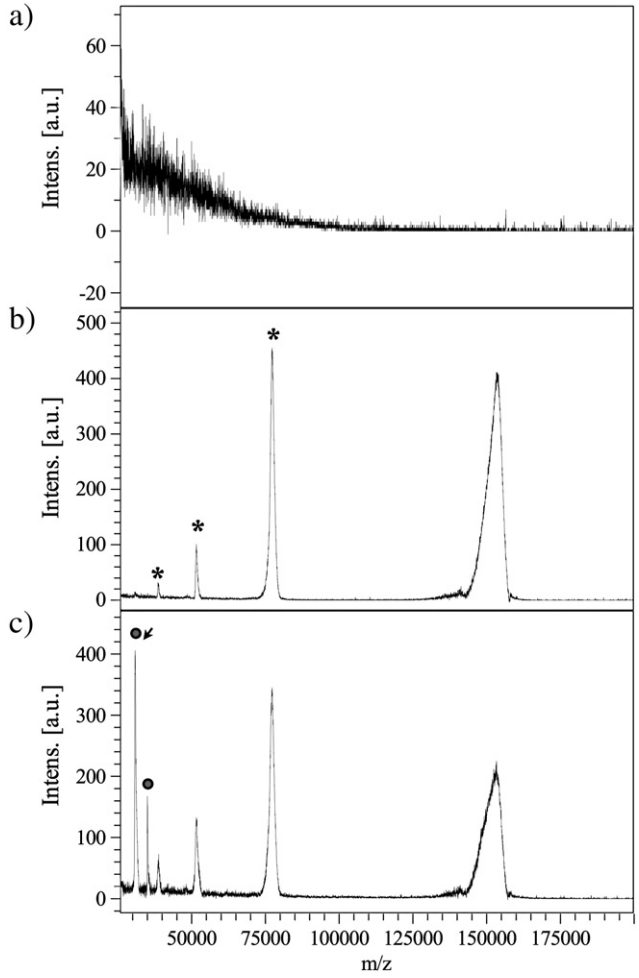

Fig. 6 – Use of modified wash and elution techniques in co-precipitation assay with IMAC. Ovine factor H was immobilized on Talon IMAC beads and hybridized with whole cell lysate of *Borrelia burdorferi* sensu stricto or TBS (negative control, b). After washing of the unbound proteins and desalting (washing twice with H_2O), factor H and its binding partner/s were eluted from IMAC beads with TA50 (c). a — incubation of IMAC beads with whole cell lysate of *Borrelia* only (this negative control was necessary to exclude protein peaks that may appeared due to non-specific binding of borrelial protein to Talon beads). Peak at 150 kDa is single charged ion of factor H. * — indicates double, triple and more charged ions of factor H. ● — co-precipitated putative factor H binding proteins of *Borrelia*. ↙ — this protein was identified as factor H binding BbCRASP-1 of *Borrelia* in our parallel study.

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