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P450 monooxygenase in biotechnology I. Single-step, large-scale purification method for cytochrome P450 BM-3 by anion-exchange chromatography

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Abstract

An efficient single-step purification protocol for recombinant cytochrome P450 BM-3 from *Bacillus megaterium*, expressed in *E. coli*, was developed. Functional crude protein was obtained by disintegrating induced *E. coli* DH5α and removing cell debris by centrifugation. After investigating different anion-exchange matrices, elution salts and the elution procedures involving an ÄKTA*explorer* system, adsorption of the crude extract from lysed *E. coli* to Toyopearl DEAE 650M anion exchanger, followed by a two-step elution using NaCl, proved sufficient to isolate almost pure protein without inactivation (up to 93% P450 BM-3 content) in yields that ranged between 79–86%. The purification method could be scaled up 1500-fold and higher without further optimization to a 6-l production-scale column containing Toyopearl DEAE 650M anion exchanger.

Keywords: Preparative chromatography; Cytochromes; Proteins

1. Introduction

Cytochromes P450 are heme–thiolate proteins widely distributed in prokaryotic and eukaryotic organisms. Many P450-monooxygenases act on inactive carbon–hydrogen bonds such as alkanes, fatty acids, terpenes and steroids, and usually exhibit high regio- and stereoselectivity.

Cytochrome P450 BM-3 (CYP102) is a water-soluble, NADPH-dependent fatty acid hydroxylase from *Bacillus megaterium* [1,2] that catalyzes the subterminal oxidation of saturated and unsaturated fatty acids. Compared to eukaryotic fatty acid hydroxylases, such as CYP52A4 from *Candida maltosa*, its turnover rate is about 100-fold higher [3]. The *Bacillus* enzyme with a molecular mass (M_r) of 120 000 consists of two distinct domains, a FAD- and FMN-containing reductase and a hemoprotein, fused into a single polypeptide. Its unusually high catalytic activity, the efficient electron utilization in strong coupled reactions [4] and information about its crystal structure in the presence and absence of substrate [5,6] make this evolutionarily advanced

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P450 monooxygenase system [7] a suitable candidate for biotechnological applications [8–10].

The gene coding for P450 BM-3 has been cloned from *Bacillus megaterium* and expressed in *E. coli* strains [1,2]. For its purification, essentially two possible methods have been described. The first is based on affinity chromatography using 2′,5′-adenosine diphosphate agarose [11] as a ligand for the NADPH binding site. This procedure is highly effective, but unsuitable for the isolation of larger amounts of protein due to the disproportionate cost of adenosine-2-monophosphate required for elution. The second method comprises four steps that begin with ammonium sulfate precipitation followed by anion-exchange chromatography on DEAE-Sephacel [12] or MonoQ [13], adsorption chromatography on hydroxylapatite and finally gel filtration on Sephacryl S300. A combination of both methods has also been reported [12], but it is not only laborious, but also time-consuming.

Here, we describe a rapid, one-step purification method for recombinant cytochrome P450 BM-3 using a commercially available, cost-effective anion-exchange resin which can be scaled up to purify this enzyme in gram quantities. This procedure enhances the prospect of obtaining sufficient amounts of pure protein for biotechnological applications such as the development of an enzyme membrane reactor, for example.

2. Experimental

2.1. Materials

All chemicals were of analytical-reagent grade or of higher quality and were purchased from Fluka (Neu-Ulm, Germany) or Sigma (Deisenhofen, Germany). The resins were supplied by TosoHaas (Stuttgart, Germany).

2.2. Bacterial strains and vector construction

E. coli strain DH5α (*supE*44, *lacU*169 [80*lacZ* M15] *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1 from Clontech, Heidelberg, Germany) was used for cloning and protein expression. Standard recombinant techniques were carried out according to Sambrook et al. [14]. The P450 BM-3 gene was isolated by polymerase chain reaction (PCR) using genomic DNA of *Bacillus megaterium* (DSM 32^T) as template, and primers introducing a *Bam*HI site (5′-gtgaaagagggatcccatgac-aattaaagaaatgcc) at the 5′ end and a His-Tag and *Eco*RI site (5′-cggaattcttaatgat-gatgatgatgat-gcccagcccacacgtcttttg) at the 3′ end of the gene for subsequent cloning. The PCR-fragment obtained was inserted downstream of the strong, temperature inducible P_RP_L-promoter of pCYTEXP1 [15] resulting in the plasmid pT-USC1BM3. After sequence check (Applied Biosystems Model 373A, Weiterstadt, Germany) a single point mutation F87A using the Stratagene QuikChange Kit was introduced with an additional *Eae*I restriction site for screening. Further details are to be described elsewhere [16].

2.3. Large-scale production of P450 BM-3

For the pilot production of P450 BM-3 in a 30-l fermenter, 500 ml Luria–Bertani medium (LB) supplemented with 100 μg/ml ampicillin for plasmid selection was inoculated with 1 ml of an overnight culture of recombinant *E. coli* DH5α pT-USC1BM3 and cultivated at 37°C and 200 rpm. After reaching an A_{578nm} of 0.8–1.0, this culture was used to inoculate 25 l of LB-medium containing 100 μg/ml ampicillin. The fermentation was carried out in a 30-l bioengineering fermenter, type LP351, with an aeration of 3.5 l/min and a stirrer speed of 300 rpm at 37°C, and an initial pH of 7.5. In order to avoid foam accumulation and to enhance P450 BM-3 production, 2.0 ml of sterile Contraspum 210 (ZSchimmer and Schwarz, Lahnstein, Germany) and 0.1 mg/l $FeCl_3$ were added prior to inoculation [17]. After reaching an $A_{578\ nm}$ of 1.0, P450 expression was induced by a temperature shift from 37°C to 42°C for 5 h. P450 BM-3, or its mutant F87A, were expressed in *E. coli* DH5α in yields of 300 to 400 nmol/l fermenter broth. *E. coli* cells containing the P450 BM-3 were harvested by cross-flow filtration using a Millipore stainless steel holder (Eschborn, Germany) with a Filtron (Dreieich, Germany) Centrasette OMEGA (0.3 μm) membrane followed by centrifugation of the 15-fold concentrated suspension at 9200 *g* for 20 min. Cells were stored for three weeks until required for further use at −20°C

