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Novel purification system for 6xHis-tagged proteins by magnetic affinity separation

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Abstract

We have developed a novel nickel–silica matrix for the generation of magnetic beads for metal-ion affinity chromatography. In contrast to magnetic Ni–NTA agarose beads, the novel particle type (SiMAC) consists of a magnetic core and a nickel–silica composite matrix with the nickel ions tightly integrated in the silica. This results in a much higher number of chelating groups compared with Ni–NTA agarose beads. With the SiMAC beads, greatly improved purification of histidine-tagged proteins from crude bacterial extracts was achieved. The yield was at least twice as high as with conventional materials, the method is faster, since the coupling step is omitted and there is no need for handling toxic Ni^{2+} salts.

Keywords: Purification; Magnetic affinity separation; Proteins, 6xHis-tagged

1. Introduction

The expression of recombinant proteins with histidine fusion tags is widely used to ease their purification from a variety of prokaryotic and eukaryotic expression systems [1–3]. Typically, purification of histidine-tagged proteins can be achieved in a single capture step using immobilized metal-ion affinity chromatography (IMAC) [4–7]. This method has been used successfully for the purification of proteins expressed in bacterial, mammalian, and insect systems. The most commonly used solid supports for IMAC purification are agarose beads with covalently immobilized iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA). With these chelating agents, four of the six coordination sites of nickel are occupied, allowing the tight binding of nickel ions. Several supporting matrices are commercially available, including agarose, Sepharose, silica and even microtiter plates. Other chelating groups for IMAC purification were described such as Tris-(carboxylmethyl)ethylenediamine [8] or iminodimethylphosphonic acid [9]. The use of magnetic agarose beads is often advantageous to chromatographic procedures allowing a fast purification without the need of prior protein fractionation. Furthermore, a wide range of applications up-scalable from micro scale in high throughput and highly parallel manner is feasible. Because the magnetic beads can

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be reused several times, this approach is also cost-saving.

However, the yields have usually been lower than with chromatographic systems and a still time-consuming coupling step of the Ni^{2+} ions to the beads before use has been required. Therefore we developed a new matrix for the purification of 6xHis-tagged proteins, which possesses substantial advantages compared with the conventional Ni–NTA-based methods.

2. Experimental

2.1. Magnetic beads

The SiMAC beads (Chemicell, Berlin, Germany) consist of a magnetic core and a nickel–silica composite matrix with the nickel ions tightly integrated in the silica. Consequently, the beads can be used directly without prior activation with Ni^{2+} ions. Additionally, modified versions of the SiMAC were produced, such as SiMAC-444 with a porous matrix surface, SiMAC-445 with a non-porous surface and SiMAC-446 with 60% less Ni^{2+}. Furthermore, reused and autoclaved SiMAC beads were tested. The ''Magnetic Agarose Beads'' (Ni–NTA MABs, Qiagen, Hilden, Germany) are supplied as 5% suspension. Each run was performed with 200 μl of the suspension.

2.2. Recombinant proteins to be purified

Two different 6xHis-tagged proteins were chosen to be separated using the magnetic beads. Recombinant cytoplasmatic aldolase 2 from pea (Rosidi, Köhl, Jacobsen, Reinard, unpublished), accession No.: X89829, is expressed into the bacterial cytosol. The molecular mass of the complete peptide is about 56 500 (38 500 aldolase and 18 000 tag sequences of the vector pET32a, Novagen). To test, whether the beads can be used as a general purification tool for 6xHis-tagged proteins, we have chosen the recombinant 6xHis-tagged NIa-protease from potyvirus PPV (Plum Pox Virus) [10] as second protein to be purified.

2.3. Growth of bacteria and induction of gene expression and isolation of recombinant proteins

Both recombinant proteins were expressed in BLR(DE3) (Novagen) grown in LB media at 37 °C up to an $OD_{600}=0.6$ [11]. After induction with 0.4 m*M* isopropyl β-D-thiogalactopranoside (IPTG) for 3 h the cells were transferred to ice for 10 min. The bacteria were harvested by 10 min of centrifugation at 5400×*g* and 4 °C and resuspended in 20 μl lysis buffer/ml bacteria culture (lysis buffer contains 20 m*M* Tris–HCl, pH 7.5, 10 m*M* $MgCl_2$, 1 mg/ml lysozyme, 20 μg/ml DNaseI, 0.1% Triton X-100), and incubated for 30 min on ice. After 1 min sonication the cells were centrifuged for 10 min at 4000×*g* and 4 °C and the supernatant was used for 6xHis-tag-based purification.

