Journal of Chromatography, 521 (1990) 211–220
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1990

Efficient high-performance liquid chromatographic system for the purification of a halobacterial serine protease

WILMA SCHMITT*, URSULA RDEST and WERNER GOEBEL
Institut für Mikrobiologie und Genetik, Röntgenring 11, D-8700 Würzburg (F.R.G.)

ABSTRACT

Many of the extreme halophiles which belong to the archaebacteria produce extracellular proteases. The extracellular serine protease (designated ESP4) of *Halobacterium* sp. strain TuA4 was isolated in a pure state with a fast protein liquid chromatographic (FPLC) system. Because the enzyme is only stable at high ionic strength, it was necessary to develop a procedure that would allow a minimum Na^+ ion concentration of 0.3 *M* in each step. This is the first halophilic salt-dependent enzyme purified with FPLC. Two precipitation steps with PEG 6000 and acetone in combination with ion-exchange chromatography (CM-Sephadex, Mono Q HR 5/5) and hydrophobic interaction chromatography (phenyl-Superose HR 5/5) permitted the isolation of 216-fold purified ESP4 with a total recovery of 3%. The purified ESP4 was shown to possess a molecular weight of 60 000 dalton in sodium dodecyl sulphate–polyacrylamide gel electrophoresis, which correlates very well with the native molecular weight determined for this enzyme.

INTRODUCTION

Extremely halophilic bacteria which grow best in 20–30% NaCl belong to the third bacterial kingdom recently named archaebacteria. In certain respects these bacteria differ from other prokaryotes (eubacteria) and from eukaryotes [1].

Extracellular proteases are widely distributed among halophilic bacteria. Because they metabolize carbohydrates only slightly, these bacteria secrete proteolytic enzymes to degrade proteins from dead organisms in the natural salt environment [2]. Enzymes from extremely halophilic bacteria show a high degree of adaptation to extreme salt concentrations (4–5 *M* NaCl or KCl) and lose their activity at low ionic strength. This fact makes the development of a purification scheme very difficult, as one can only use procedures that are unaffected by high salt concentrations. The most highly purified and best characterized halobacterial proteins are structural proteins, such as the bacterioopsin [3], the gas vesicle protein [4], the cell envelope glycoprotein [5] and the ribosomal proteins [6]. These proteins are made in large amounts and can be isolated very easily. Only a few halobacterial enzymes have been purified because most of them are irreversibly denatured and inactivated after exposure to low salt concentrations. The purification methods that have generally been applied are salting-out mediated chromatography [7] and hydroxyapatite [8] and affinity chromatography [9], which allow high concentrations of salt. The first protease isolated from

halobacteria was the extracellular serine protease from *H. halobium* [9]. The method described here for the isolation of the extracellular serine protease (termed ESP4) from the mesophilic halobacterial isolate *Halobacterium* sp. strain TuA4 is new. In order to shorten the purification time we developed an efficient five-step procedure with two high-resolution steps using an automated fast protein liquid chromatographic (FPLC) system as last step of the purification scheme.

EXPERIMENTAL

Materials

All chemicals were of analytical-reagent grade and were purchased from Merck (Darmstadt, F.R.G.), Roth (Karlsruhe, F.R.G.) and Sigma (Deisenhofen, F.R.G.). DEAE-Sephadex A-50, CM-Sephadex C-50, PD 10 columns (Sephadex G-25 M), arginine-Sepharose 4B, benzamidine-Sepharose 6B and the prepacked FPLC columns Superose 12 HR 10/30, Mono Q HR 5/5, phenyl-Superose HR 5/5 and Q-Sepharose HiLoad 16/10 were obtained from Pharmacia–LKB (Freiburg, F.R.G.). Sterile filters were from Sartorius (Göttingen, F.R.G.) and Gelman (Dreieich, F.R.G.).