without activity loss, as demonstrated by the pNCA assay in potassium phosphate (50 m*M*, pH 7.5) or Tris–HCl buffer (50 m*M*, pH 7.5).

2.4. Preparation of E. coli DH5α cell extracts

The cells were stored in 0.1 *M* potassium phosphate buffer or 0.1 *M* Tris–HCl (pH corresponding to purification between 7.0 and 8.0) containing 0.1 m*M* EDTA and 0.1 m*M* PMSF (phenylmethansulfonyl fluoride) at −20°C. Up to 15 g (wet mass) DH5α in 25 ml potassium phosphate buffer (50 m*M*, pH 7.5) or Tris–HCl buffer (50 m*M*, pH 7.5) were thawed on ice. After sonifying the cells for 3 min in an ice bath (Branson Sonifier W250, Dietzenbach, Germany; output level 80 W, duty cycle 20%) in the presence of 0.1 m*M* EDTA (pH 7.5) and 0.1 m*M* PMSF, the suspension was centrifuged for 20 min at 32 500 *g*. Prior to loading the anion-exchange column via a superloop [Amersham Pharmacia Biotech (APB), Uppsala, Sweden], the crude extract was passed through a 0.22 μm Sterivex-GP filter (Millipore) and the P450 BM-3 content was measured by CO-differential spectra [18].

2.5. Spectrophotometric enzyme assays

All spectral assays were carried out under aerobic conditions. UV–Vis measurements were performed in a Pharmacia spectrophotometer, Model BioChrom4060 (APB, Uppsala, Sweden), with BioChrom4060 Windows 3.11 software v2.0.

P450 BM-3 concentrations were measured by the CO-differential spectra method as described by Omura and Sato [18], using ε:=91 mM^{-1} cm^{-1} with a scan rate of 125 nm/min in 0.1 nm intervals. The protein content was determined by the bicinchoninic acid (BCA) method [19] with bovine serum as standard (Mirco BCA protein assay reagent kit, Pierce, St. Augustin, Germany). Activity of the P450 BM-3 reductase (BMR) was determined by cytochrome *c* assay [20] using an extinction coefficient of ε:=21 mM^{-1} cm^{-1} at 550 nm, and P450 BM-3 activity was measured by NADPH consumption [21] at 340 nm based on an extinction coefficient of ε:=6.66 mM^{-1} cm^{-1}.

2.6. Chromatographic equipment

To develop the purification protocol, an APB ÄKTA*explorer* system was used with a personal computer-based UNICORN control system v2.1 and a Frac-900 collector. Continuous monitoring of P450 BM-3 in the column effluent was carried out by simultaneously determining absorption at 280 nm (protein) and 417 nm (heme). The scale-up was undertaken with a 2 ml flow-through cell with a 1/20 split stream to the ÄKTA*explorer* system and a spectrophotometer (Shimadzu UV1202, Duisburg, Germany). All chromatographic resins were obtained from TosoHaas (Stuttgart, Germany). A pre-packed MPD-DEAE 650S [85 mm×7.5 mm, particle size 35 μm, asymmetry factor (Af)=1.1, 4100 theoretical plates (TP)/m], along with self-packed XK16/20 (100 mm×16 mm, APB) columns containing Toyopearl DEAE 650M (Af=1.1, 1610 TP/m), SuperQ 650M, (Af=1.2, 1840 TP/m) and QAE 550M (Af=1.0, 1780 TP/m) with a particle size of 65 μm were used. In the trial run, an INDEX200 (APB) glass column (19 cm×20 cm, Af=1.4, 3800 TP/m) and a gearwheel pump (ISMATEC, PB, USA), Toyopearl DEAE 650M (65 μm) were tested. In contrast to the buffer preparation methods used for the small columns, the buffers for the trial run were prepared manually prior to use. They consist of 0.1 *M* Tris–HCl, pH 7.8, containing 0.1 m*M* EDTA, 0.1 m*M* PMSF and 150 m*M* NaCl for the first, 250 m*M* NaCl for the second, and 1 *M* NaCl for the third step.

A load of 8±0.2 mg P450 BM-3 per 100 ml matrix was used in all columns. As the expression level of the recombinant protein depends on fermentation conditions, the total amount of protein differs by about 25% if same amount of P450 BM-3 is used. It was shown, however, that a 25-fold excess of the above load onto a DEAE 650S MDP column (85 mm×7.5 mm) did not exceed its capacity.

All analytical and trial runs were performed at 200 cm/h and at ambient temperature. For the required purification period, temperatures <30°C are recommend to avoid P450 BM-3 inactivation [22].