2.4. Procedures for magnetic beads separation

For the purification of His-tagged proteins differently modified SiMAC beads were used, and Ni–NTA MABs were used as a control. The Ni–NTA MABs were already supplied Ni^{2+}-activated. For the second run, the beads had to be reloaded with Ni^{2+}. All procedures were done as described by the manufacturer.

The SiMAC beads do not require Ni^{2+}-coupling at all. The suspension (70 mg/ml) was washed three times with 1 ml 20 m*M* Na_2HPO_4, pH 7.0.

All purification procedures were done as follows: the bacterial protein extract was split into aliquots of 1 ml added to 200 μl SiMAC beads (concentration: 70 mg/ml, if not stated otherwise) and incubated for 1 h on a shaker (Rotamix RM1, ELMI, Latvia) at 4 °C. Qiagen supplies the Ni–NTA MABs as 5% solution out of which 200 μl were used. Incubation and washing procedures were the same as described for the SiMACs. After usually three washing steps with 500 μl wash buffer each (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 10 m*M* imidazole, pH 8.0), the His-tagged proteins were eluted with an elution buffer (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 250 m*M* imidazole, pH 8.0). The elution step was repeated once. When SiMACs are used, the elution step is crucial for the yield of purified protein. It is performed best by using a pipette for resuspending the pellet.

All magnetic beads were reused after treatment with Strip Buffer (100 m*M* EDTA, 500 m*M* NaCl, 20 m*M* Tris–HCl, pH 7.9) and—in case of Ni–NTA MABs—after reloading the beads with Ni^{2+}.

2.5. Sodium dodecyl sulfate–polyacrylamide (SDS–PAGE), blotting and immunostaining

SDS–PAGE, blotting and immunostaining were carried out as described previously [12]. After western transfer of His-tagged proteins onto poly-(vinylidene fluoride) (PVDF) membranes 6xHis tags were detected using mouse anti-6xHis IgG (Amersham Pharmacia, 1:1000) followed by rabbit anti-mouse IgG-alkaline phosphatase (Sigma–Aldrich, 1:1000). The NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3 indolyl phosphate) system [12] was used for the detection. All antibodies were diluted in blotto as described by [10].

2.6. Computational methods

Signal strength of the Coomassie-stained eluates from different purification steps was analyzed densitometrically using the program Scanpack II (Biometra, Göttingen, Germany) with standard settings. The value for SiMAC-444 eluates was set 100%.

3. Results and discussion

Recombinant cytoplasmatic aldolase was extracted from IPTG-induced bacteria. Before loading to the different beads, the extract was split into aliquots. This procedure ensured that equal amounts of protein were loaded onto each type of beads. As shown in Figs. 1 and 2, the yield of purified protein depends on the protein itself. Best yields for both proteins were obtained when using SiMAC-445 with a non-porous matrix surface. This type was also found suited best for the purification of other 6xHis-tagged proteins, like scFvs (single-chain variable fragments, e.g., scFv102 anti NIa [10]). Therefore we conclude that SiMAC-445 is best to get uniform results for different 6xHis-tagged proteins. The cytoplasmic aldolase was efficiently purified from SiMAC-446 beads as well, but the yield of NIa protease using SiMAC-446 was much lower. Similar results were obtained for Ni–NTA MABs from Qiagen, which were not suitable for the purification of aldolase, but good for the purification of NIa protease. These data

