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Rapid evaluation of nickel binding properties of His-tagged lactate dehydrogenases using surface plasmon resonance

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Abstract

The use of surface plasmon resonance (SPR), for the comparison of metal binding properties of polyhistidine tags, was evaluated. Six different tags containing various number of histidines, either none (tags n and t), three (tags H_3A_3 and HA_2HA_2H) or six (tags H_6 and His_6), were genetically fused to the N-terminal of lactate dehydrogenase (LDH). The binding ability of these constructs to nickel ions, immobilised with nitrilotriacetic acid (NTA), was tested both by conventional immobilised metal ion affinity chromatography (IMAC) and SPR. The relative binding strengths of the tags to nickel were identical using both methods ($n \approx t < HA_2HA_2H < H_3A_3 < His_6 < H_6$), confirming the value of the SPR technique for investigating metal–protein interactions. Protein modelling has also proved to be useful in supporting the experimental results.

Keywords: Immobilised metal ion affinity chromatography; Nickel; Affinity tags; Nitrilotriacetic acid; Surface plasmon resonance; Lactate dehydrogenase

1. Introduction

Since the introduction of immobilised metal ion affinity chromatography (IMAC) by Porath and co-workers [1,2], it has been extensively used for protein purification. The most commonly used metals for IMAC are Co^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} chelated to iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA) [3] columns. Since only a limited number of wild-type proteins can bind selectively to metal ions, the production of recombinant proteins carrying hexa-histidine tags has proved to be very efficient [4–6], and several novel metal binding tags have been developed [7–10]. However, only limited knowledge is available on the degree of affinity between the immobilised metal ions and the histidine-containing tags on the proteins. Particularly surface plasmon resonance (SPR) has proved useful for monitoring such biomolecular interactions [11]. SPR systems are optical biosensors, which have the ability to follow the interaction between two biomolecules such as proteins, oligonucleotides, lipids, sugars or even cells in real-time [12–16]. In order to perform an SPR analysis, the ligand must first be immobilised on the sensor chip. The ligand immobilisation can be performed in different ways, mostly covalently, using different kind of chemistries depending on the nature of the ligand. Sensor chips carrying the metal-chelating agent NTA have been developed [17–19], that are able to adsorb histidine-tagged proteins on the chip, for further ligand interaction analysis.

In this study, we have explored the possibility of utilising SPR to evaluate and compare the influence of the amino acid sequence of histidine-containing tags on the binding properties to nickel ions. The different tags were attached to the N-terminal of the thermostable enzyme lactate dehydrogenase (LDH) (*Bacillus stearothermophilus*). The results obtained from SPR were compared with those using a conventional IMAC column, in order to confirm that SPR can be used as a practical tool for affinity comparison even in the case of metal–protein interactions. The use of protein modelling was also shown to be helpful, to support hypothesis raised by experimental results.

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2. Experimental

2.1. Reagents

Restriction endonucleases, T4 DNA ligase and the DNA size ladder (λ/*Eco*RI + *Hind*III) were purchased from MBI Fermentas (Vilnius, Lithuania) and used according to the supplier's recommendations. The Qiaprep spin miniprep kit and the Qiaquick gel extraction kit from Qiagen (Basel, Switzerland) were used for plasmid DNA purification and restricted DNA purification from agarose gels, respectively. The chelating Sepharose fast flow was obtained from Amersham Biosciences AB (Uppsala, Sweden) and Ni-NTA agarose from Qiagen (Basel, Switzerland). All other chemicals were of analytical grade and commercially available.

2.2. Bacterial strain and plasmids

The *Escherichia coli* strain TG1 [*supE thi-1 Δ*(*lac-proAB*)*Δ*(*mcrB-hsdSM*) *5* (r_K^- m_K^-) *F′ traD36 proAB lacI*^{q}*ZΔM15*] was used as the host in all cloning procedures. Plasmid pTrc99A [20] was used as cloning vector. The native *ldh* gene from *B. stearothermophilus* [21] was introduced into pUC18 by Carlsson et al. [22] expressing the native form of LDH carrying three additional residues, n-LDH (EC 1.1.1.27) (Table 1). Plasmid pBLH62 was used for preparing a his_6-tagged protein, His_6-LDH [23] (Table 1). All cloning procedures were performed according to Sambrook et al. [24].

