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Strategy for the isolation of native dehydrogenases with potential for biosensor development from the organism *Hyphomicrobium zavarzinii* ZV580

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ABSTRACT

Dehydrogenases are interesting candidates for the development of electrochemical biosensors. Most dehydrogenases are characterised by a comparatively broad substrate spectrum, yet highly specific enzymes exist as well. A specific formaldehyde dehydrogenase has, e.g., been described for the organism *Hyphomicrobium zavarzinii* ZV580. Isolation of enzymes from their natural source instead of a recombinant expression renders the isolation more challenging, as common tools such as affinity tags are no longer available. In this contribution, we develop chromatographic procedures for such isolation tasks. The previously described formaldehyde dehydrogenase was isolated by two procedures, one based on affinity chromatography, the other on hydroxyapatite. Neither procedure yielded an active enzyme. In addition two dehydrogenases, a formaldehyde and a methylamine dehydrogenase, were found in the cell free extract, which had not been described previously. Both enzymes could be isolated to near purity by a sequence of hydroxyapatite and anion exchange chromatography. The new formaldehyde dehydrogenase requires reconstitution with calcium and pyrroloquinoline quinone in order to become active. The enzyme shows no cross-reactivity with methylamine or methanol. The methylamine dehydrogenase catalyses the conversion of methylamine into formaldehyde, hence it could become a technical catalyst for the inverse reaction. This enzyme consists of two types of subunit and may be one of the rare α,β-methylamine dehydrogenases.

1. Introduction

Nature provides many specific biocatalysts that could become interesting tools in industrial biotechnology. While the DNA-based search of genomes and metagenomes may provide potential candidates, such searches are largely based on homologies. Whenever the metabolic capability is instead used as starting point for the isolation of the corresponding biocatalysts, the protein has to be isolated from its native environment before further research can be carried out. Compared to the isolation of many recombinantly produced proteins such isolation can be quite challenging, as some of the established tools, such as the use of affinity tags, are no longer applicable. Moreover, some of the more complex enzymes, e.g. those bearing covalently bound co-factors, are not easy to express recombinantly. In such cases isolation from the native production organism may be the only option. In this contribution we describe innovative approaches to such isolation challenges.

The organism *Hyphomicrobium zavarzinii* ZV580 has been described to produce a NAD^+-independent, highly specific formaldehyde dehydrogenase ("FalDH") [1,2]. According to the authors, the enzyme is produced when *H. zavarzinii* ZV580 is grown on methylamine as sole C-source. The enzyme is described as a homo-tetramer with a molecular weight of 195 200 Da with a subunit weight of 48 800 Da [2]. Contrary to many other dehydrogenases, even after purification, the enzyme does apparently not require the addition of NAD^+, or any other cofactor, to be active. It was hence assumed that the native enzyme contains a covalently bound redox entity ("cofactor") [1]. The exact nature of the covalent cofactor has still to be elucidated, although a couple of possibilities could be ruled out. Pyridoxal phosphate, pyrroloquinoline quinone and other cofactors would give the enzyme a distinct absorption spectrum, which is not observed [2]. Some evidence has nevertheless been given to support the notion that the redox-active cofactor is a quinone residue [1]. Most importantly, FalDH is one of only two highly specific formaldehyde dehydrogenases described so far [1,3].

Its unusually high specificity for formaldehyde, but also the fact that the redox-active cofactor putatively is a covalently linked one instead of the soluble and costly NAD^+, makes FalDH from *H. zavarzinii* ZV580 an interesting candidate for biosensor development. Such an application would require the efficient purification of the target enzyme, since *H. zavarzinii* ZV580 contains several

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enzymes for the conversion of a variety of C1 substrates. Peptide and DNA sequences for FalDH have been published in 2004 [2] and recombinant production of the target enzyme was attempted, yet did not yield an active enzyme [4]. It was thus necessary to isolate the native enzyme from *H. zavarzinii* ZV580 cultures.

In our hands, the previously published purification procedure based on $(NH_4)_2SO_4$ precipitation, dialysis, DEAE-Sephacel-, Sepharcyl S-300-, Phenyl-Sepharose-, and finally DEAE-Trisacyl-chromatography (yield 8%) [1], did not yield an active enzyme in appreciable yields. Hence the isolation strategy was redesigned, based on the determination of formaldehyde and methylamine dehydrogenase (DH) activities in the various chromatographic fractions.

2. Materials and methods

2.1. Materials

Chemicals including minerals for culture media were from established suppliers such as Sigma–Aldrich and used as obtained. Milli-Q water was used for the preparation of all aqueous solutions. The strain *H. zavarzinii* ZV580 is obtainable from the collection of the "Institute für Allgemeine Mikrobiologie" (University of Kiel, Germany, accession number IFAM ZV-580, EMBL accession number Y14306) and was kindly provided by Dr. Vorholt (INRA/CNRS, Castanet-Tolosan, France). Prior to production and molecular biology studies, enrichment cultures were obtained by repeated selection of appropriate colonies, whose morphological characteristics were verified via microscope. Polyclonal anti-FalDH antibodies recognising two peptide sequences (linear epitopes: FHPKFKENGHFFVS and WEGDPLDAGQRKDV) found according to computer simulations (threading model, http://www.cse.ucsc.edu/reserch/compbio/HMM-apps/T02-query.html) on the surface of FalDH, were produced according to our specifications by Eurogentec, Cologne, Germany (polyclonal rabbit IgG).

2.2. Bioprocess and cell free extract

Bacteria were grown as described previously [5] in a mineral medium containing 10 mM K_2HPO_4, 14 mM NaH_2PO_4, 10 mM $MgSO_4$, 15 mM $(NH_4)_2SO_4$, 4 μM $FeSO_4$, 1 μM $ZnSO_4$, 10 μM $CaCl_2$, 1 μM $CuSO_4$, 1 μM $CoCl_2$, 186 μM $(NH_4)_6Mo_7O_{24}$, 5 μM $MnCl_2$, 4 μM EDTA and supplemented, unless indicated otherwise, with methylamine hydrochloride as sole carbon source. The pH of the medium was adjusted to 7.0 before autoclaving. Bacterial growth was monitored by measuring the absorbance density of the culture at 600 nm (A_{600}). The bioreactor was a NLF22 (Bioengineering, Wald, Switzerland), the inoculation cell density was $A_{600} = 0.1$, the initial culture volume 7.9 L, and the initial C1 source concentration in the medium was 10 mM. The cultivation temperature was 30 °C. The stirrer speed and the airflow were programmed in cascade with the dissolved oxygen in such a way that the set point of 30% oxygen saturation was maintained (air flow between 0 and 11.5 L/min, stirrer speed between 92 and 200 rpm). For higher output signals a constant stirrer speed of 200 rpm was adjusted. The culture was run in the fed-batch mode, the feed pump (5 M methylamine hydrochloride, flow rate: 30 mL/min) was activated for 0.7 min whenever the dissolved oxygen value surpassed 30%. The pH of the culture was maintained constant at pH 7.0 ± 0.1 by automatic titration with 2 M NaOH. After the fourth feeding the A_{600} had typically reached 1.6 and the biomass was harvested by centrifugation (Sorval EvolutionRC, 13 000 × *g*, 4 °C, 40 min). A typical culture yielded ca. 17 g wet paste, which was subsequently stored at −80 °C.

