Immobilised metal affinity chromatography for the capture of hydroxamate-containing siderophores and other Fe(III)-binding metabolites directly from bacterial culture supernatants

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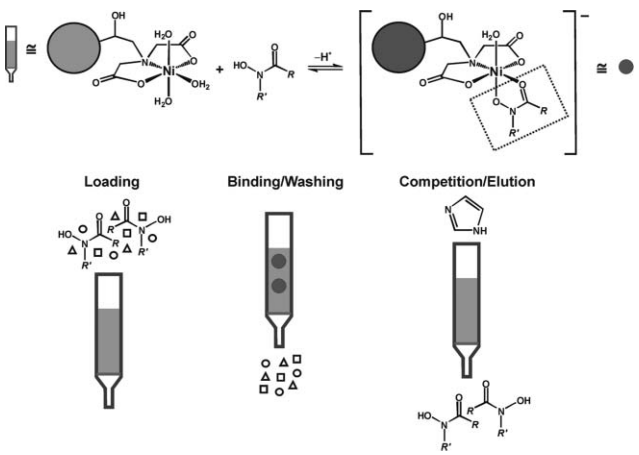
Nickel(II)-based immobilized metal affinity chromatography (IMAC) has been used to capture from standard samples the hydroxamate-containing siderophores, acetohydroxamic acid (ahaH), suberodihydroxamic acid ($shaH_2$) or desferrioxamine B (DFOB) in recoveries ranging between 35–90%. The capacity of a 1 mL Ni(II)-charged IMAC column towards DFOB capture at the pH optima of 8.9 is approximately 3000 nmol. This method has been used for the direct capture of DFOB (~65% recovery) from the untreated supernatant of iron-deprived cultures of *Streptomyces pilosus*, the soil bacterium from which DFOB was first discovered. In addition to selecting for DFOB and a related siderophore, two other Fe(III)-responsive species have been identified from RP-HPLC analysis of the IMAC-processed eluant from the *S. pilosus* supernatant. Since the characterisation of siderophores from natural systems is hampered by the low yields obtained from traditional purification methods, this IMAC-based affinity method offers significant potential for improving yields of this key class of bioligands and other small molecule metabolites with affinities to IMAC-compatible metal ions.

Introduction

Siderophores (Gr 'iron carrier') are small organic molecules with high Fe(III) affinities ($K_{aff} \sim 10^{40}$ M^{-1}) produced by marine and terrestrial bacteria in response to the poor availability of soluble Fe(III), which in an aqueous, oxic and pH-neutral environment, exists at concentrations significantly below that required for viable microbial growth.[1,2] Siderophores have been classified according to the Fe(III)-coordinating groups: catecholate (*e.g.*; enterobactin from *Escherichia coli*), hydroxamate (*e.g.*; desferrioxamine B (DFOB) from *Streptomyces pilosus*) or hydroxycarboxylate (*e.g.*; achromobactin from *Erwinia chrysanthemi*).[3] Traditional isolation procedures of hydroxamate-containing siderophores from bacterial isolates studied in our laboratory[4] have given yields that preclude complete characterisation. Indeed, although about 500 different types of siderophores have been documented,[3] only about 30 have been spectroscopically characterised and fewer still characterised by X-ray crystallography. Taken together with the importance of hydroxamate-containing siderophores in human medicine (DFOB·mesylate is used for the treatment of patients with β-thalassemia who suffer from transfusional-dependent iron-overload disease),[5] we were prompted to develop an affinity purification technique for hydroxamate-containing siderophores (Scheme 1) which draws upon immobilised metal affinity chromatography (IMAC) used for the isolation of recombinant histidine-tagged (His-tag) proteins.



Scheme 1 Ni(II)-based immobilised metal affinity chromatography (IMAC) for the purification of hydroxamate-containing siderophores.