2.3. Plasmid constructions

New plasmids were constructed by using the following oligonucleotides, which were synthesised by MWG Biotech (Ebersberg, Germany):

h6_for	5′-CATGGCTCACCATCACCATCACCA-TGCTAGCG-3′
h6_rev	5′-GATCCGCTAGCATGGTGATGGTGA-TGGTGAGC-3′
h3a3_for	5′-CATGGCTCACGCGCACGCGCACG-CGGCTAGCG-3′
h3a3_rev	5′-GATCCGCTAGCCGCGTGCGCGTG-CGCGTGAGC-3′
haahaah_for	5′-CATGGCTCACGCGGCTCACGCGG-CTCACGCTAGCG-3
haahaah_rev	5′-GATCCGCTAGCGTGAGCCGCGTG-AGCCGCGTGAGC-3

Complementary mixtures of 0.1 μM forward and reverse oligonucleotides were hybridised at 90 °C for 15 min and slowly cooled down to room temperature. The hybridised DNA fragment was introduced into pTrc99A between the *Nco*I site at the 5′-end and the *Bam*HI. The insert carries a unique *Nhe*I site to facilitate the screening of the insertion. The *ldh* gene was subsequently inserted using the *Bam*HI and *Pst*I sites, resulting in plasmids pTrcH_6LDH, pTrcH_3A_3LDH and pTrcHA_2HA_2HLDH, respectively. Plasmid pTrctLDH was obtained by directly introducing the *ldh* gene into pTrc99A using *Bam*HI and *Pst*I. This vector encodes t-LDH, which carries a random peptide tag useful for controlling the behaviour of SPR and IMAC. The sequences of the final constructs were confirmed using Big Dye v3.0 DNA sequencing kit from Applied Biosystems (Warrington, UK) and analysed by CyberGene AB (Huddinge, Sweden). The amino acid sequences of the different tags are given in Table 1.

Table 1
Amino acid sequence of the different tags

Construct	Tag sequence
n-LDH	MNA-LDH
His_6-LDH	MTMITNSHHHHHHGSNA-LDH
t-LDH	MEFELGTRGSNA-LDH
H_6-LDH	MAHHHHHHASGSNA-LDH
H_3A_3-LDH	MAHAHAHAASGSNA-LDH
HA_2HA_2H-LDH	MAHAAHAAHASGSNA-LDH

2.4. Protein expression and purification

All cells were grown in Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl). Two hundred millilitres of LB medium containing 100 μg/mL of ampicillin, was inoculated by 0.5 mL of an overnight culture and gene expression was induced directly by 0.2 mM IPTG (isopropyl-β-D-thiogalactoside). The cells were harvested (3000 × *g*, 5 min) at late log phase and re-suspended in buffer A (50 mM sodium phosphate, 0.5 M NaCl, pH 7.5). The cell suspension was then sonicated and centrifuged (20,000 × *g*, 15 min), followed by a heat treatment of the supernatant (65 °C, 15 min) to denature most of the host cell proteins, followed by a second centrifugation (16,000 × *g*, 15 min).