For the preparation of the cell free extracts, frozen cells (2.5 g) were resuspended in 10 mL of a 50 mM Tris–HCl buffer, pH 7.4, containing 2.5 U/mL DNase, 0.2 mM phenylmethanesulphonyl fluoride (PMSF), 3.0 mM $MgCl_2$, and 50 μg lysozyme, followed by sonication on ice (five times 3 min, Sonoplus GM 2200 (Bandelin, Berlin, Germany)). Cell debris was removed by centrifugation (13 000 × *g*, 4 °C, 40 min). The cell free extract (9.5 mL) was subjected to sterile filtration (0.2 μm sterile filter, Sartorius Minisart, CE, Sartorius, Göttingen, Germany) and the protein content was determined via Bradford assay (supplier: Bio-Rad Labs., Hercules, CA, USA) according to the manufacturer's instructions.

2.3. Analytical methods

Fractions obtained during chromatography were analysed by sodium docecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using Criterion XT pre-cast gels (4–12% Bis–Tris) from Bio-Rad according to the manufacturer's instructions. Precision Blue Protein Standards, All Blue from Bio-Rad was used as molecular weight marker. Protein bands of interest were cut out from the gels and subjected to peptide digestion and sequencing (Proteome Factory, Berlin, Germany). Gels separating proteins intended for sequencing were Coomassie-stained, all other gels were silver-stained unless mentioned otherwise. Stains were from Bio-Rad and used as indicated by the manufacturer.

For the determination of dehydrogenase (DH) activity, a spectrophotometric enzyme assay for formaldehyde dehydrogenase scaled down to the microtitre plate format ('redox cycling microassay') was used as described previously [5]. This test relies on a redox reaction converting the substrate (formaldehyde, methylamine, methanol) into the corresponding product (formate, formaldehyde), thereby releasing 2 electrons, which are transferred first to a putative cofactor, then to PMS (phenazine methosulphate), and finally to the dye DCPIP (dichlorophenolindophenol), which becomes yellow upon reduction, leading to a decrease of the A_{595}. For analysis, 50- to 100-μL aliquots of the chromatographic fractions of interest were mixed with phosphate buffer pH 8.0 (final concentration 80 mM, final volume 200 μL), containing 2 mM PMS, and 0.2 mM DCPIP. The reaction was started by the addition of 10 mM of substrate. The assay was performed at 30 °C. If indicated 50 μM Ca^{2+} and/or 1 μM PQQ were also added to reconstitute the enzyme (incubation for ca. 30 min at ambient temperature). The assay was performed in flat-bottom microtitre plates (Greiner Bio-One, Frickenhausen, Germany, optical path length 5.9 mm, temperature 30 °C, detection 595 nm) in a Genios Pro plate reader (Tecan, Crailsheim, Germany).

Interaction with the anti-FalDH antibody was determined by dot and Western blot assay. Nitrocellulose membranes for the blots were Optitran BA-S 83 from Schleicher and Schuell (Dassel, Germany). For immobilisation the membranes were incubated with TBS (0.02 M Tris–HCl pH 7.5, 150 mM NaCl) and the samples diluted in 50 mM Tris–HCl pH 7.5 (total volume 200 μL) were applied in four quantities (30, 10, 5, and 2.5 μg) by micropipette. Afterwards the membranes were washed (twice) with TBS-Tween (TBS + 0.05% Tween 20) and the remaining active sites were blocked by incubation for 2 h with bovine serum albumin (BSA) (0.1%)-containing TBS-Tween, followed by 3 washings with TBS-Tween. The primary antibody (anti-FalDH antibody) was dissolved 1:24 600 in TBS-Tween/1% BSA and the membrane was incubated for 2 h at ambient temperature. Afterwards membranes were washed 3× with TBS-Tween. For detection, the membrane was incubated for 2 h at room temperature with the detection antibody (anti-rabbit mouse-antibody, horse radish peroxidase (HRP) labelled, diluted 1:62 500 in TBS-Tween/1% BSA). The membrane was washed again 3× with TBS-Tween and the freshly prepared chromophore substrate solu-

tion (75 mg DAB (3,3-diaminobenzidine), 75 mL 50 mM Tris–HCl pH 7.6, 750 μL 3% H_2O_2) was added for an incubation of 5–10 min. Afterwards the blot was rinsed gently with deionised water and photographed.

2.4. Chromatography

A BioLogic DuoFlow system (Bio-Rad) was used for all preparative chromatography experiments, except for the affinity chromatography, where an Äkta Purifier from GE Healthcare (Uppsala, Sweden) was used. The separations were performed at ambient temperature. Unless indicated otherwise, the following conditions were used.

The gel filtration column was a Superdex 75 10/300 GL (exclusion limit: 100 kDa, GE Healthcare), 0.5 mL of desalted (HiTrap Desalting Column, GE Healthcare, used in the minimum dilution mode) cell free extract was injected for each separation. The mobile phase was a 50 mM phosphate buffer, pH 7.0 (flow rate 0.4 mL/min). 0.5 mL fractions were collected.

The anion exchange column was a Resource Q (6 mL, GE Healthcare), the mobile phase a gradient (0–100% B, flow rate 1 mL/min, gradient volume as indicated) of buffer A (25 mM Tris–HCl, pH 8.0) to buffer B (25 mM Tris–HCl + 1 M NaCl, pH 8.0). 1 mL fractions were collected. The samples (2.5 mL) were desalted prior to injection.