2.7. Choice of buffer

The experimental conditions involved the purifica-

tion of recombinant P450 BM-3 from lysed crude cell extracts of *E. coli* without further ammonium sulfate precipitation [11] on anion-exchange resins using linear salt gradients at a pH range between 7.0 and 8.0 for elution, confirmed Tris buffer (0.1 *M*, pH 7.8) containing 0.1 m*M* EDTA, to be the most suitable. For this reason it was employed for all subsequent experiments. P450 BM-3 can be stored in this buffer for more than 2 h at ambient temperature without loss of activity.

3. Results and discussion

3.1. Linear and scaled gradient

The separation results of P450 BM-3 on four anion exchangers DEAE 650S (35 μm), DEAE 650M (65 μm), SuperQ 650M (65 μm) and QAE 550M (65 μm) are summarized in Table 1 and Fig. 1. In each experiment, a linear gradient (ten column volumes, CV) of 0–1 *M* NaCl in 0.1 *M* Tris–HCl buffer at pH 7.8 was used. In all cases, recovery of P450 BM-3 was between 80% and 84%. The highest yield of P450 BM-3 (39–42% of the total protein content) was obtained with the DEAE resins, followed by SuperQ 650M and QAE 550M. The differences in resolution of the four materials are illustrated in Fig. 1 by absorption at a wavelength of 280 nm (protein) and 417 nm (heme of P450 BM-3). Further improvement of the resolution could be achieved by scaled gradients. The ability of the ÄKTA*explorer* to monitor the elution of cytochrome P450 BM-3 (417 nm) and the conductivity of the salt gradient online simultaneously proved very useful for these experiments. Fig. 2A, for example, shows the optimized two-step gradient for the 20 ml DEAE 650 M column, first using 150 m*M* NaCl and then 250 m*M* NaCl. P450 BM-3 is eluted when NaCl is increased from 150 m*M* to 170 m*M*. This behavior can be predicted from the conductivity values of the linear gradient shown in Fig. 1B. The appropriate NaCl concentration for the elution is 250 m*M*; concentrations $<$230 m*M* cause a broadening of the P450 BM-3 elution peak and concentrations$>$300 m*M* result in a loss in resolution (data not shown). A similar pattern was also observed for the Toyopearl resins SuperQ 650M (Figs. 1C and 2B) and QAE 550M.

The elution protocols for the four anion-exchange materials and the improvements in separation which can be achieved by a scaled rather than linear salt elution profile are presented in Tables 1 and 2 as well as in the corresponding Figs. 1 and 2, by comparing the protein content and peak area quotient $Q_{280/417}$. Thus, if a 10 CV linear gradient is used on column DEAE 650M (Table 1) a content of approximately 39% P450 BM-3 of the total protein in the elution peak can be achieved, whereas application of an optimized scaled gradient on the same material in the column results in a P450 BM-3 protein content of 84% of the total protein (Table 2). Using a scaled gradient, the collected fractions containing P450 BM-3 reveal contents $>$80% of the total protein if DEAE 650M, DEAE 650S or SuperQ 650M anion exchanger material is used. The best results were obtained with a DEAE 650S column, which resulted in a content of 93% P450 BM-3 in the elution peak (Table 2). Table 2 contains further the final conditions used for the optimize two salt step gradients

Table 1
Comparison of 0–1 *M* linear NaCl gradients in 10 column volumes (CVs) on Toyopearl DEAE 650M, DEAE 650S, SuperQ 650M and QAE 550M

Anion-exchange matrix	Yield BM (%)	Protein ratio P450/total protein[a]	Peak area ratio 280/417 nm[b]
DEAE 650M	81	0.39±0.05	7.7
DEAE 650S	83	0.42±0.05	6.0
SuperQ 650M	80	0.31±0.05	8.2
QAE 550M	84	0.27±0.05	10.2

[a] Total protein content of the P450 BM-3 peak determined with BCA method [19].
[b] Wavelength of the P450 BM-3 heme absorption [2].