Isolation and purification of ESP4

Halobacterium sp. strain TuA4 was isolated from Chott-el-Djerid, Tunisia, and purified in this laboratory. The cells were grown in rich medium containing 4.3 *M* NaCl, 0.12 *M* $MgSO_4$, 0.03 *M* KCl, 0.01 *M* trisodium citrate and 1% peptone (Oxoid) (pH 7.2) with shaking and illumination at 37°C to a cell density of 10^9 cells/ml. After 5 days the cells were harvested by centrifugation at 11 000 *g* for 20 min at 4°C. A 5-l volume of culture supernatant was filtered with a 0.45-μm Sartorius filter and then concentrated 100-fold by a Diaflow HP 10-20 hollow-fibre cartridge from Amicon (10 000-dalton cut-off). To 30 ml of the concentrated supernatant PEG 6000 was added from 60% stock solution in 3 *M* NaCl–10 m*M* $CaCl_2$–20 m*M* $MgCl_2$–50 m*M* Tris–HCl (pH 9.0) to a final concentration of 10%. After 30 min on ice, nucleic acids and membrane compounds were pelleted at 17 000 *g* for 15 min at 4°C. The PEG supernatant, which contained the protease activity, was then mixed with two volumes of acetone. The precipitation was complete after 20 min at −20°C and the precipitate was then centrifuged at 24 000 *g* for 20 min at 4°C. The pellet was suspended in 18 ml of 20% glycerol and desalted on PD 10 columns. The final buffer composition was 0.3 *M* NaCl–10 m*M* $CaCl_2$–50 m*M* 2-(N-morpholino)ethanesulphonic acid (MES) (pH 6.0)–20% glycerol. The eluate was then adsorbed in the batch on CM-Sephadex C-50 (50 ml in a beaker) equilibrated with the same buffer. After 30 min of adsorption the supernatant containing the protease activity was centrifuged at 39 000 *g* at 2°C for 20 min to remove remaining CM-Sephadex particles, desalted on PD 10 columns to 0.3 *M* NaCl–10 m*M* $CaCl_2$–50 m*M* Tris–HCl (pH 7.0)–20% glycerol, filtered with a 0.45-μm Acrodisc sterile filter and applied to a Mono Q HR 5/5 column (50 mm × 5 mm I.D.) equilibrated in 0.3 *M* NaCl–50 m*M* Tris–HCl (pH 7.0)–20% glycerol. All buffers used for FPLC were prepared with triply distilled water, filtered with a 0.22-μm Sartorius filter and then autoclaved. The protease activity was eluted with 28 ml of an increasing gradient of NaCl [0.3 to 1.5 *M* in 50 m*M* Tris–HCl (pH 7.0)–20% glycerol]. Protease-containing fractions were pooled, dialysed against 1.3 *M* Na_2SO_4–50 m*M* Tris–HCl (pH 7.0) and loaded onto a phenyl-Superose HR 5/5 column (50 mm ×

5 mm I.D.) that had been equilibrated in the same buffer. Protease activity was eluted with 18 ml of a decreasing linear gradient of Na_2SO_4 [1.3 *M* Na_2SO_4 to 10% ethylene glycol in 50 m*M* Tris–HCl (pH 7.0)]. After the final purification step phenylmethylsulphonyl fluoride (PMSF) (0.5 *M* in methanol) was added (final concentration 5 m*M*) to the purified enzyme to prevent autoproteolysis. The protease was precipitated by adding four volumes of saturated ammonium sulphate solution (69.7 g in 100 ml of triply distilled water). After 90 min on ice, the precipitate was centrifuged at 24 000 *g* at 4°C for 30 min. The samples were further analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

Protease assay

Enzyme activity was determined by a modification of the Azocoll method described by Tsuboi *et al.* [10]. To 5 mg of Azocoll in 3 *M* NaCl–10 m*M* $CaCl_2$–20 m*M* $MgCl_2$–50 m*M* Tris–HCl (pH 7.0) were added 5–50 μl of enzyme solution. The samples were incubated at 56°C for 2 h and, after centrifugation at 12 000 *g* for 10 min, the released dye was measured at 520 nm with a Zeiss Model DM 4 spectrophotometer. One arbitrary unit (AU) of protease activity was defined as the amount of enzyme which causes an increase in absorbance of 0.1 under these conditions.