The hydroxyapatite column was packed from Macro-Prep Hydroxyapatite Type I (Bio-Rad), column dimensions: 7 mm × 50 mm (2 mL). The mobile phase was a double gradient (0–100% B in 10 min, back to 100% A, 0–100% C in 60 min, flow rate 1 mL/min). Buffer A was 20 mM phosphate pH 7.0, buffer B 20 mM phosphate + 1 M NaCl pH 7.0, and buffer C 400 mM phosphate pH 7.0. 1 mL fractions were collected. The samples (2.0 mL) were desalted prior to injection.

For the affinity chromatography, the polyclonal anti-FalDH antibody was immobilised on Affi-Gel Hz gel (Bio-Rad) according to the manufacturer's instructions. For affinity chromatography the cell free extract was diluted 1:10 in 20 mM sodium phosphate buffer pH 7.5 containing 150 mM NaCl, followed by concentration to ca. 65 mL via cross flow filtration (Millipore Labscale TFF system, cut off: 19 kDa, Millipore, Schwalbach, Germany). The loading buffer was 20 mM sodium phosphate buffer pH 7.5 containing 150 mM NaCl, the elution buffer was 0.1 M glycine pH 3.0. The flow rate was 1 mL/min, 1 mL fractions were collected and immediately neutralised by addition of 200 μL of 1 M Tris–HCl pH 9.0.

In case of multistep chromatographic procedures, fractions containing the protein of interest were identified via the activity assay and pooled (unless indicated otherwise). The pooled fractions were desalted (HiTrap Desalting Column, GE Healthcare, used in the minimum dilution mode) before injection into the next column.

3. Results and discussion

A protocol for the isolation of FalDH from *H. zavarzinii* ZV580 lysates has been published by Klein et al. in 1994 [1], which according of the authors resulted in yields of maximal 8%. A direct reproduction of this protocol was not possible, since some of the column materials were no longer available. An approximate reproduction of the procedure did not yield a protein at appreciable yield. The *de novo* design of a purification strategy from cell free extract was therefore necessary.

The development of the isolation procedure for DHs from *H. zavarzinii* ZV580 started with a standardized cell free extract produced as published previously [5], which typically contained 8.6 mg/mL of protein. Assigning a particular DH activity to the extract, was impossible, due to the known cross-reactivity of some

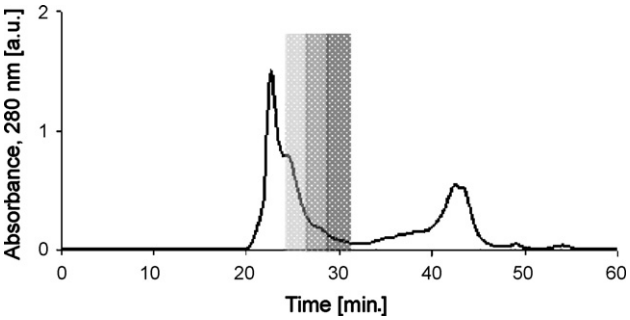

Fig. 1. Gel filtration of the desalted cell free extract. Column: Superdex 75 10/300 GL, mobile phase: 50 mM phosphate buffer pH 7.0, flow rate 0.4 mL/min, fraction size: 0.5 mL. Three fractions of interest are indicated by hatching, from left to right: FalDH, methylamine DH, formaldehyde DH.

of the enzymes presumably present in the extract, but also due to the putative presence of, e.g., more than one formaldehyde DH [6]. As a consequence it is not possible to calculate a global yield of the procedures discussed below. When the cell free extract was investigated for DH activity in general, formaldehyde DH activity, methylamine DH activity, and methanol-DH activity were determined. Contrarily to the previously published results [1], these activities were determined independent of whether the cultures were grown on methanol or methylamine as C1 source. Again this may be due to the presence of several enzymes with overlapping activities and metabolic functions.

The design of a isolation procedure for a native enzyme from the corresponding cell free lysate can follow several strategies. In our case the DNA sequence of the gene encoding for the previously described FalDH was known [2] and based on homology considerations with already characterised dehydrogenases, a computer simulation of the structure was possible. From this simulation two peptide sequences (liner epitopes) could be derived, which should be present on the surface of our enzyme. With this information, the production of a polyclonal antiserum ("anti-FalFH antibody") specific for these epitopes became possible. When this antibody preparation was used in standard blotting assays, positive signals could be detected in the cell free extract of *H. zavarzinii* ZV580. It thus seemed likely that FalDH was present in the cell free extract, while a means for the specific detection of the enzyme even in complex solutions was available. In addition to the immunological detection of FalDH, however, the subsequent isolation was also to be based on the detection of enzymatic activity in the various fractions.

3.1. Initial separation of the cell free extract

Most previous attempts to isolate FalDH from *H. zavarzinii* ZV580 lysates started with a capturing step based on $(NH_4)_2SO_4$-precipitation [1,5]. However, the purification/concentration factor of this step is minimal, while the yield is quite low (76% in [1]) and stringent desalting is necessary before the subsequent (ion-exchange) chromatographic steps can be undertaken. Therefore the possibility of direct chromatographic processing of the cell free extract by gel filtration was investigated. The result is shown in Fig. 1. Analysis of the protein-containing fractions by the DH-activity assay and by immunoblotting showed three consecutive yet distinct areas of interest. Fractions 25 and 26 contained a protein interacting with the anti-FalDH antibody. These fractions were followed by two fractions containing the majority of a methylamine DH activity (no activity towards methanol, formaldehyde). Finally a formaldehyde DH activity was found mainly in fractions 29 and 30. Formaldehyde DH-activity could only be detected in these frac-

tions after the addition of calcium (ca. 50 μM) to the samples. This incidentally supports the notion of a PQQ-related molecule as cofactor, since PQQ usually requires calcium for activity [7]. Although the formaldehyde DH-, the methylamine DH-, and the FalDH-containing fraction were thus clearly separated after the gel filtration, none of them were pure according to the analysis by SDS-PAGE (silver-stained), data not shown. Both the retention in the gel filtration column and the position of the corresponding band in the SDS-PAGE argue for a molecular weight of approximately 42 kDa in case of the formaldehyde DH. All three proteins of interest (FalDH, methylamine DH, and formaldehyde DH) eluted well after the upper exclusion limit of 100 kDa of the gel filtration column.