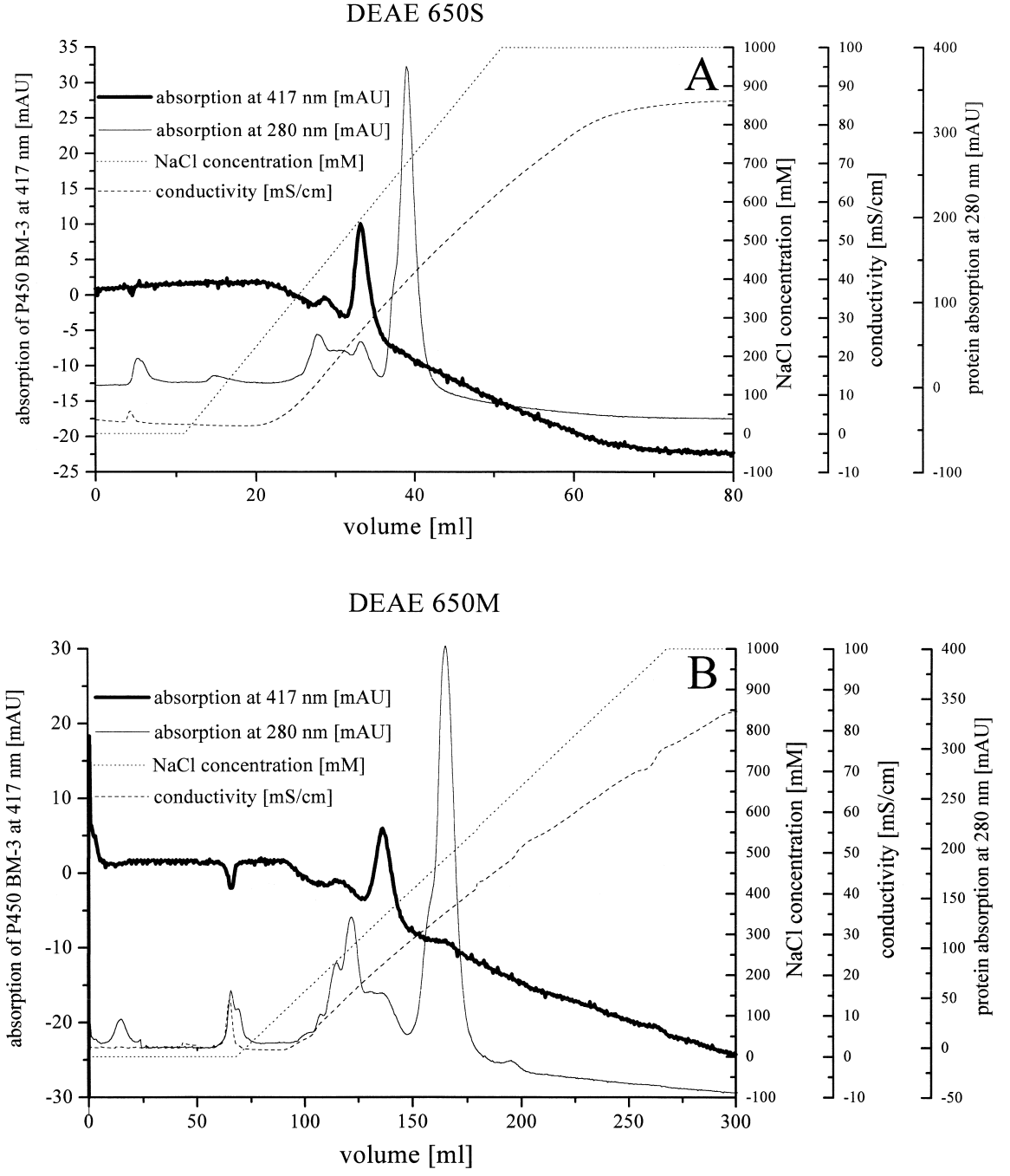
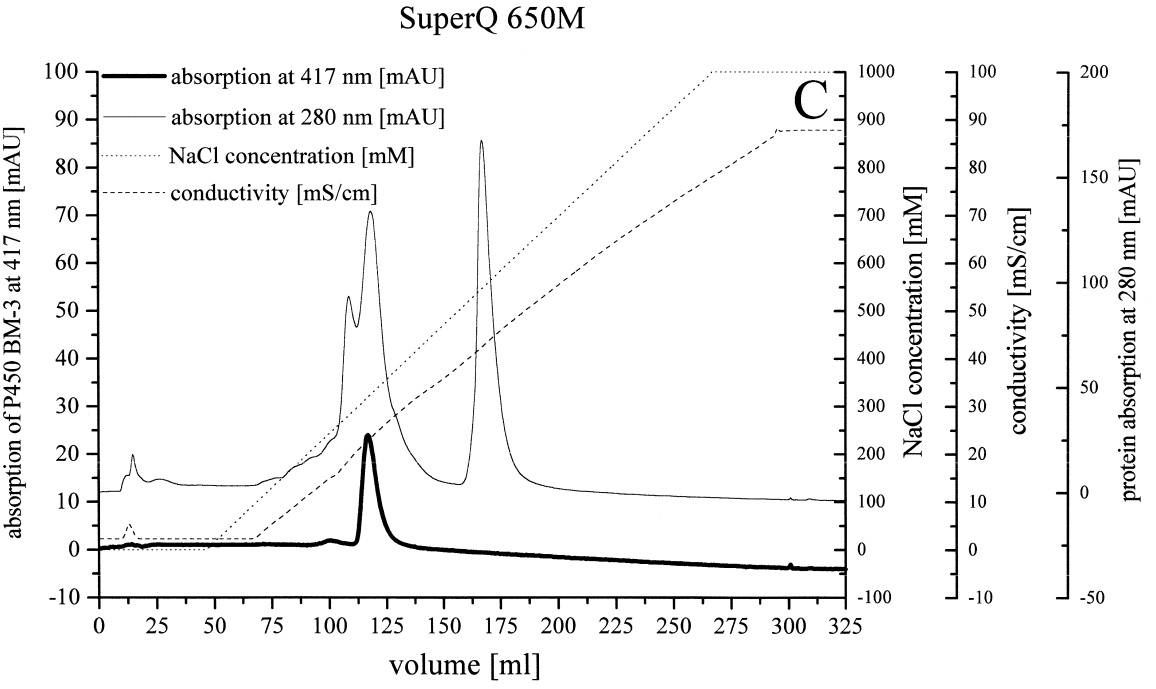


Fig. 1. Separation of P450 BM-3 crude extract using a linear 0–1 *M* sodium chloride gradient in 10 column volumes on Toyopearl (A) DEAE 650S (MDP 85×7.5 mm), (B) DEAE 650M (XK 16/10 100×16 mm), (C) SuperQ 650M (XK 16/10 100×16 mm) and (D) QAE 550M (XK 16/10 100×16 mm) columns in 0.1 *M* Tris buffer, pH 7.8. A load of 8±0.2 mg P450 BM-3 per 100 ml matrix was used in all columns. The elution of P450 BM-3 was monitored at a wavelength of 417 nm. The dotted lines indicate the sodium chloride concentration loaded on the columns and the conductivity achieved afterwards.