Batch adsorption

Adsorption of ESP4 to arginine-Sepharose 4B and benzamidine-Sepharose 6B was performed utilizing batch adsorption. A 100-μl volume of the chromatographic material was added to a series of Eppendorf tubes and equilibrated by washing three times with 1 ml of the following buffers: 3 *M* NaCl–10 m*M* $CaCl_2$–50 m*M* Tris–HCl (pH 6.0, 7.0, 8.0) and 0.3 *M* NaCl–10 m*M* $CaCl_2$–50 m*M* Tris–HCl (pH 6.0, 7.0, 8.0). After centrifugation, each tube was filled with the equilibration buffer to 200 μl and 50 μl of concentrated supernatant were then added. The gel was mixed for 10 min at room temperature and centrifuged at 12 000 *g* for 10 min. The protease activity in the supernatant was determined with Azocoll as previously described.

Chromatography on Q-Sepharose

The Q-Sepharose 16/10 HiLoad column (200 mm × 16 mm I.D.) was equilibrated with five bed volumes (100 ml) of 0.3 *M* NaCl–10 m*M* $CaCl_2$–50 m*M* Tris–HCl (pH 7.0)–20% glycerol (buffer A). Acetone precipitate was dissolved in the above buffer and 5 ml were applied to the column, after which the column was further washed with 20 ml of buffer A. The protease was eluted with 280 ml of an increasing gradient of NaCl from 0.3 to 1.5 *M* in 50 m*M* Tris–HCl (pH 7.0)–10 m*M* $CaCl_2$–20% glycerol at a flow-rate of 2.5 ml/min.

Test-tube method for selecting starting pH

The starting pH for anion-exchange chromatography was selected according to the method described by Pharmacia–LKB [11]. A 1-ml volume of enzyme solution in the appropriate buffer was added to each tube. After 30 min 100 μl of the supernatant were tested for enzyme activity. Desorption was carried out in 3 *M* NaCl–10 m*M* $CaCl_2$–50 m*M* Tris–HCl (pH 7.2). Volumes of 100 μl of the eluted samples were then tested for protease activity.

Protein assay

Protein concentration was determined by a modification of Bradford's method [12]. A 100-μl volume of 0.06% Coomassie Brilliant Blue G250 in 1.9% perchloric acid was mixed with 80 μl of distilled water and 20 μl of sample. After 2 min the absorbance was measured with an enzyme-linked immunoadsorbent assay (ELISA) reader (transmission wavelength 630 nm; reference wavelength 405 nm). Bovine serum albumin was used as a standard in the range 0–20 μg.

Gel electrophoresis

Proteins were analysed by discontinuous SDS-PAGE as described by Laemmli [13]. Silver staining of the gels was carried out according to the method of Blum *et al.* [14].

RESULTS AND DISCUSSION

As the extracellular protease of *Halobacterium* sp. strain TuA4 is a serine protease (see Table I), first attempts to purify the enzyme were made with affinity chromatography. Arginine-Sepharose 4B was shown to bind enzymes which contain serine at their active sites and which cleave proteins at the carboxyl end of arginine residues such as prekallikrein from bovine plasma [15]. Benzamidine-Sepharose 6B is another useful adsorbent specific for serine proteases such as trypsin and trypsin-related enzymes [16]. Both materials were tested with different ionic strength and pH conditions for binding of ESP4, but insufficient adsorption was obtained under the conditions tested, suggesting that the halophilic protease is not arginine/lysine-specific.