His-tag protein purification uses a resin with covalently bound tridentate organic acids, such as iminodiacetic acid (IDA) to which transition metal ions (usually Ni(II)) are coordinated. The recombinant protein binds to the immobilised Ni(II)-IDA complex *via* interactions between the engineered *N*-terminal hexameric histidine tag and the three vacant Ni(II) coordination sites. Unwanted proteins are washed from the column and the target protein is eluted upon the addition of an excess of ligand (histidine or imidazole) which competes for the Ni(II) binding sites.

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Based upon the affinity between Ni(II) and hydroxamic acids evident from coordination chemistry and chemical biology,[6–9] we report here of the successful use of Ni(II)-based IMAC for the capture of pure hydroxamic acids and for the capture of hydroxamate-containing siderophores, such as DFOB, directly from the supernatant of the bacterium, *S. pilosus*, when cultured under Fe(III)-deprived conditions. Other Fe(III)-responsive metabolites have also been captured using this technique.

Material and methods

IMAC procedure

A solution of the trihydroxamic acid DFOB (*e.g.*, 300 nmol) in binding buffer (20 mM Na_2HPO_4/NaH_2PO_4, containing 500 mM NaCl, pH 8.0) was sorbed onto an IMAC column (His GraviTrap, GE Healthcare Biosciences; column volume = 1 mL; metal ion = Ni(II)) which had been subject to washing with 10 × column volumes (CV) of elution buffer (binding buffer containing 0.5 M imidazole) to remove non-specifically bound Ni(II) and equilibrated with binding buffer (20 × CV). After loading the sample, the column was washed with binding buffer (9 × CV) and then elution buffer (11 × CV) and fractions were assayed for the presence of siderophores using an Fe(III) addition assay ($\lambda = 450$ nm).[10]

Cultivation of *Streptomyces pilosus*

YM broth (Difco; 2.1% w/v) was prepared in Milli-Q water in glassware which had been soaked overnight in 3 M HNO_3. Chelex 100 (Na^+ form) was added to the broth (10% w/v) with stirring for 15–20 min and the pH value adjusted to pH 7.2. The broth was decanted, autoclaved and transferred to 1 L flasks which were inoculated with *Streptomyces pilosus* (ATCC 19797). Cultures were grown for 20 d at room temperature with orbital shaking (Ratek OM6) at 120 rpm.

RP-HPLC

Analytical RP-HPLC was performed using a Waters 600 Controller and a Waters Tunable Absorbance detector with a Waters Sunfire™ C18 column (particle size: 5 μM; column dimensions: 150 × 4.8 mm id) and a flow rate of 0.2 mL min^{-1} and solvents A: 90 : 10 H_2O/CH_3CN; B: 100 : 0 CH_3CN/H_2O, both containing 0.05% TFA (gradient: 0 to 40% B over 25 min). An aliquot of Fe(III) (10 mM in 0.1 M HCl) was added to the sample (1 : 1 volume ratio) and subject to RP-HPLC, as above.

Results and discussion

Method development

The DFOB standard bound to the column and was eluted upon the addition of imidazole-containing buffer (Fig. 1a). In the presence of excess imidazole, DFOB does not bind to the resin (Fig. 1b). When Ni(II) was stripped from the column using EDTA, DFOB did not bind to the resin (trace similar to Fig. 1b) which shows that non-specific binding is not a significant problem in this IMAC application. Nickel (<10%) was leached from the resin (as determined by graphite furnace AAS) in cases