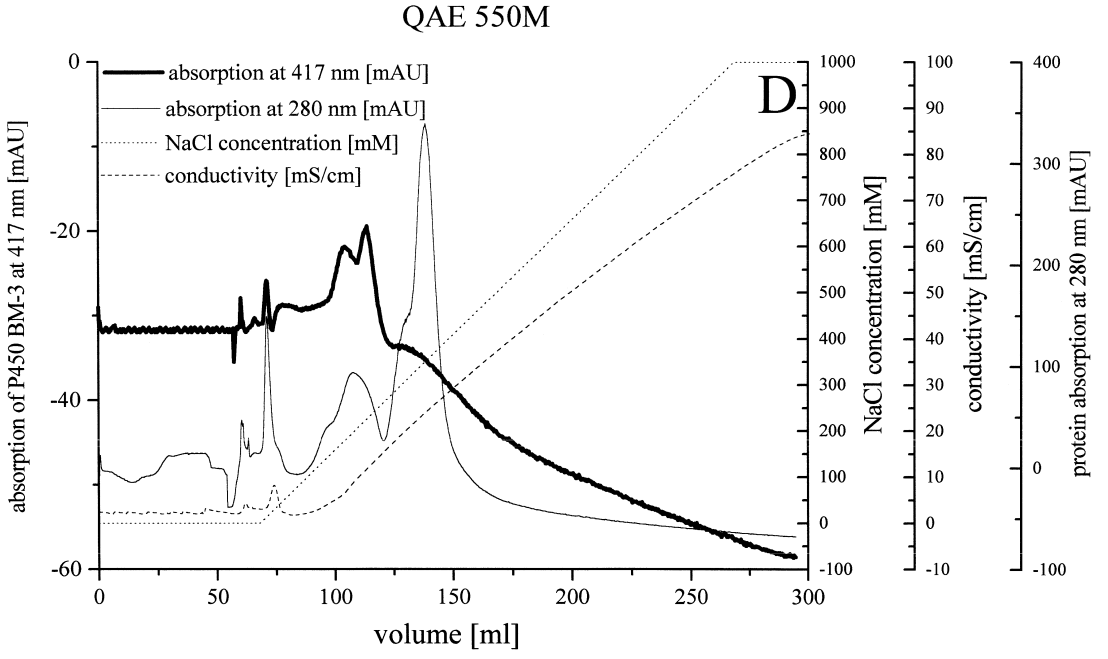


Fig. 1. (*continued*)