3.2. Isolation of FalDH

In case of the FalDH isolation, a slightly deviating protocol was used to prepare the cell free extract. In particular, 26 g of frozen cells were resuspended in 50 mL saline (0.9% NaCl), washed twice (4000 × *g*, 20 min, 4 °C), and resuspended (30 min incubation) in 25 mL of a 50 mM Tris–HCl buffer, pH 7.5, containing 10 μg/mL DNase I, ca. 100 ng/mL RNase, and 10 μg/mL lysozyme, followed by sonication (5× for 3 min) on ice. Cell debris was removed by centrifugation (13 000 × *g*, 4 °C, 60 min). Then the cell free extract was subjected to sterile filtration and the protein content was determined via the Micro BCA Protein Assay Reagent Kit from Pierce (Rockford, IL, USA).

In a first attempt to directly isolate FalDH from this cell free extract, the anti-FalDH antibody was immobilised on an agarose support matrix and used as affinity ligand for FalDH, Fig. 2a. The protein recognised by the anti-FalDH antibody bound to the column in sodium phosphate buffer pH 7.5 containing 150 mM NaCl to suppress non-specific interactions. According to the Western blot no anti-FalDH antibody interactive proteins were found in the flow through or the washing buffer, data not shown. Elution was possible with a standard glycine pH 3.0 step gradient. The protein containing fractions were pooled. As the SDS-PAGE and Western blot demonstrate, Fig. 2b, the protein fraction contains several proteins, of which one at approximately 48 kDa is recognised in the Western blot by the anti-FalDH antibody. Moreover, the co-eluting proteins tend to be smaller than 48 kDa, it is hence possible that these are fragments of FalDH. When the pooled fractions were investigated for DH activity (formaldehyde, methylamine, methanol, various reconstitution attempts), however, no activity could be observed. Eluting fractions had been immediately neutralised. In spite of this, however, we could not exclude that the observed lack of enzymatic activity in the FalDH fractions was due to the harsh elution conditions. Therefore, we attempted to establish more physiological, if less specific, purification strategies. Many dehydrogenases can, e.g., be purified by dye affinity chromatography where competitive elution is an option. However, since FalDH is an NAD-independent enzyme, this approach was considered unlikely to succeed for this particular enzyme.

A direct separation of a cell free extract by anion exchange chromatography is shown in Fig. 3. According to the results from the immunoblot, FalDH eluted in a very broad zone over fractions 32–39. Moreover, in none of these fraction was DH activity detected. Instead, the majority of the enzymatic activity (formaldehyde, methylamine, methanol DH activity) was found in fractions 28 and 29, i.e. well separated from the FalDH zone. According to the corresponding analysis by SDS-PAGE, all fractions collected after anion exchange chromatography still contained more than one protein.

Since neither gel filtration nor anion-exchange chromatography showed satisfactory enrichment of the FalDH protein, while affinity chromatography may have been too harsh in terms of elution conditions, we resorted to somewhat unusual material, namely hydroxyapatite (HA), for the initial capturing step. Due to its complex interaction mode, which allows considerable fine-tuning of the interaction of a particular protein [8], HA chromatography has been called a "pseudo affinity" form of chromatography. In our case the protein recognised by the anti-FalDH antibody eluted in fractions 24–29 of the phosphate gradient, although traces were found in the NaCl-gradient and the flow through. This argues for C-site interaction as the main mechanism of retention. Positively charged proteins interact mainly electrostatically (ion-exchange mode) with the negatively charged phosphate groups on the surface of the HA (so called P-site interaction). Such proteins can be eluted in a standard NaCl gradient from 0 to 1 M. C-site interaction, on the other hand, is more complex and involves the calcium sites of the HA. Elution of C-site interacting proteins requires strongly calcium interacting agents such as phosphate. Given the presumed structure of FalDH, an acidic isoelectric point of 6.02 can be predicted

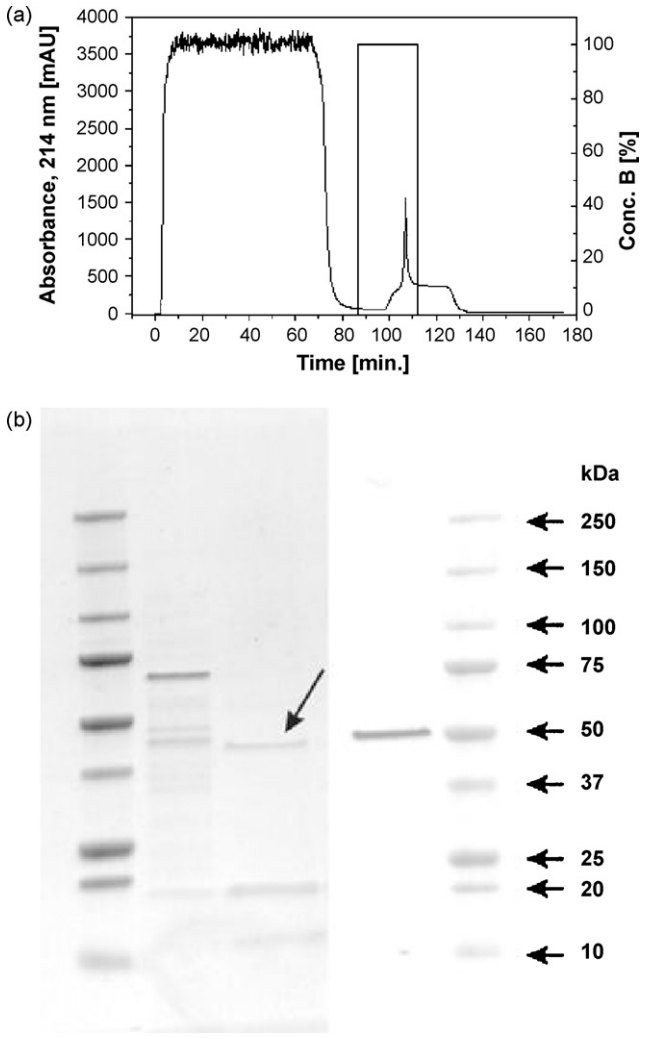

Fig. 2. (a) Affinity chromatography of the desalted cell free extract. Column: Affi-Gel-Hz bearing anti-FalDH antibodies as affinity ligands, loading buffer: 20 mM phosphate buffer pH 7.5 containing 150 mM NaCl, elution agent: 100 mM glycine–HCl pH 3, flow rate 1.0 mL/min, fraction size: 1 mL. Fractions were immediately neutralised by the addition of 200 μL 1 M Tris–HCl pH 9.0. (b) SDS-PAGE (4–12% Bis–Tris) and Western blot of the fractions of interest obtained by affinity chromatography. SDS-PAGE lane 1: molecular weight marker (Precision Blue Protein Standards, All Blue, Bio-Rad), lane 2: flow through, lane 3: protein fraction eluted in the glycine step, Western blot lane 4: protein fraction eluted in the glycine step, lane 5: molecular weight markers.