Fortunately, ESP4 is active and relatively stable in lower ionic strength buffers (0.3 *M* Na^+) if 20% gycerol is added as a stabilizing agent. As most of the halophilic proteins have a high content of acidic amino acids, anion-exchange chromatography seemed to be suitable for the purification of ESP4. The starting pH for anion-exchange chromatography was selected using the test-tube method as described by Pharmacia–LKB [11]. Adsorption on DEAE-Sephadex occurred within the pH range 5.0–7.5, suggesting that the isoelectric point of ESP4 is below 5.0 and the desorption was optimum at pH 7.0 (see Table II); thus anion-exchange chromatography was carried out in 50 m*M* Tris–HCl (pH 7.0) with the addition of 0.3 *M* NaCl and 20% glycerol. Binding of proteins to an anion-exchange material in the presence of 0.3 *M* NaCl has not been described previously, as most proteins elute under such conditions with the exception of highly acidic halobacterial proteins, which still bind to anion-exchangers in the presence of 0.3 *M* NaCl.

TABLE I

INHIBITION OF ESP4 WITH VARIOUS SERINE PROTEASE INHIBITORS

Inhibitor	Concentration (m*M*)	Inhibition (%)
PMSF	5	95
Diisopropyl fluorophosphate	5	100
Leupeptin	20	82

TABLE II

TEST-TUBE METHOD FOR SELECTING THE STARTING pH FOR ANION-EXCHANGE CHROMATOGRAPHY

pH	Enzyme activity after adsorption on DEAE-Sephadex (AU)	Enzyme activity without DEAE-Sephadex (AU)	Enzyme activity after desorption from DEAE-Sephadex (AU)
5.0	0.17	2.80	0.33
5.5	0.27	2.80	0.52
6.0	0.34	2.80	1.00
6.5	0.39	2.80	2.00
7.0	0.42	2.80	2.70
7.5	0.47	2.80	1.90

Furthermore, sodium chloride in the buffer could be replaced with sodium sulphate with little loss of activity, so that hydrophobic interaction chromatography could be applied for the purification of ESP4. The prepacked alkyl-Superose HR 5/5 FPLC column (50 mm × 5 mm I.D.) was tested first; ESP4 did not bind to the column in 1.3 *M* Na_2SO_4–50 m*M* Tris–HCl (pH 7.0), suggesting that the enzyme is not strongly hydrophobic. However, under the same conditions ESP4 was adsorbed on the more hydrophobic agent phenyl-Superose HR 5/5 (50 mm × 5 mm I.D.) column.

Based on these findings, a purification procedure could be developed that would maintain the protease in a minimum sodium ion concentration of 0.3 *M*. This is essential, because below 0.3 *M* Na^+ the protease activity is irreversibly lost. The crude concentrated supernatant is very complex and contains many proteins and peptones from the medium and nucleic acids and membrane compounds from "leaky" cells which lyse during the preparation. The nucleic acids and membrane compounds which can interfere with optimum separation on high-resolution FPLC columns were precipitated with 10% PEG 6000 in 3 *M* NaCl–10 m*M* $CaCl_2$–20 m*M* $MgCl_2$–50 m*M* Tris–HCl (pH 9.0). During this step the total enzyme activity increases about 1.5 fold, indicating that a more or less specific inhibitor is separated from the protease by PEG precipitation. About 48% of the protease activity could be precipitated by acetone precipitation (2:1, v/v) whereas other soluble proteins, especially smaller peptides, remained in the acetone supernatant. This step led to a 1.5-fold purification with a 48% total recovery. The loss of activity is high because the protease is exposed to low ionic strength in this step, but acetone precipitation is necessary at this point to remove the protease from PEG and peptone contamination.

The third step in the purification was the binding of contaminating proteins to CM-Sephadex C-50 in 0.3 *M* NaCl–10 m*M* $CaCl_2$–50 m*M* MES (pH 6.0)–20% glycerol. ESP4 has a net negative charge at pH 6.0 and did not adsorb on the column. Cation-exchange chromatography resulted in a 2.6-fold purification with a 28% total recovery.