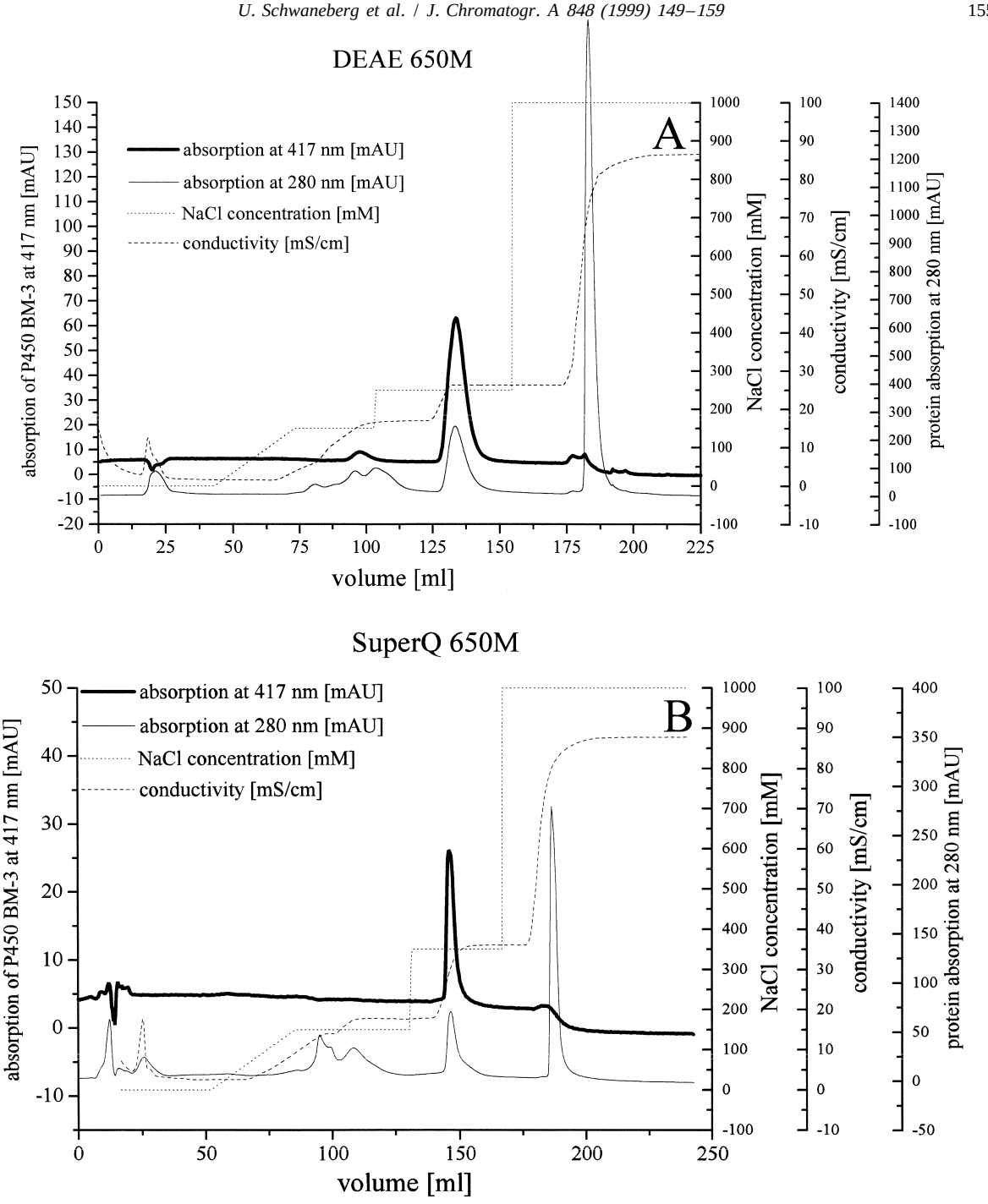


Fig. 2. Separation of P450 BM-3 crude extract using a two-step sodium chloride elution on Toyopearl (A) DEAE 650M (first step 150 m*M*/second 250 m*M*) and (B) SuperQ 650M (first step 160 m*M*/second 340 m*M*) in 0.1 *M* Tris buffer, pH 7.8. A load of 8±0.2 mg P450 BM-3 per 100 ml matrix was used in all columns. The elution of P450 BM-3 was monitored at a wavelength of 417 nm. The dotted lines indicate the sodium chloride concentration loaded on the columns and the conductivity achieved afterwards. The coomassie Brilliant Blue R250 stained SDS–PAGE in (C) visualizes the achievable purification with the DEAE 650M and SuperQ 650M using the scaled methods. Lane 1 shows the purification result, lane 2 the crude protein extract and lane 3 the marker proteins ($M_r \cdot 10^{-3}$).

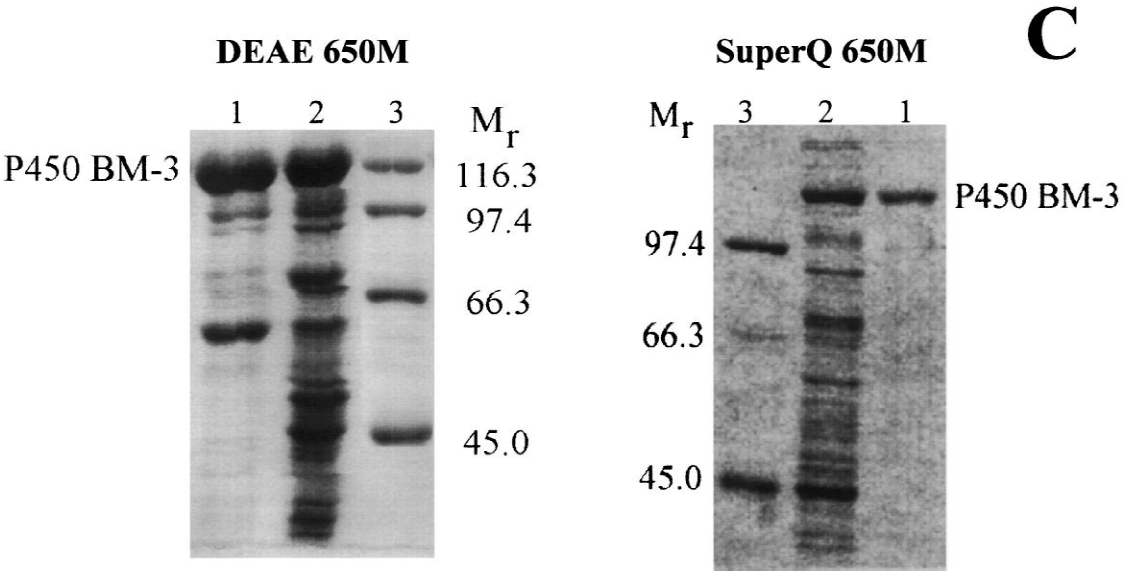


Fig. 2. (*continued*)

and Fig. 2C, the corresponding sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel, visualizes the achievable purification.

As Tris is the most expensive constituent of used buffer, its concentration was gradually reduced. No difference in resolution was observed with the DEAE 650M scaled method down to the concentration of 10 m*M* Tris.

Following the Hofmeister series of anion binding strength, Cl^- was replaced by Ac^- and Br^- and using scaled methods the resolution was again determined by comparing peak areas quotient $Q_{280/417}$ of the elution peak at 280 nm (total protein) and 417 nm (P450 BM-3). If sodium acetate was used, $Q_{280/417}$ relative to sodium chloride differed only slightly (0.03 to 0.05) for SuperQ 650M and QAE 550M, but was registered at 0.6 for DEAE 650M. In the case of sodium bromide, poorer resolution was observed ($Q_{280/417}$ 3.9 for SuperQ 650M, 4.9 for DEAE650M, and 14.8 for QAE 550M). In conclusion, acetate may be used as an alternative to chloride for the graduated elution of P450 BM-3 in Tris–HCl buffer at pH 7.8, and sodium chloride and sodium acetate are more suitable than sodium bromide. In our studies, we selected sodium chloride as elution salt for the trial runs.