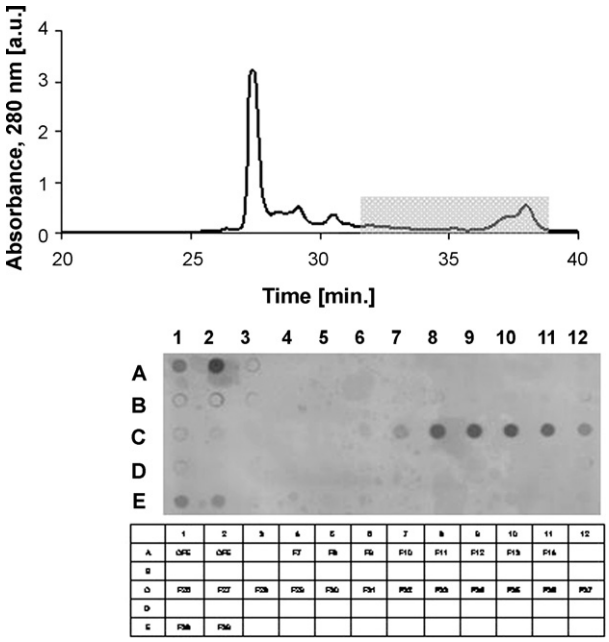

Fig. 3. Anion-exchange chromatography of the desalted cell free extract. Column: Resource Q (6 mL), buffer A: 25 mM Tris–HCl pH 8, buffer B: 25 mM Tris–HCl + 1 M NaCl pH 8, gradient 0–100% B in 20 min, flow rate 1.0 mL/min, fraction size: 1.0 mL. Bottom: dot blot analysis of the fractions of interest.

for the protein without the signal sequence and C-site interaction becomes hence a possibility.

However, when the corresponding pooled fractions were tested for enzymatic activity, again no DH activity could be observed. We are at present confident to have isolated a protein corresponding to the FalDH described by Klein et al. [1,2] by two different chromatographic strategies. The reason why the purified protein did not show the expected DH activity can at present only be speculated upon. Although we took care, especially in the second chromatographic process, to maintain gently conditions, it is possible that the yet unknown co-factor was removed form the enzyme. Even though according to Klein et al. the co-factor is supposed to be covalently bound, this has not been proven. It is also possible that the *H. zavarzinii* ZV580 strain used in our study produced an enzyme that in inactive due to a mutation. Irrespective of the cause, however, the isolated "FalDH" was not a suitable candidate for biosensor development. On the other hand, two of the DH-activities detected in the cell free extract during the initial experiments could become interesting candidates for a technical application, namely the formaldehyde DH and the methylamine DH. Methylamine DH converts methylamine into formaldehyde and ammonium, it could therefore also be used to catalyse the inverse reaction and thereby become a part of a technical formaldehyde biosensor.

3.3. Isolation of the formaldehyde dehydrogenase activity

Since for the formaldehyde DH and the methylamine DH no affinity ligands were available, we designed an isolation procedure based on multiple step chromatography, guided by the activity assay. The initial separation of the cell free extract by anion exchange chromatography, Fig. 3, had resulted in a mixture of DH-activities in fractions 28 and 29. By increasing the gradient volume from 20 to 240 min (the flow rate remained at 1 mL/min), the separation could be improved, Fig. 4a. Under these conditions, the formaldehyde DH-activity was found in a fairly broad peak collected in fractions 44 and 45. Both fractions contained several proteins as shown in the SDS-PAGE gel, Fig. 4a insert. In fact, an identification of the bands corresponding to the formaldehyde DH was not possible based on this gel. When fractions 44 and 45 were pooled and re-chromatographed on the anion exchanger under