The most effective purification steps were the anion-exchange chromatography on Mono Q HR 5/5 (Fig. 1) and hydrophobic interaction chromatography on phenyl-Superose HR 5/5 (Fig. 2) using an automated FPLC system from Pharmacia–

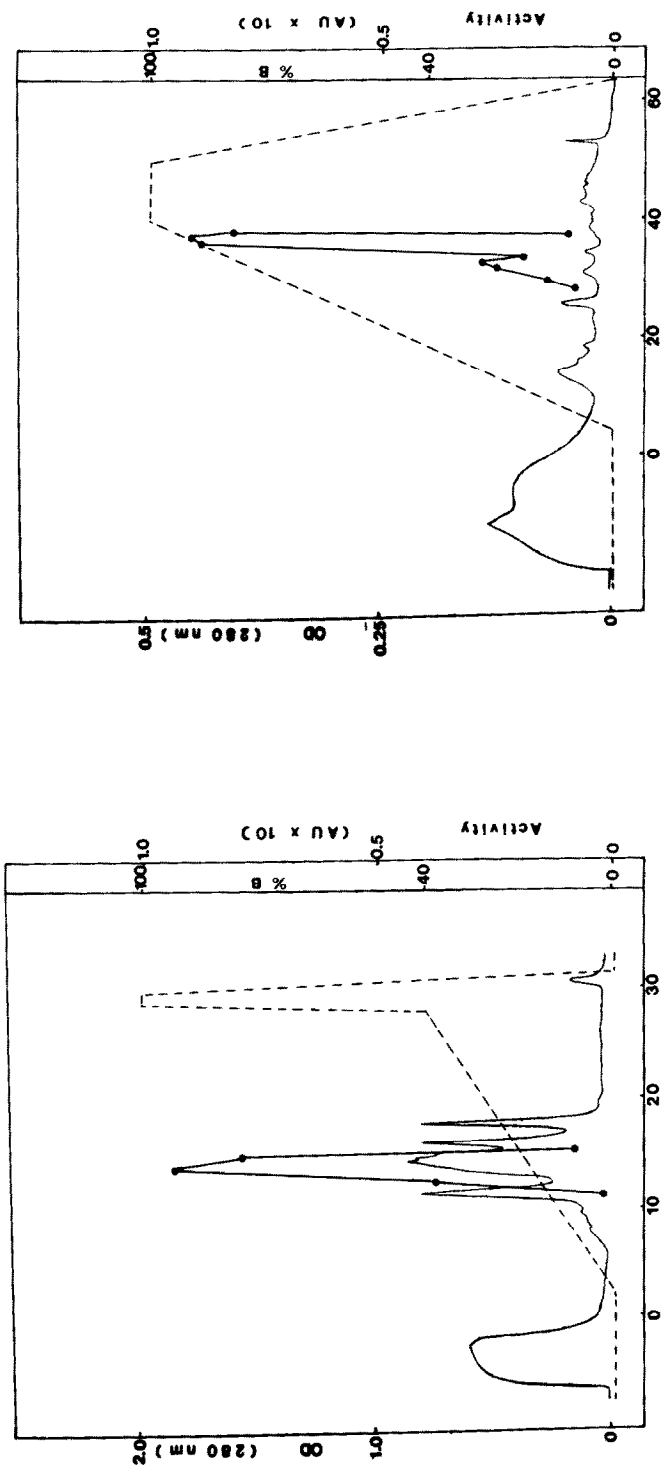

Fig. 1. Anion-exchange chromatography on Mono Q. 4 ml of enzyme solution were loaded onto the column and eluted in 1-ml fractions with an increasing sodium chloride gradient (– – –) at a flow-rate of 1 ml/min. A 50-μl volume of each fraction was assayed for protease activity (●—●). Protein absorbance was measured at 280 nm (———).