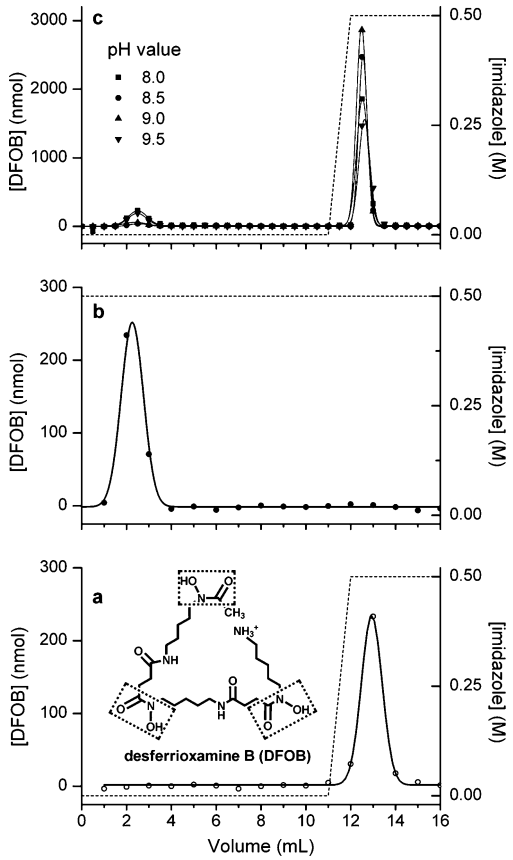

Fig. 1 DFOB (300 nmol) bound to a 1 mL Ni(II)-charged IMAC column and eluted with 0.5 M imidazole (a). DFOB is not bound to a column equilibrated with imidazole-containing buffer (b). The pH dependence of DFOB binding (3000 nmol) to a 1 mL Ni(II)-charged IMAC column (c).

where a large excess of DFOB (>>3000 nmol) was applied to the column.

When 1 μmol of acetohydroxamic acid (ahaH) was sorbed onto a 1 mL Ni(II)-charged IMAC column at pH 8.0, ~65% was not retained by the resin; 35% was recovered upon the addition of elution buffer. Significantly greater binding to the Ni(II)-charged IMAC resin is observed with the dihydroxamic acid, suberodihydroxamic acid ($shaH_2$) or DFOB (Table 1). There is a small difference in the pH optima for the capture of $shaH_2$ (pH = 8.4) and DFOB (pH = 8.9: Fig. 1c). The binding capacity of the IMAC technique towards $shaH_2$ and DFOB is similar, which correlates with the ability of these ligands to donate to the three vacant coordination sites of the immobilized Ni(II)-IDA complex as tridentate chelates, rather than as the bidentate ahaH. The IMAC-based binding affinity between Ni(II) and ahaH, $shaH_2$ or DFOB reflects the 1 : 1 Ni(II) : hydroxamate binding constants (Table 1).

Bacterial culture supernatant

The capacity of the Ni(II)-based IMAC technique to directly capture hydroxamate-containing siderophores from a bacterial culture supernatant was examined using *S. pilosus*, which is known to produce DFOB under iron-deficient conditions.[13] DFOB production was measured from a culture of *S. pilosus*

Table 1 Ni(II)-hydroxamate (1 : 1) formation constants and % binding at pH 8.0 on Ni(II)-charged IMAC resin

Hydroxamic acid	Equilibrium[a]	Log *K*	Load/μmol	% bound
ahaH (LH)	$Ni^{2+} + L^{-}/NiL^{+}$	5.3[b]	1	35
$shaH_2$ (LH_2)	$Ni^{2+} + L^{2-}/NiL$	7.4[c]	3	92
DFOB (LH_4^{+})	$Ni^{2+} + LH_2^{-}/NiLH_2^{+}$	7.7[b]	3	84

[a] Reactants/products. [b] 20 °C, ionic strength = 0.1 M, ref. 11. [c] Log *K* for the dihydroxamic acid, *N*,*N*′-dihydroxyheptanediamide, given according to ref. 12 (temperature and ionic strength unspecified), as an analogue of $shaH_2$ (*N*,*N*′-dihydroxyoctanediamide).

using the chrome-azurol shuttle (CAS) assay, which is reported as being specific for the detection of siderophores,[14] and the culture supernatant harvested after ~20 d. An aliquot (13.5 mL) of a batch of *S. pilosus* culture supernatant containing ~350 nmol DFOB (as determined using the CAS assay) was adjusted to pH = 9 and sorbed onto a 5 mL Ni(II)-loaded IMAC column and processed as described for the DFOB standard. This experiment shows the capture of ~65% CAS-positive species, which includes DFOB (Fig. 2).