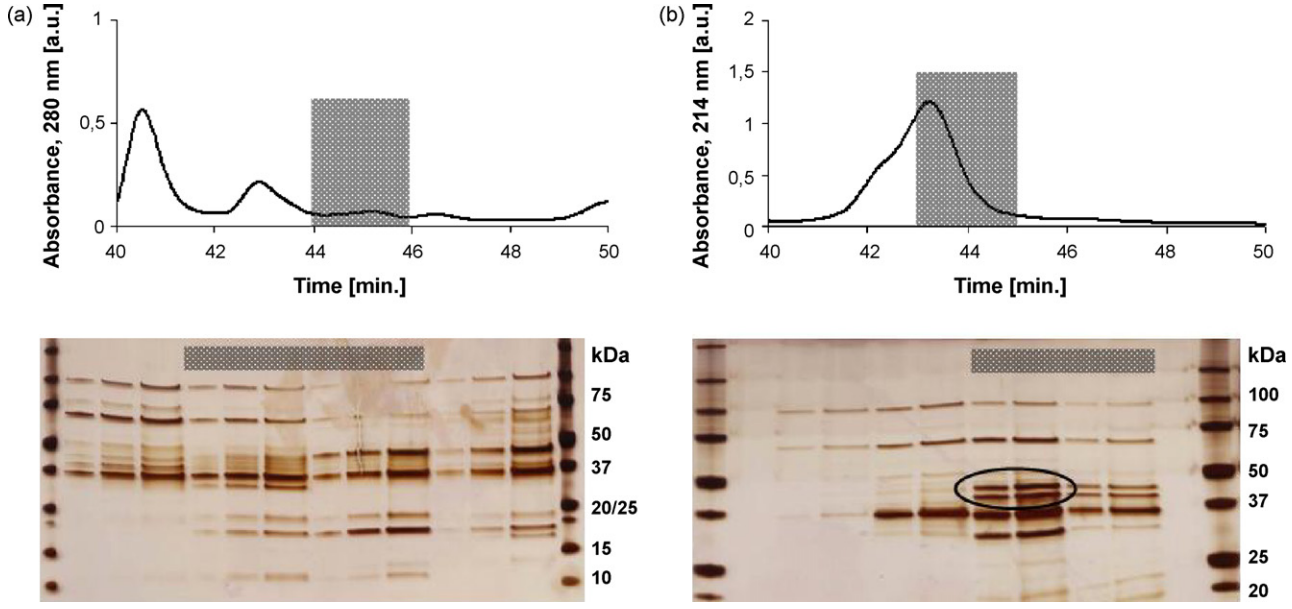

Fig. 4. (a) Anion-exchange chromatography of the desalted cell free extract. Column: Resource Q (6 mL), buffer A: 25 mM Tris–HCl pH 8, buffer B: 25 mM Tris–HCl + 1 M NaCl pH 8, gradient 0–100% B in 240 min, flow rate 1.0 mL/min, fraction size: 1.0 mL. Fractions containing the formaldehyde DH activity are hatched. (a insert) SDS-PAGE (4–12% Bis–Tris) of the fractions of interest from (a). Left and right lane: molecular weight marker (Precision Blue Protein Standards, All Blue, Bio-Rad), lanes 2–4: fraction 43, lanes 5–7: fraction 44, lanes 8–10: fraction 45, lanes 11–13: fractions 46. Fractions containing the formaldehyde DH activity are indicated by the hatched band. (b) Anion-exchange chromatography of the pooled fractions 44 and 45 from (a). Column: Resource Q (6 mL), buffer A: 25 mM Tris–HCl pH 8, buffer B: 25 mM Tris–HCl + 1 M NaCl pH 8, gradient 0–100% B in 240 min, flow rate 1.0 mL/min, fraction size: 1.0 mL. Fractions containing the formaldehyde DH activity are hatched. (b insert) SDS-PAGE (4–12% Bis–Tris) of the fractions of interest from (b). Left and right lane: molecular weight marker (Precision Blue Protein Standards, All Blue, Bio-Rad), lanes 3, 4: fraction 41, lanes 5, 6: fraction 42, lanes 7, 8: fraction 43, lanes 9, 10: fractions 44. The bands of interest are indicated by a circle. Fractions containing the formaldehyde DH activity are indicated by the hatched band.

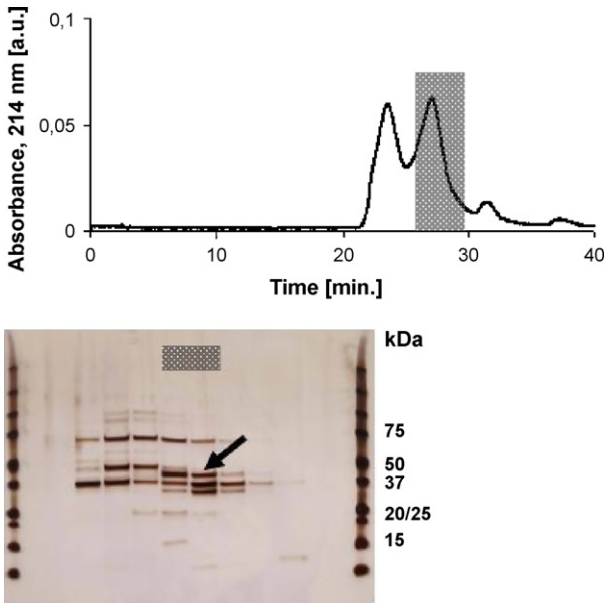

Fig. 5. Gel filtration of fraction 43 from Fig. 4b. Column: Superdex 75 10/300 GL, mobile phase: 50 mM phosphate buffer pH 7.0, flow rate 0.4 mL/min. Fractions containing the formaldehyde DH activity are hatched. (Insert) SDS-PAGE (4–12% Bis–Tris) of the fractions of interest from the main figure. Left and right lane: molecular weight marker (Precision Blue Protein Standards, All Blue, Bio-Rad), the indicated lanes cover fractions collected between minutes 22.5 and 32.5. The band of interest is indicated by an arrow. Fractions containing the formaldehyde DH activity are indicated by the hatched band.

identical conditions, Fig. 4b, the protein zone spread over 4 fractions (41–44), but formaldehyde DH-activity was found mainly in fractions 43 and 44. Although the SDS-PAGE gel showed again that these fractions were not pure, two bands could be identified that putatively corresponded to the target protein, Fig. 4b insert.

Since these bands appeared to be well separated from each other but also from the rest of the contaminants, fraction 43 containing the majority of the formaldehyde DH activity was subjected to a polishing step by gel filtration. Although no baseline separation of the protein mixture was possible, Fig. 5, a single band could be identified in the corresponding SDS-PAGE, Fig. 5 insert, that occurred exclusively in the fractions with formaldehyde DH activity and seemed to correspond in strength to the observed activity. Based on the gel, it appears that the protein is a monomer with a molar mass between 37 and 50 kDa, considering the position of the protein's band in relation to that of the molecular weight markers of 37 and 50 kDa. It is thus unlikely that the protein corresponds to the one described by Klein et al., for which a molar mass of approximately 200 kDa and a subunit mass of ca. 50 kDa has been reported. In the activity assay the protein showed no cross-reactivity with either methylamine or methanol, Fig. 6. For the activity assay the sample had to be desalted, while some calcium had to be added. The addition of an electron transfer agent such as NAD^+ or PQQ, however, was not necessary, arguing that either such an agent was still present in the sample in sufficient quantity or that the formaldehyde DH isolated by us also contained a covalently bound agent as had previously been postulated for FalDH.

Since repeated anion exchange chromatography followed by gel filtration did not yield a pure protein, a second multi-step separation process was designed based again on hydroxyapatite chromatography. When a desalted cell free extract was subjected to double gradient HA chromatography, the formaldehyde DH activity was found in two fractions (fractions 8 and 9) towards the end of the flow through, Fig. 7. The protein thus appears to interact

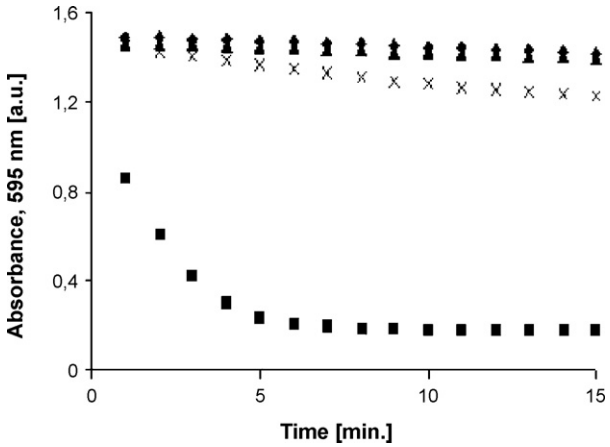

Fig. 6. Redox enzyme assay performed with the protein-containing fraction (formaldehyde DH activity) using water (♦), methanol (■), methylamine (▲), and formaldehyde (×) as substrate.

under the chosen conditions only weakly with the HA and to elute isocratically in the loading buffer (20 mM phosphate pH 7.0). When the fractions containing the formaldehyde DH-activity were pooled and subjected to anion exchange chromatography, the protein of interest could be found in a small shoulder (fraction 44) following the first major peak, Fig. 8. SDS-PAGE analysis showed, Fig. 8 insert, that this fraction contained at least 2 proteins and that the protein corresponding to the formaldehyde DH activity was not the major one. The molar mass of the protein was again between 37 and 50 kDa.