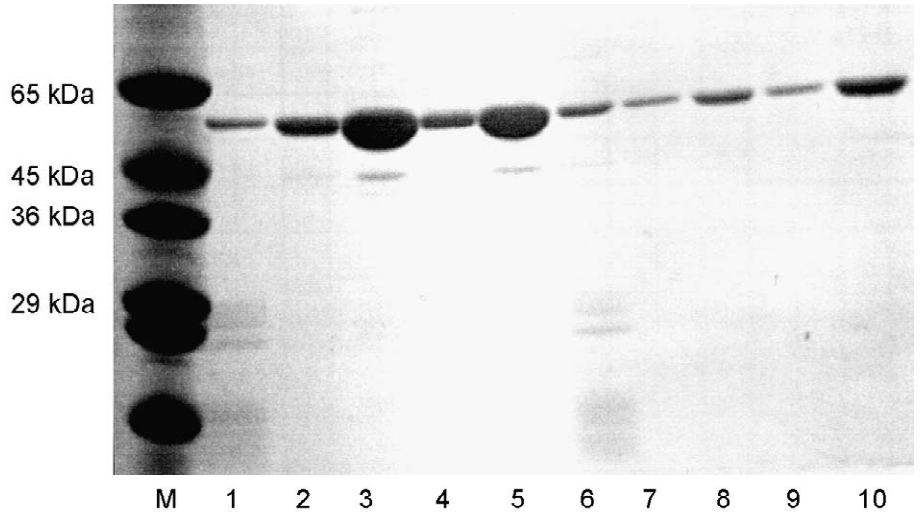

Fig. 1. Crude protein extract from a cytoplasmatic aldolase expressing *Escherichia coli* strain was divided into aliquots and incubated with different beads. Eluates were separated on SDS–PAGE and stained with Coomassie. Lanes: M=marker (from top: 65 000, 45 000, 36 000, and 29 000), 1=Qiagen Ni–NTA MABs, 2=SiMAC-444, 3=SiMAC-445, with a non-porous matrix surface, 4=autoclaved SiMAC-444, and 5=SiMAC-446, which contains 60% less Ni^{2+} ions. Lanes 6–10 contain the same samples, but from reused magnetic beads.

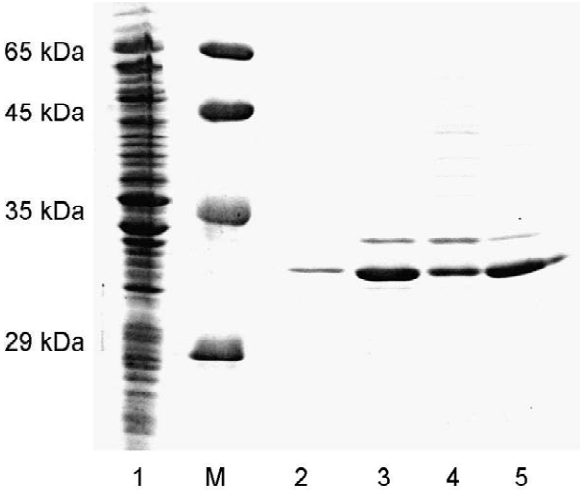

Fig. 2. Crude protein extract from an *E. coli* strain, expressing recombinant potyviral NIa protease was divided into aliquots and incubated with different beads. Eluates were separated on SDS–PAGE and stained with Coomassie. Bacterial crude extract was applied on lane 1, M contains marker protein (from top: 65 000, 45 000, 36 000, and 29 000). Lanes 2–5 were loaded with eluate from SiMAC-444 (lane 2), SiMAC-445 (lane 3), SiMAC-446 (lane 4), and Qiagen Ni–NTA MABs (lane 5).

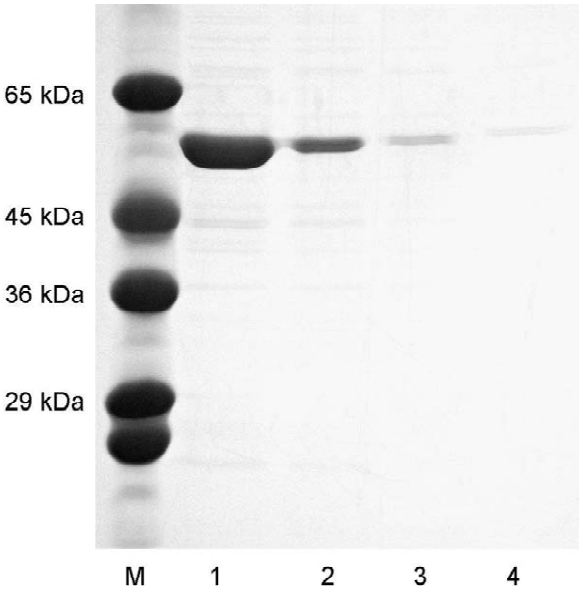

Fig. 3. To evaluate the optimal concentration of beads, needed for the purification, 6xHis-tagged cytoplasmatic aldolase (as used in Fig. 1) was applied to different amounts of SiMAC-445 beads: lanes: M=marker, 1=200 mg/ml, 2=100 mg/ml, 3=50 mg/ml, and 4=25 mg/ml beads.

show, that Ni–NTA MABs and SiMAC-446 are influenced by the type of protein, which has to be separated. Compared with the other materials, the yield obtained from SiMAC-444 was much lower for both proteins tested. Autoclaving of SiMAC beads did not influence the yield. Reused SiMAC beads are more difficult to handle, since the beads tend to stick to each other during the second usage. This makes their handling more difficult. Nevertheless, yields of purified proteins from reused SiMAC are still as high as from Ni–NTA MABs.

In some lanes, further peptides can be detected. Usually these additional bands only occur, when a high yield of purified protein was obtained (Fig. 1, lanes 3 and 5, Fig. 2, lanes 3–5). This shows that the binding capacity of the beads was exceeded.