The supernatant was purified using IMAC in batch format. Samples n-LDH and t-LDH were treated using Sepharose fast flow IDA gel immobilised with nickel ions (Ni^{2+}-IDA), whereas all other samples were purified on NTA agarose gel immobilised with nickel ions (Ni^{2+}-NTA). The purifications were performed in 1.5 mL tubes, using 250 μL of immobilised chelating gel, to which was applied 1 mL of heat-treated protein extract. The suspension was mixed gently for 1 h at room temperature. The gel was subsequently spun down, the unbound fraction removed and the gel washed with 3 × 1 mL of buffer A. Native bacterial *E. coli* proteins were removed by gently mixing the gel for 15 min with 1 mL of 10 mM imidazole solution, except for His_6-LDH and H_6-LDH where a 50 mM imidazole solution was used. The bound LDHs were finally eluted from the gel using 2 × 0.5 mL of a 100 mM imidazole solution apart for the two hexa-histidines constructs for which a 150 mM imidazole solution was used.

Before SPR measurements, the imidazole was removed by dialysis using Spectra/Por tubing from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA) soaked into buffer B (0.01 M Hepes, 0.15 M NaCl, pH 7.4). The purity of the samples was checked on 12% Tris–HCl SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using the Mark12 protein molecular mass standard from Invitrogen Corp. (Carlsbad, CA, USA) and Coomassie brilliant blue staining. Protein concentrations were measured using the Protein Assay from Bio-Rad Laboratories AB (Sundbyberg, Sweden) [25].

2.5. Enzyme activity assay

The determinations of LDH activity were performed in 0.1 M 2-[*N*-morpholino]-ethanesulfonic acid (MES) buffer (pH 6.5), containing 30 mM pyruvate and 0.2 mM NADH, by monitoring the absorbance decrease of NADH at 340 nm. One enzyme unit (U) represents the reduction of 1 μmol of pyruvate per minute.

2.6. IMAC experiments

The chromatography experiments were performed at room temperature in a C10 column (10 cm × 1 cm) using an AC10 adaptor, both purchased from Amersham Biosciences AB (Uppsala, Sweden). A 0.6 mL of Ni-NTA agarose gel was packed in the column and 1 mL of heat-treated samples were applied at a flow rate of 0.75 mL/min. Elution was carried out by a continuous gradient of imidazole, ranging from 0 to 150 mM and 0.75 mL fractions were collected. LDH was followed by measuring its enzymatic activity in the different fractions.

2.7. Protein modelling

Protein models of the three-dimensional structure of clones His_6-LDH and H_6-LDH were produced and visualised using the program DeepView/Swiss-PdbViewer v3.7b2. [26] in combination with POV-Ray v3.5 software. The structural data used for LDH were obtained from the Protein Data Bank (PDB) [27] under the reference ID: 1LDN [28]. The amino acids of the tag were added one by one at the N-terminal by choosing the conformation of each side chain that was most probable according to DeepView.

2.8. SPR measurements

All measurements were performed on a Biacore 3000 system, using a Sensor Chip NTA, Biacore AB (Uppsala, Sweden). All the buffers and solutions used in the experiments were filtered (0.20 μm) and degassed before use into the Biacore.

The running buffer was composed of 50 μM EDTA in buffer B. The nickel ions were first immobilised on the chip by a 60 s injection at a flow rate of 10 μL/min. The different samples were then applied to the chip, using 3 min injections of a 200 nM purified protein extract, at a flow rate of 10 μL/min. Between each sample injections, the chip was regenerated by a 3 min injection of regeneration buffer (0.01 M Hepes, 0.15 M NaCl, 0.35 M EDTA, 0.005% surfactant P20, pH 8.3) at a flow rate of 10 μL/min and re-immobilised with nickel ions (500 μM $NiCl_2$ in buffer B).

3. Results and discussion

3.1. LDH constructs

The sequence and structure of the affinity tags used in IMAC affect their ability to bind to metal ions. In order to evaluate these effects, four different tags were genetically engineered and fused to the N-terminal of LDH (Table 1). These new constructs were compared with each other, together with two other constructs (n-LDH and His_6-LDH) prepared previously [22,23].