When the enzyme was investigated in the activity assay, as before some calcium had to be added to obtain the activity, Fig. 9. For the first time, however, it was also necessary to add an electron transfer agent, namely PQQ, to the assay mix to obtain an active enzyme. When PQQ was added, however, we obtained a very high specific activity. From that we concluded that the enzyme had been purified for the first time to this purity by the sequence of HA and anion exchange chromatography and that in particular whatever electron transfer agent had still been present after 2× anion exchange chromatography/gel filtration had been removed. Note that the addition of PQQ was necessary only after the anion exchange step. After the HA chromatography, formaldehyde DH activity could be detected in the fractions without this addition. Again the enzyme was highly specific accepting only formaldehyde and neither methylamine nor methanol as substrate. Other aldehydes have to date not been tested. In addition, the enzyme showed a pronounced sensitivity to salts and in particular to NaCl, Fig. 10.

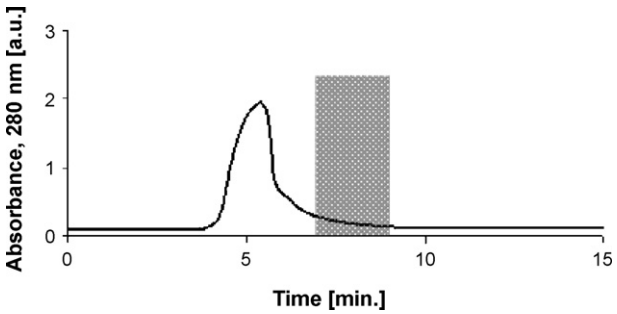

Fig. 7. Hydroxyapatite chromatography of a desalted cell free extract (flow through). Column: Macro-Prep HA type I, 20 μm (2 mL column), buffer A: 20 mM phosphate pH 7.0, flow rate 1.0 mL/min, fraction size: 1.0 mL. Fractions containing the formaldehyde DH activity are hatched.

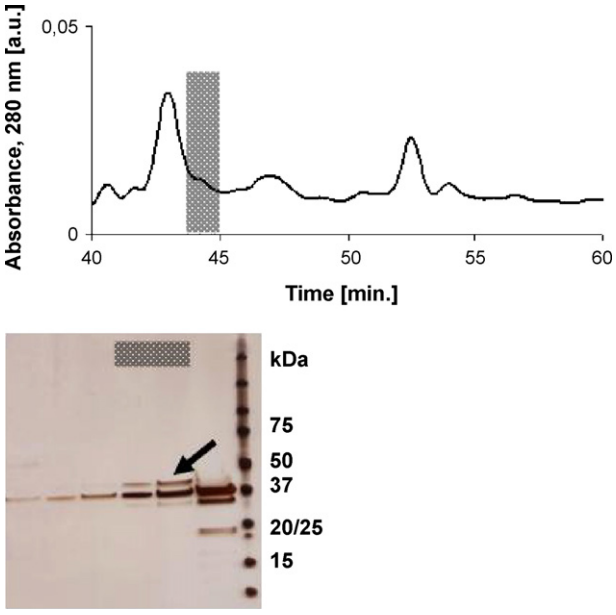

Fig. 8. Anion-exchange chromatography of the pooled fractions 8 and 9 from Fig. 7. Column: Resource Q (6 mL), buffer A: 25 mM Tris–HCl pH 8, buffer B: 25 mM Tris–HCl + 1 M NaCl pH 8, gradient 0–100% B in 240 min, flow rate 1.0 mL/min, fraction size: 1.0 mL. Fractions containing the formaldehyde DH activity are hatched. (Insert) SDS-PAGE (4–12% Bis–Tris) of the fractions of interest from the main figure. Right lane: molecular weight marker (Precision Blue Protein Standards, All Blue, Bio-Rad), lane 1: fraction 46, lanes 2, 3: fraction 45, lanes 4, 5: fraction 44, lane 6: 43. The band of interest is indicated by an arrow. Fractions containing the formaldehyde DH activity are indicated by the hatched band.

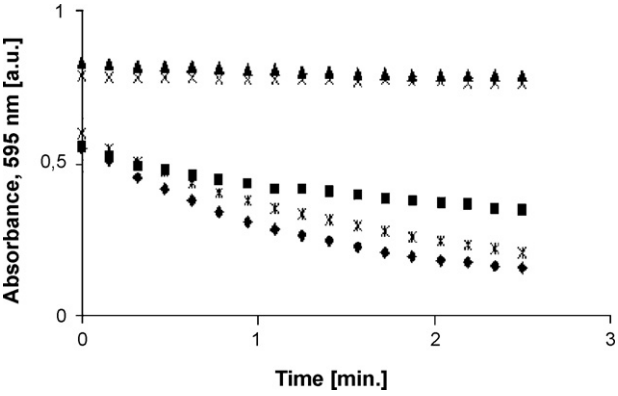

Fig. 9. Redox enzyme assay performed with fraction 44 from Fig. 8. (♦) Enzyme + 2 mM Ca^{2+} + PQQ, (*) enzyme + 50 μM Ca^{2+} + PQQ, (■) enzyme + PQQ, (×) Ca^{2+} + PQQ, (▲) enzyme + Ca^{2+}.

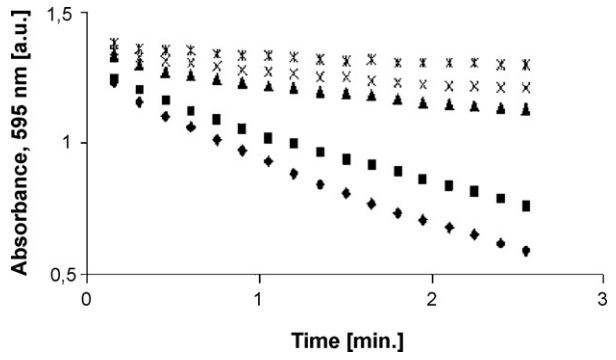

Fig. 10. Redox enzyme assay performed with the newly isolated formaldehyde DH in the presence of the indicated amounts of NaCl. (♦) 0 mM NaCl, (■) 10 mM NaCl, (▲) 50 mM NaCl, (×) 100 mM NaCl, (*) 200 mM NaCl.