Fig. 2. Hydrophobic interaction chromatography on phenyl-Superose. 3.5 ml of the pooled activity from Mono Q was applied to the column. ESP4 was eluted in 0.4-ml fractions with an increasing ethylene glycol gradient (– – –; 0–10%) at a flow-rate of 0.4 ml/min. A 50-μl volume of each fraction was assayed for protease activity (●—●). Protein absorbance was measured at 280 nm (———).

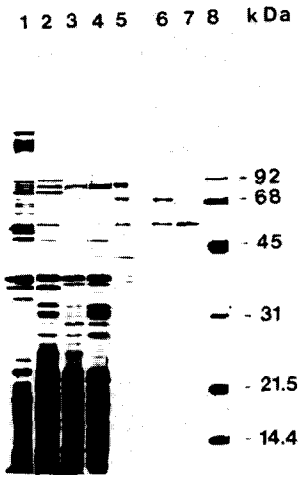

Fig. 3. Purification of ESP4 as monitored by SDS-PAGE. Proteins were removed at various steps of the purification procedure and loaded onto a 12% SDS polyacrylamide gel. Lanes: 1 = crude supernatant (2.5 μg); 2 = PEG supernatant (2.5 μg); 3 = acetone pellet (4.0 μg); 4 = CM-Sephadex eluate(3.0 μg); 5 = activity pool from Mono Q (1.0 μg); 6 = activity peak 1 from phenyl-Superose (1.0 μg); 7 = activity peak 2 from phenyl-Superose (0.5 μg); 8 = molecular weight markers (kDa = kilodalton).

LKB. These techniques resulted in a 177-fold purification with a 24% total recovery for Mono Q (only a 4% loss of activity in relation to the previous step) and a 216-fold purification with a 3% total recovery for phenyl-Superose. Such a degree of purification has never been achieved previously for halophilic enzymes purified using other procedures [9,17]. Especially the chromatography on Mono Q resulted in a 70-fold purification as this was the step where most of the contaminating non-halophilic proteins originating from the medium were separated from the protease. After chromatography on phenyl-Superose two protease activities were present, as shown in Fig. 2. The first peak is not pure, as shown in a 12% SDS polyacrylamide gel (Fig. 3), whereas from peak 2 only one protein band with a molecular weight of 60 000 dalton was detected in the gel. This value correlates very well with the native

TABLE III

PURIFICATION OF ESP4 FROM *HALOBACTERIUM* SP. STRAIN TuA4

Step	Volume (ml)	Total activity (AU)	Amount of protein (mg)	Specific activity (AU/mg)	Yield (%)	Purification factor
(1) Concentrated supernatant	30	4752	150	31.7	100	1
(2) PEG supernatant	36	7092	144	49.2	150	1.5
(3) Acetone pellet	18	2286	49.5	46.2	48	1.5
(4) CM-Sephadex	25	1320	16.25	81.2	28	2.6
(5) Mono Q	20	1120	0.2	5600	24	177
(6) Phenyl-Superose	6	144	0.02	6858	3	216

TABLE IV

COMPARISON OF ESP4 WITH THE EXTRACELLULAR SERINE PROTEASE OF *H. HALOBIUM* WITH REGARD TO THE SODIUM CHLORIDE CONCENTRATION

NaCl (*M*)	Enzyme activity (%)	
	H. halobium protease	ESP4
0	n.d.	0
0.3–20% glycerol	n.d.	70
0.5–20% glycerol	n.d.	86
0.5	n.d.	70
1	0	100
2	20	100
3	34	100
4.3	100	100