The modified enzymes were expressed into *E. coli* by IPTG induction and purified from the cells using sonication, heat treatment and IMAC in batch format. The cells were grown until late log phase, harvested and re-suspended into buffer to the same concentration for each clone. The heat treatment step was used to remove most of the bacterial host proteins. However, since the *ldh* gene originates from a thermophilic organism, this step only has a small influence on the native form of the enzyme. In order to dissect the influence of the tag on the thermal stability of the protein, the remaining enzymatic activity after heating for 15 min at 65 °C was evaluated. None of the tags had any significant influence on the thermal stability of the enzyme. All activities thus remained within 92–96% of the original activity (data not shown).

The relative levels of expression of soluble LDH were also determined by analysing the heat-treated fractions by SDS-PAGE gels (Fig. 1). The relative levels of expression of soluble LDH obtained were of 100, 382, 135, 243, 73 and 25 for n-LDH, His_6-LDH, t-LDH, H_6-LDH, H_3A_3-LDH and HA_2HA_2H-LDH, respectively. The differences in expression levels were surprisingly large. The presence of a hexa-histidine sequence in the tag appeared to be beneficial, as in both cases the expression was greatly increased. This beneficial effect is probably related to a more favourable transcript structure [29].

The protein extracts were further purified by IMAC using Ni^{2+}-IDA Sepharose for the two non-His-tagged proteins, n-LDH and t-LDH, and Ni^{2+}-NTA agarose for all the other constructs. The reason why a different gel was used for the non-his-tagged constructs is that they have no affinity to the NTA ligand. IDA is a tridentate chelator, leaving three available ligand positions on the co-ordination sphere of the nickel ions, which therefore can bind to non-vicinal histidine residues on the surface of native LDH. In the case of NTA, which is a tetradentate chelator, the purification is more selective requiring interactions with neighbouring histidines [30]. The purity of the different clones after IMAC was also

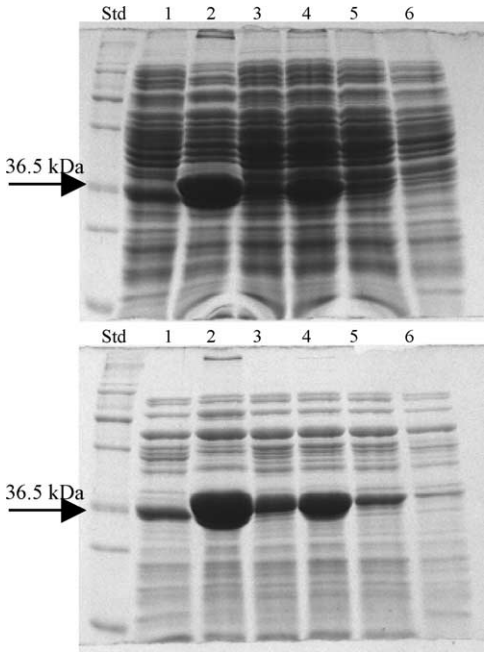

Fig. 1. A 12% gel SDS-PAGE analysis of the clones before (top gel) and after (bottom gel) heat treatment at 65 °C for 15 min. Molecular mass standard (Std), n-LDH (1), His_6-LDH (2), t-LDH (3), H_6-LDH (4), H_3A_3-LDH (5), HA_2HA_2H-LDH (6).

examined by SDS-PAGE (Fig. 2). After the chromatographic step, the specific activity of every clone was measured in order to evaluate the influence of the tags on the enzymatic activity (Fig. 3). The lower specific activities observed for some of the constructs were linked to the lower degree of purity of these samples. When corrected, the specific activities of all clones were similar (data not shown) and no remarkable effects could be attributed to any of the tags.