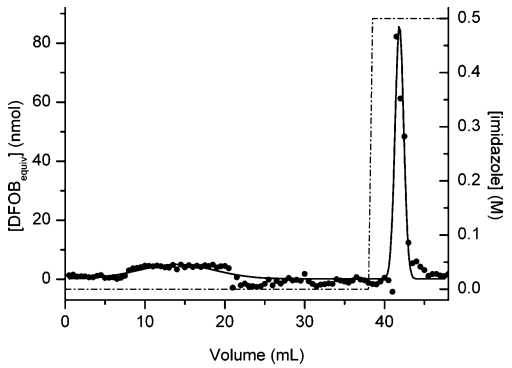

Fig. 2 Direct capture of CAS-positive species using Ni(II)-based IMAC (5 mL column) at pH 9.0 from untreated *S. pilosus* culture supernatant.

In addition to DFOB, the culture supernatant contains alternative non-proteinaceous and proteinaceous species biosynthesized by *S. pilosus*, together with the base media components (vitamins, proteins and amino acids) that may potentially compete for Ni(II)-binding sites. Compared to the results using pure DFOB (Fig. 1), the presence of these alternative ligands in the culture supernatant attenuates the capacity of Ni(II)-based IMAC towards DFOB capture. Apart from the pH adjustment step, no other pre-treatment is required for the high-yielding capture of CAS-positive species from *S. pilosus* bacterial culture supernatant. DFOB can also be eluted from the Ni(II)-loaded IMAC column by using a low-pH elution buffer (pH = 5.7) and omitting imidazole, which is preferrable for downstream purification processes where high imidazole concentrations are problematic.

Reverse-phase HPLC

Using reverse-phase HPLC, we examined the distribution of species in *S. pilosus* culture supernatant purified from Ni(II)-based IMAC. The RP-HPLC trace of crude *S. pilosus* supernatant shows at least 50 species that elute between 15–35% B (Fig. 3a). A sharp peak occurs in this trace at t_R = 13.9 min and is attributed to a species(s) of uncertain nature (species X). An aliquot (5 mL) of *S. pilosus* supernatant

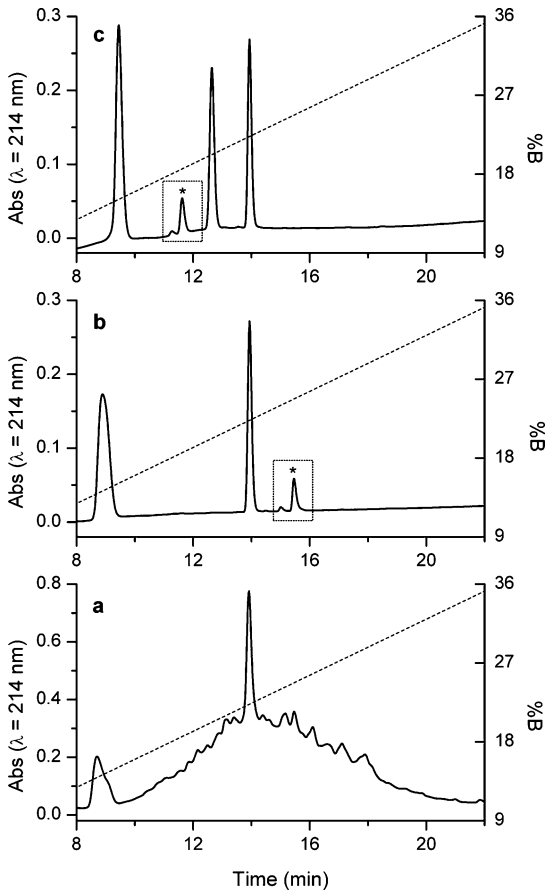

Fig. 3 RP-HPLC traces from (a) crude supernatant from an Fe(III)-deprived culture of *S. pilosus*; and (b) the CAS-positive eluant from the supernatant following a Ni(II)-based IMAC procedure (DFOB marked with *); and (c) the sample from (b) in the presence of Fe(III) (1 : 1) ratio (Fe(III)-DFOB marked with *). The trace in (c) has been multiplied by 2 to correct for the dilution effect from the addition of Fe(III).

containing ~300 nmol DFOB equivalents (CAS assay) was subject to the IMAC procedure using a 5 mL Ni(II)-loaded IMAC column with elution using a low pH/low salt buffer (20 mM Na_2HPO_4/NaH_2PO_4, 200 mM NaCl, pH 5.7).