Different concentrations of magnetic beads were tested, ranging from 25 to 200 mg/ml. As shown in Fig. 3, yields increased with increasing amounts of beads used. Nevertheless, usually a concentration of 100 mg/ml was found to be the best suited. First, concentrations higher than 100 mg/ml are difficult to handle during washing and elution steps. Secondly, usually 100 mg/ml beads were found to be sufficient. Only when a high amount of 6xHis tag protein has to be purified, as it was done in Fig. 3, an improvement by using higher concentrated beads (200 mg/ml) was observed. We also tested the suitability of the beads for reuse in a second purification procedure. Upon reuse the yield of purified protein was usually lower, independent of the type of beads.

Varying numbers of washing steps were carried out (3–20). We found three washing steps sufficient (data not shown). It has to be emphasized that the condition of resolubilisation of the magnetic bead pellet affected the yield. We got best results when resolubilisation was done by pipetting.

Finally, we proved that the protein isolated using the magnetic beads was in fact the desired 6xHis-tagged protein to be purified. For this purpose, the NIa protease containing eluates from the different types of magnetic beads were separated by SDS–PAGE and blotted. The immunostaining was performed using the specific anti-NIa scFv 102 [10]. Fig. 4 shows, that the purified band definitely represents the potyviral protease NIa. Fig. 5 summa-

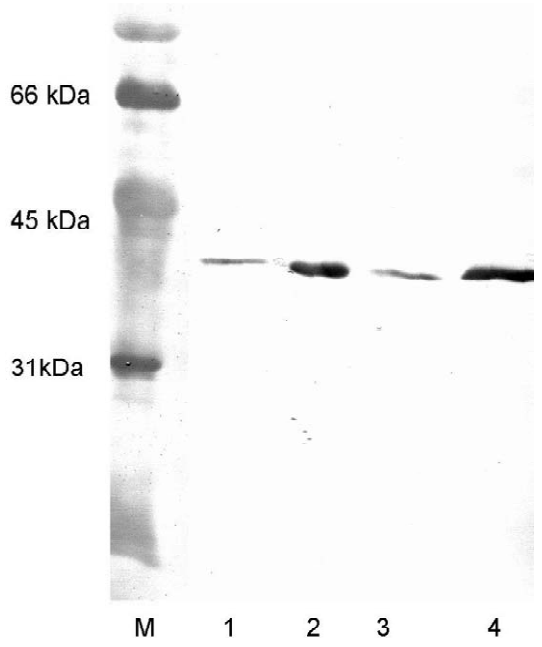

Fig. 4. To verify that the purified proteins are the recombinant 6xHis-tagged proteins, eluates from Fig. 3 (NIa protease) were applied on SDS–PAGE and blotted onto PVDF. After blocking with blotto, 6xHis tags were detected using an antibody directed against 6xHis tag followed by a secondary antibody. NBT/BCIP was used as substrate for the staining reaction as described [11]. Lane M contains molecular mass marker proteins (Bio-Rad) of which sizes in kDa are indicated at the left. Eluates from different beads were loaded on the following lanes: SiMAC-444 (lane 1), SiMAC-445 (lane 2), SiMAC-446 (lane 3), and Qiagen Ni–NTA MABs (lane 4).

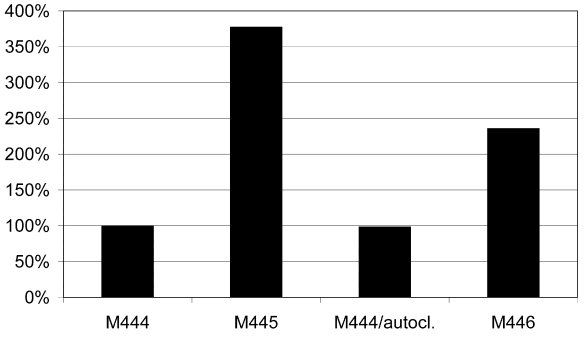

Fig. 5. SDS–PAGE-separated eluates from different experiments were analyzed densitometrically. Grey shades (scanner units) were measured using the programs standard settings. Every SDS–PAGE contained eluates from SiMAC-444, therefore we set this value to 100%. All other values are referred to the amount of SiMAC-444. NIa and aldolase eluates from 6 to 11 different purification procedures were considered (autoclaved SiMAC-444 only three experiments).

rizes a quantitative assessment of all data obtained. We analyzed the SDS–PAGE-separated eluates from the different materials densitometrically. Since all gels contained eluates from SiMAC-444, this value was set 100% and all other data were correlated to this. Fig. 5 confirms data presented in Figs. 1–4.

Here we show that the SiMAC-445 beads can be widely used for different 6xHis-tagged proteins. The new system offers several advantages: the yield was at least twice as high compared to Qiagen Ni^{2+}–NTA MABs. The method is faster, the time needed for the purification can be reduced by almost 1 h since the coupling step is omitted and there is no need to work with toxic Ni^{2+} salts.

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