3.2. IMAC experiments

In order to compare the ability of the different tags to bind to nickel ions, the different LDH constructs were initially examined on Ni^{2+}-NTA agarose gels and eluted by a continuous gradient of imidazole. The elution fractions were screened for LDH activity (Fig. 4). The variation between

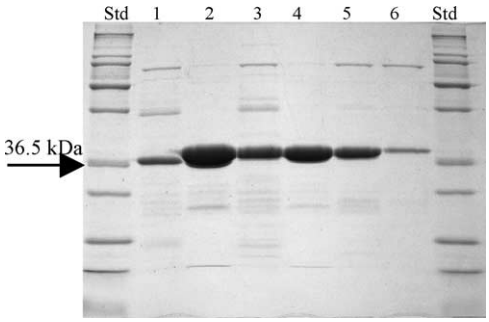

Fig. 2. A 12% gel SDS-PAGE analysis of the clones after purification by IMAC. Molecular mass standard (Std), n-LDH (1), His_6-LDH (2), t-LDH (3), H_6-LDH (4), H_3A_3-LDH (5), HA_2HA_2H-LDH (6).

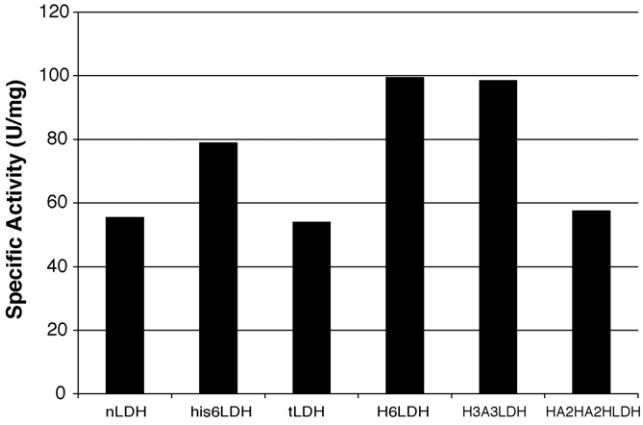

Fig. 3. Specific enzymatic activity in U/mg of all constructs after IMAC purification.

the elution profiles of His_6-LDH and H_6-LDH can be explained by the fact that the hexa-histidine sequence probably sticks out more from the protein surface in the H_6-LDH construct than in His_6-LDH. The histidines are separated by two additional amino acids from the native protein in H_6-LDH. In addition, the hexa-histidine sequence of H_6-LDH starts also closer to the N-terminal of the protein. Therefore, the histidines are probably more exposed and show a stronger interaction with the Ni^{2+}-NTA binding sites. This was also verified by protein modelling. Proteins models of constructs His_6-LDH and H_6-LDH were designed and visualised using the DeepView/Swiss-PdbViewer and POV-Ray softwares (Fig. 5). The tags are closely associated in space into pairs which point in opposite directions from either side of the protein. In the case of H_6-LDH, the hexa-histidine sequence is clearly more exposed than in His_6-LDH, supporting thereby the experimental results and the hypothesis mentioned previously.

H_3A_3-LDH showed a higher affinity to nickel than HA_2HA_2H-LDH, even though they both have the same

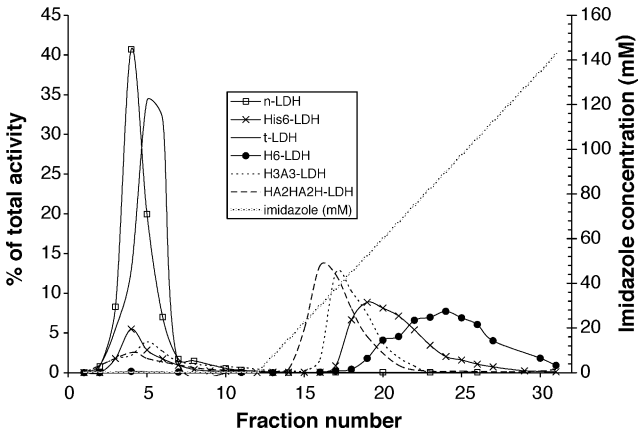

Fig. 4. Elution profile of the different constructs from the Ni-NTA agarose gel. The percentage of total enzymatic activity in each collected fractions, is normalised against the total activity measured in the fraction applied to the gel (heat-treated fraction).