3.2. Scale-up studies and preparative runs

As our initial experiments based on DEAE 650M

Table 2
Comparison of elution steps of P450 BM-3 on Toyopearl DEAE 650M, DEAE 650S, SuperQ 650M and QAE 550M

Anion-exchange matrix	NaCl 1st step/2nd step	Yield BM-3 in 2nd step (%)	Protein ratio P-450/total protein[a]	Peak area ratio 280/417 nm[b]
DEAE 650M	150 m*M*/250 m*M*	85	0.84±0.05	3.1
DEAE 650S	150 m*M*/250 m*M*	82	0.93±0.05	2.7
SuperQ 650M	160 m*M*/340 m*M*	79	0.86±0.05	2.7
QAE 550M	230 m*M*/420 m*M*	80	0.41±0.05	5.7
Upscale DEAE 650M	150 m*M*/250 m*M*	86	0.78±0.05	3.3

[a] Total protein content of the P450 BM-3 peak determined with BCA method [19].
[b] Wavelength of the P450 BM-3 heme absorption [2].

and SuperQ 650M anion exchangers resulted in similar yields and purities of P450 BM-3, the weaker and less expensive anion exchanger DEAE 650M was favored. For the purification of cytochrome P450 BM-3 in the preparatory scale, a 19 cm×20 cm INDEX200 (APB) glass column, filled with Toyopearl m-grade DEAE with a particle size of 65 μm, was used and a backpressure of 2 bar during the runs was observed. The finer s-grade material with a particle size of 35 μm was not used because it generated an even higher backpressure. The UV signal at 280 nm was monitored directly by a flow-through cell, and UV 280 nm and Vis 417 nm were measured additionally via a 1/20 split to the ÄKTA*explorer* (Fig. 3).

In a total of three runs carried out with this system, 480±30 mg of almost pure P450 BM-3 were obtained. As described previously, a 25-fold higher load onto the small column is possible without exceeding the column capacity but as this increases backpressure, DNAse treatment of the crude lysed cell extract prior to use proved to be suitable. A comparison of Figs. 2 and 3 and Tables 1 and 2 reveals that the transfer of the two-step elution protocol from a 3.8 ml DEAE 650S column to a 6-l Toyopearl DEAE 650M column resulted in essentially the same amount of recovered protein.

3.3. Comparison to other purification methods

The resin and buffer costs of the scaled DEAE 650M/650S and Super 650M methods are lower compared to the affinity chromatography using 2′,5′-adenosine diphosphate agarose [11] or multiple-step methods [1,12] and amenable to purify P450 BM-3 in gram scale for biotechnological applications. The overall yields of the scaled DEAE 650M method (85%) and affinity chromatography (85–87%) [11] and the time-requirement of both methods are similar and superior to those of multiple step methods (overall yield 6% [1]). The purity of P450 BM-3 attainable with the scaled DEAE 650M/650S and

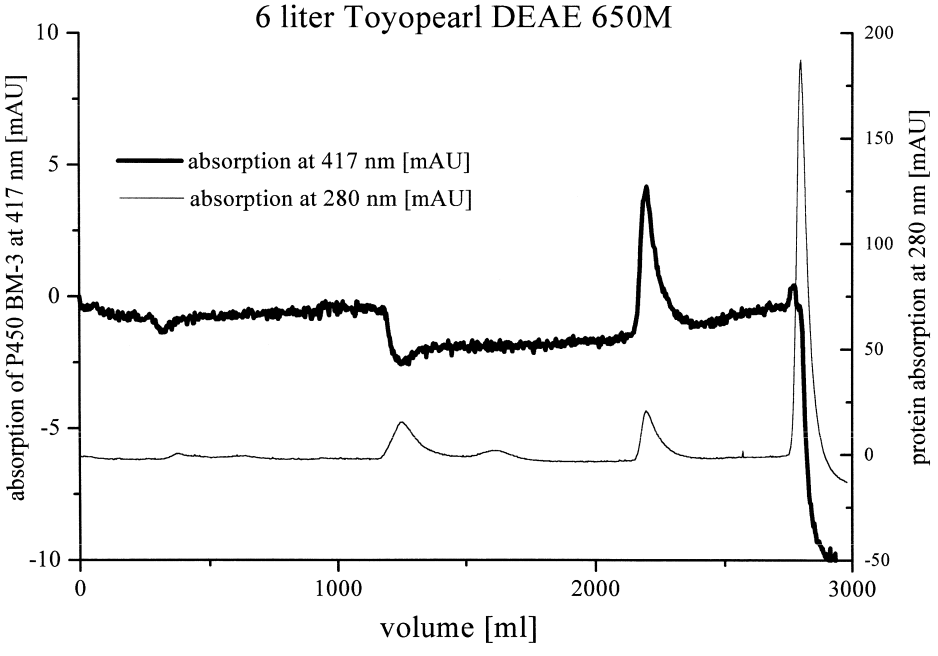


Fig. 3. Trial separation of P450 BM-3 crude extract was set up using first 150 m*M* sodium chloride (3 CVs) and second 250 m*M* sodium chloride (2 CVs) on an INDEX200 (APB) glass column (20×19 cm, Af=1.4, 3800 TP/m) with Toyopearl DEAE 650 M (65 μm), an ISMATEC gearwheel pump and a spectraphotometer (Shimadzu UV1202) with a 2-ml flow-through cell. A 1/20 split stream to the ÄKTA*explorer* System was used to monitor the protein absorption simultaneously at 280 nm and P450 BM-3 absorption at 417 nm during the run in 0.1 *M* Tris buffer, pH 7.8. A load of 8±0.2 mg P450 BM-3 per 100 ml matrix was used.