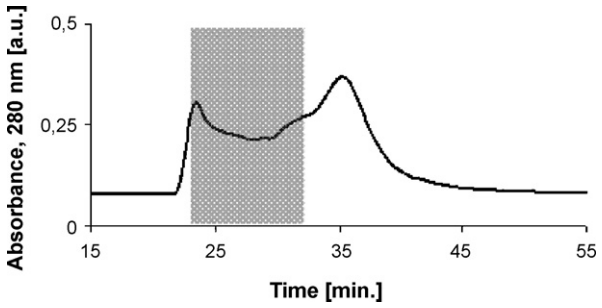

Fig. 11. Hydroxyapatite chromatography of a desalted cell free extract (phosphate gradient). Column: Macro-Prep HA type I, 20 μm (2 mL column), buffer A: 20 mM phosphate pH 7.0, buffer C: 400 mM phosphate pH 7.0, gradient 0–100% C in 60 min, flow rate 1.0 mL/min, fraction size: 1.0 mL. Fractions containing the methylamine DH activity are hatched.

The band presumably corresponding to the formaldehyde DH was cut from the gel and subjected to peptide mapping. The obtained map was compared to the sequence published for FalDH. No correlation was found. As already assumed, the formaldehyde DH isolated by us from *H. zavarzinii* ZV580 extracts is not identical to the previously described FalDH. Moreover, when we compared the obtained map to protein structures in general using MASCOT search (www.matrixscience.com), no significant hits were found. It appears that the isolated DH is an unknown protein. Moreover, due to the lack of homology with already described enzymes it is unlikely that this particular protein would have been found using standard DNA-based strategies. The enzyme is currently characterised in terms of catalytic activity and molecular biology in our laboratory.

3.4. Isolation of the methylamine DH activity

In order to isolate the methylamine DH activity, fractions obtained by double gradient HA chromatography were directly

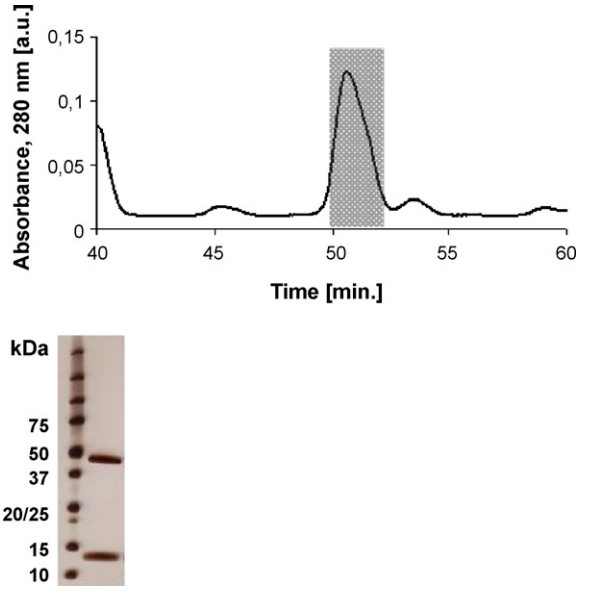

Fig. 12. Anion-exchange chromatography of the pooled methylamine DH fractions from Fig. 11. Column: Resource Q (6 mL), buffer A: 25 mM Tris–HCl pH 8, buffer B: 25 mM Tris–HCl + 1 M NaCl pH 8, gradient 0–100% B in 240 min, flow rate 1.0 mL/min, fraction size: 1.0 mL. Fractions containing the methylamine DH activity are hatched. (Insert) SDS-PAGE (4–12% Bis–Tris) of the hatched fraction of the main figure. Left lane: molecular weight marker (Precision Blue Protein Standards, All Blue, Bio-Rad), right lane: methylamine DH. The protein is constituted of two subunits differing in mass.

assayed for the corresponding activity. The methylamine DH eluted in a broad and ill-separated zone in the phosphate gradient, Fig. 11. This argues for a strong C-site interaction. When the fractions containing the activities were pooled and subjected to anion exchange chromatography, the methylamine DH activity was obtained in pure form in a well-separated peak, Fig. 12. In its native form the protein seems to be composed of at least two types of subunit, with molecular masses of approximately 12 and 43 kDa. In the DH-activity assay the enzyme showed no cross-reactivity with formaldehyde and methanol. Given the position of the methylamine DH-containing fractions in the initial gel filtration experiment, Fig. 1, it is hence likely that the enzyme isolated by us is one of the rare α,β-methylamine dehydrogenases [9]. According to [9] and papers cited within, most of the methylamine DHs reported so far have an α_2,β_2-structure, safe for a methylamine DH from *Methylobacillus flagellatum* strain KT, which also has an α,β-structure.

Further characterisation of the enzyme is currently under way in our laboratory. In particular it would be interesting to see if the enzyme can be used to catalyse the inverse reaction, i.e. the reduction of formaldehyde to methylamine, and thus become part of the envisioned biosensor. The fact that the methylamine DH is much less sensitive than the formaldehyde DH to environmental conditions such as the salt content would be of advantage in this context.

4. Conclusions

In spite of the considerable progress in molecular biology, the isolation of an active protein from a complex sources such as cell lysates based on its activity will continue to play an important role in bioprocess development. This is especially the case for proteins (enzymes) that cannot straightforwardly be expressed in recombinant form, e.g. in case of the FalDH considered in this publication most likely due to the necessity of a specific co-factor, or for proteins that for various reasons cannot be produced as fusion proteins bearing an affinity tag. Novel options exits that may aid such process development. In cases where some information about the primary and tertiary structure is availably, the production of specific antibodies based only on this structural information and computer simulations of the protein surface is possible, which extends the scope of affinity chromatography. In cases where the elution form an affinity column is too harsh or affinity ligands cannot be identified due to lack of structural information concerning the protein of interest, the use of hydroxyapatite may allow the set up of very specific "pseudo affinity" separations for such native enzymes. Finally, by using a chromatographic strategy based on activity (usually easy to do for an enzyme) it is possible to find also those proteins of a given catalytic ability that do not show pronounced homology to the already known ones.

Acknowledgements

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