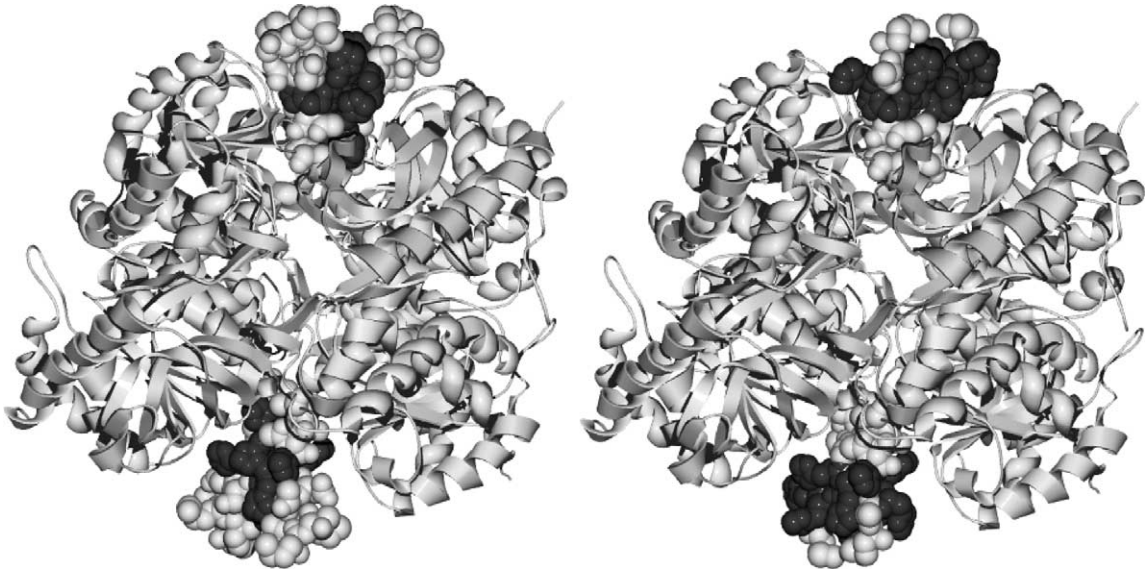

Fig. 5. Protein models of constructs His_6-LDH (left) and H_6-LDH (right). The different subunits are represented in light grey using ribbon style. The tags are represented in space-filled atoms style, also coloured in light grey, except for the histidines which are coloured in dark grey.

number of histidines. This observation clearly shows that the HXXH sequence is less favourable than HXH or HXXXH, where X represents any amino acid. According to Haymore et al. [31], HXXH sequences present at the surface of proteins have to be found in a reverse β-turn conformation, in order for the side chains of the histidines to point out in the same direction and to form possible metal-chelation sites. On the other hand, a β-strand structure is necessary for HXH sequences and an α-helix structure for HXXXH. Starting from the N-terminal, the tag in the H_3A_3-LDH construct can form metal-chelation sites of both type HXH, between the first and second histidine or between the second and third, and of type HXXXH between the first and third histidine residue. On the other hand, the tag in HA_2HA_2H-LDH can only form HXXH chelating sites. Indeed, using the Chou–Fasman model for protein secondary structure prediction [32], both tags were determined to adopt an helical conformation. Because of the nature of histidine and alanine residues, the flexible tag in H_3A_3-LDH is much more likely to adopt an α-helix or on lower level a β-strand conformation, than the HAAH sequences in HA_2HA_2H-LDH are to adopt reverse β-turn structures, thereby generating stronger metal affinity.

3.3. SPR measurements

The use of the surface plasmon resonance technique, for the affinity comparison of the different tags to nickel ions, was also evaluated. The experiments were conducted in a Biacore 3000 system. Nickel ions were first immobilised on a chip covered with NTA, and then the different protein solutions were adsorbed on the metal ions using a 3 min injection of a 200 nM enzyme purified extract. The binding response was monitored using two flow cells, by subtracting the signal measured on a reference cell, where no nickel was immobilised, in order to minimise all unspecific interactions (Fig. 6).