The RP-HPLC trace of one of the CAS-positive fractions which contained ~40 nmol DFOB equivalents (CAS assay),† shows one broad peak at t_R = 8.9 min, which is asymmetric

† The recovery of CAS-positive species with the use of the low pH/low salt elution buffer (~13%) appears to be less than that using the imidazole-containing elution buffer (65%), perhaps due to the difference in [NaCl]; the ~13% recovery rate, however, is artificially low, since it represents the DFOB equivalents in only one of the HPLC fractions.

(evident from an expanded plot) and three other peaks at t_R = 13.9 min (species X), t_R = 15.0 min and t_R = 15.5 min (Fig. 3b). As determined using a DFOB standard, the peak at t_R = 15.5 min (Fig. 3b, marked with *) is due to DFOB.

In the presence of Fe(III), which was added to the fraction in a 1 : 1 volume ratio, the DFOB peak at t_R = 15.5 min and the minor peak at t_R = 15.0 min (both boxed), shift in a systematic fashion to t_R = 11.6 min and at t_R = 11.3 min, respectively (Fig. 3c), which suggests that the minor peak may be due to an alternative siderophore of the DFO class. While DFOB is the predominant siderophore produced by *S. pilosus*,[13,15] the complete gene cluster for *S. pilosus* desferrioxamine biosynthesis is not yet known.[16] The peak assigned to species X (t_R = 13.9 min), which is present in all chromatographic traces, is not Fe(III)-responsive and is, therefore, not a siderophore. Taking into account the area and the width-at-half-height of the sharp peaks at t_R = 9.45 min (0.072/0.23 min) and t_R = 12.63 min (0.041/0.17 min) in Fig. 3c, it is suggested that these species represent Fe(III)-bound species derived from the species represented under the broad, asymmetric peak at t_R = 8.91 min (0.084/0.43 min) in the Fe(III)-free trace. These species are likely to be non-DFO-type metabolites produced by *S. pilosus* that have an affinity to transition metal ions, including Ni(II) and Fe(III). Possible candidates might include mycin-type antibiotics which are commonly produced by *Streptomyces* species.[17]

Conclusions

In this work, we aimed to study the utility of IMAC, which is used almost exclusively for the purification of His-tag recombinant proteins, for the selective capture of hydroxamate-containing siderophores. Our experiments began by studying the IMAC-based capture of commercially-available hydroxamic acid standards, ahaH, $shaH_2$ and DFOB. Ultimately, we were interested in establishing whether the technique could be used for the capture of hydroxamate-containing siderophores directly from complex mixtures such as bacterial culture supernatants.

Nickel(II)-based immobilised metal affinity chromatography of the crude, untreated supernatant from Fe(III)-deprived cultures of the desferrioxamine B-producing bacterium, *Streptomyces pilosus*, yields four unique Fe(III)-responsive species from a complex mixture, one of which is desferrioxamine B. A comparison of Fig. 3a (>50 species) and Fig. 3b (5 species, 4 of which are Fe(III)-responsive) illustrates the effectiveness of Ni(II)-based IMAC for the purification of selected metabolites, including DFOB, *directly* from untreated bacterial culture supernatant. The use of Ni(II)-based IMAC to map siderophores and alternative metabolites produced by *S. pilosus* and other culturable bacteria will provide valuable insight that complements gene sequence data and offers wide scope in facilitating drug discovery from natural products research and mutasynthesis/biocombinatoral chemistry.[18,19] This technique will significantly expedite purification of hydroxamate-containing siderophores directly from bacterial culture supernatants and provides scope for better understanding siderophore biosynthetic pathways.[20,21] We are currently exploring the use of V(IV/V)-, Co(II)- and Cu(II)-loaded IMAC resins for the capture of hydroxamate- or catecholate-containing siderophores and alternative metabolites.

Acknowledgements

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