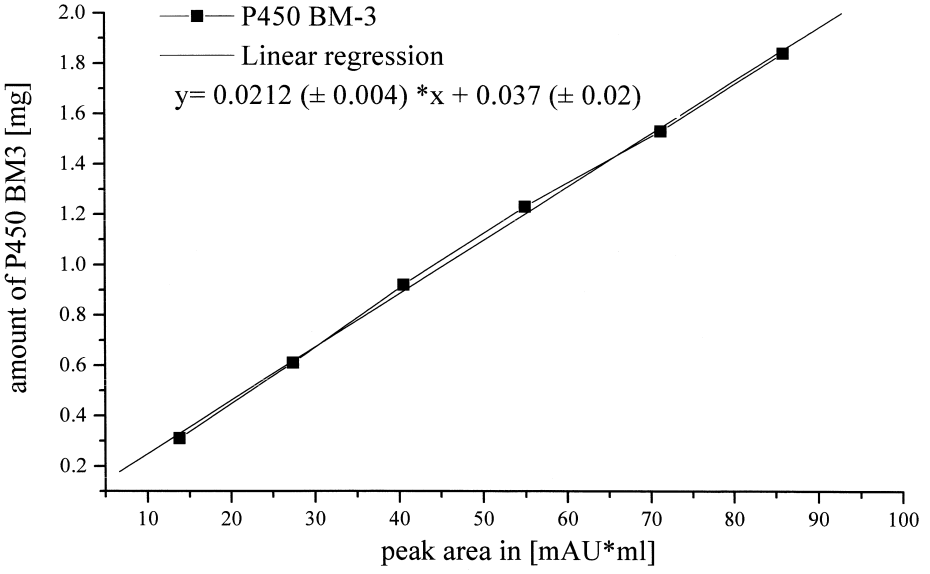


Fig. 4. Correlation between the peak area of the P450 BM-3 absorption at 417 nm and the amount of P450 BM-3 which was calculated according to the CO-differential spectra method of Omura and Sato [18] using the DEAE 650M step elution method (Fig. 2A).

Super 650M methods is not significantly poorer in comparison to other approaches [1,11].

3.4. Correlation between peak area at 417 nm and P450 content

Using different amounts of P450 BM-3, the correlation between the peak area recorded at a wavelength of 417 nm and the P450 BM-3 content determined by the CO-differential spectra method of Omura and Sato [18] were compared. As shown in Fig. 4, 1 mg of P450 BM-3 correlates to a peak area of 45 mAU.

Thus, once a calibration curve has been obtained, this method can also be used for other P450 enzymes to determine the P450 content of a sample during purification, thereby avoiding the use of the time-consuming CO-differential spectra method, which takes approximately 10 min per fraction.

4. Conclusions

A fast method for the purification of recombinant P450 BM-3 in a single step was developed that is based on the use of commercial anion exchangers (Toyopearl DEAE 650M, DEAE 650S, SuperQ 650M and QAE 550). Significantly improved results were achieved if an optimized, graduated salt elution was used instead of a linear salt gradient. An ÄKTA*explorer* system allowed the conductivity, protein and heme absorption to be monitored simultaneously, which proved very useful in optimizing the elution protocol. A simple two-step elution proved sufficient to isolate almost pure protein (up to 93% P450 BM-3) in overall yields between 79–86%. The optimized, graduated elution could be transferred directly on a preparative six liter column filled with Toyopearl DEAE 650M and allowed the purification of P450 BM-3 in the relevant scale. A rapid method for correlating the peak area of heme absorption at 417 nm with the content of P450 BM-3 in the column effluent was designed and shown to be equivalent to the cumbersome CO-differential spectra method, which is usually applied to quantify P450 enzymes in solution.

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References

[1] L.O. Narhi, A.J. Fulco, J. Biol. Chem. 261 (1986) 7160.
[2] L.O. Narhi, A.J. Fulco, J. Biol. Chem. 262 (1987) 6683.
[3] U. Scheller, T. Zimmer, E. Kargel, W.-H. Schunck, Arch. Biochem. Biophys. 328 (1996) 245.
[4] S.S. Boddupalli, R.W. Estabrook, J.A. Peterson, J. Biol. Chem. 265 (1990) 4233.
[5] K.G. Ravichandran, S.S. Boddupalli, C.A. Hasemann, J.A. Peterson, J. Deisenhofer, Science 261 (1993) 731.
[6] H. Li, T.L. Poulos, Nat. Struct. Biol. 4 (1997) 140.
[7] K.N. Degtyarenko, Protein Eng. 8 (1995) 737.
[8] Y. Dror, A. Freeman, Appl. Environ. Microbiol. 61 (1995) 855.
[9] C. Larroche, J.B. Gros, Adv. Biochem. Eng. Biotechnol. 55 (1997) 179.
[10] M. Mattey, Crit. Rev. Biotechnol. 12 (1992) 87.
[11] S.D. Black, M.H. Linger, L.C. Freck, S. Kazemi, J.A. Galbraith, Arch. Biochem. Biophys. 310 (1994) 126.
[12] A.W. Munro, S. Daff, J.R. Coggings, J.G. Lindsay, S.K. Chapman, Eur. J. Biochem. 239 (1996) 403.
[13] A.J. Fulco, M.L. Klein, J. Biol. Chem. 268 (1993) 7553.
[14] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.
[15] T.N. Belev, M. Singh, J.E.G. McCarthy, Plasmid 26 (1991) 147.
[16] U. Schwaneberg, E. Bonacker, C. Schmidt-Dannert, R.D. Schmid, in preparation.
[17] H. Nishihara, T. Okamura, R.D. Schmid, A. Hauck, M. Reuss, J. Biotech. 56 (1997) 57.
[18] T. Omura, R.J. Sato, J. Biol. Chem. 239 (1964) 2370.
[19] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 175 (1985) 231.
[20] T. Oster, S.S. Boddupalli, J.A. Peterson, J. Biol. Chem. 266 (1991) 22718.
[21] G.P. Kurzban, H.W. Strobel, J. Biol. Chem. 25 (1986) 7824.
[22] U. Schwaneberg, D. Bezdan, J. Schmitt, R.D. Schmid, submitted for publication.