The results obtained by SPR measurements were in good agreement with those obtained by IMAC. The simple observation of the SPR response curves gives clear indications about the binding abilities of the different constructs. Both hexa-histidine constructs, His_6-LDH and H_6-LDH, showed very stable binding as hardly any dissociation could be observed. These results were expected since LDH is a tetrameric enzyme, therefore holding four tags. According to Nieba et al. [19], at least two hexa-histidine tags are preferable to obtain stable binding to a NTA chelating chip. In addition, the difference in binding strength observed between our two different hexa-histidine constructs in the IMAC experiments, was also observed with SPR, as H_6-LDH was immobilised to a higher level than His_6-LDH.

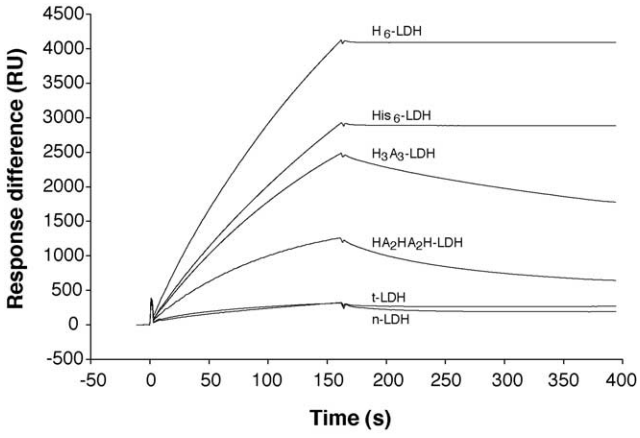

Fig. 6. SPR sensograms of the different LDH constructs (relative response units/time (s)).

Table 2
Apparent K_D values of the different constructs

Clone	k_a ($M^{-1}\,s^{-1}$)	k_d (s^{-1})	K_D (M)
His_6-LDH	2.16×10^4	5.13×10^{-8}	2.4×10^{-12}
H_6-LDH	2.82×10^4	8.83×10^{-9}	3.1×10^{-13}
H_3A_3-LDH	1.92×10^4	1.42×10^{-3}	7.4×10^{-8}
HA_2HA_2H-LDH	2.35×10^4	3×10^{-3}	1.3×10^{-7}

The two other constructs H_3A_3-LDH and HA_2HA_2H-LDH, resulted in less stable binding as they dissociated more rapidly from the surface and here again, the structure of the tag in HA_2HA_2H-LDH proved to be less beneficial as it dissociated faster from the surface. In order to estimate the differences between the constructs, the apparent K_D values for the different curves were calculated using the BIA evaluation 3.2 software (Biacore AB) and to simplify the calculations further a simple 1:1 Langmuir binding model was used (Table 2). K_D was calculated as the ratio between the dissociation and the association rate constants ($K_D = k_d/k_a$) and gives information on the strength of the binding, the lower the value, the stronger the affinity. The k_a values were similar, clearly indicating that the type of interaction studied is the same in all cases (histidine–nickel ion interaction). Thus, the differences observed are linked with differences between the dissociation rates. The K_D values obtained for the two hexa-histidine constructs are extremely small, showing the high stability of this interaction.

4. Conclusion

In this work, we have demonstrated that it is possible to correlate the affinity binding results from SPR measurements, with chromatographic procedures such as column IMAC. The ability of the SPR technique to detect small differences between metal ions and proteins interactions, makes it a suitable method to use in a tag affinity comparison study. It could, for example, therefore be used for the screening of a metal binding tag library. The SPR method presents great advantages. The protocol set-up is much faster as the analysis only takes a few minutes. The procedure is easier to operate than an IMAC experiment in column format where multiple fractions need to be handled and analysed. Finally, the method is also beneficial because only very small concentrations of protein are needed for the analysis, which can be very useful in many cases where the available amounts of sample are limited.

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