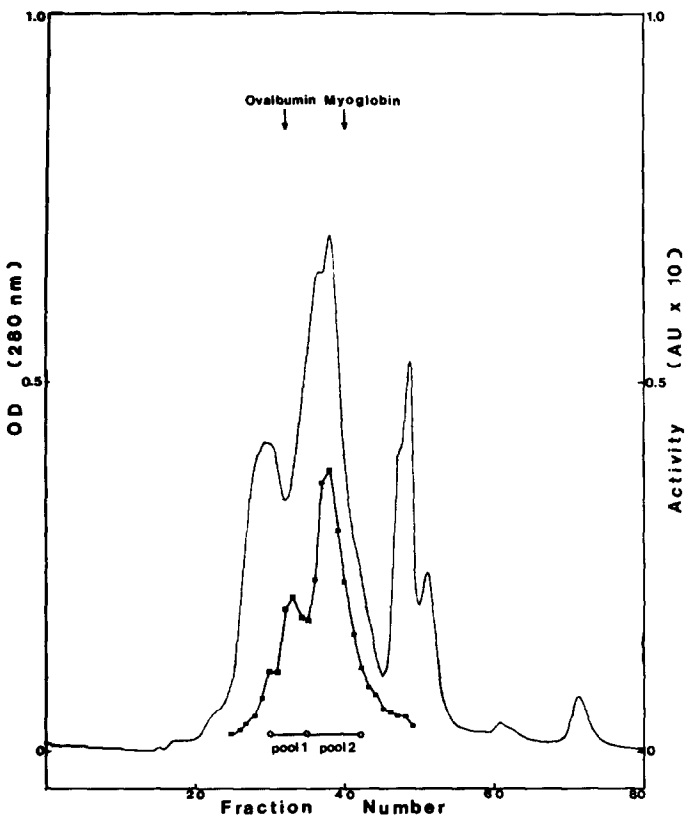

Fig. 4. Gel filtration of 100 μl of the resuspended acetone precipitate on a Superose 12 column. Fractions of 0.8 ml were collected and assayed for protease activity (■—■). The chromatography was carried out in 1 *M* NaCl–10 m*M* $CaCl_2$–50 m*M* Tris–HCl (pH 7.2) at a flow-rate of 0.4 ml/min. Protein absorbance was measured at 280 nm (———).

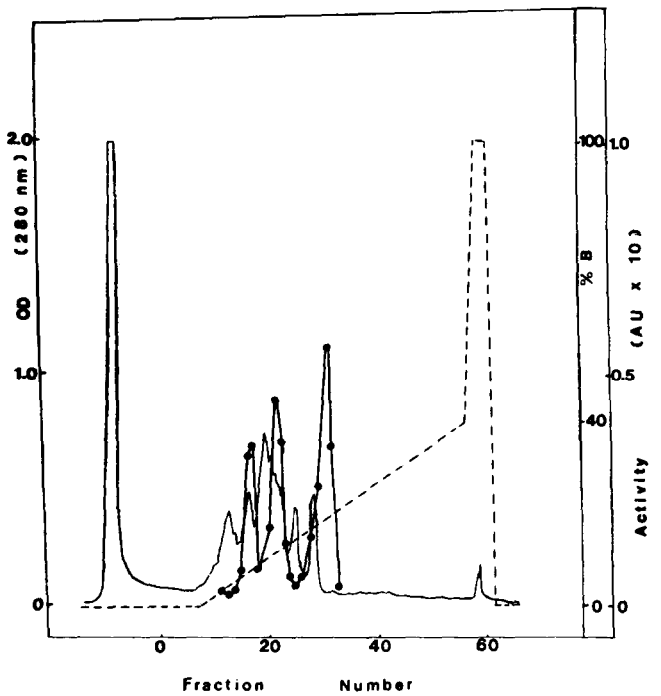

Fig. 5. Anion-exchange chromatography on Q-Sepharose. 5 ml of enzyme solution were applied to the column and eluted in 5-ml fractions with an increasing sodium chloride gradient (– – –) at a flow-rate of 2.5 ml/min. A 50-μl volume of each fraction was assayed for protease activity (●—●). Protein absorbance was measured at 280 nm (———).

molecular weight of ESP4 determined by glycerol gradient centrifugation and with the molecular weight determined by SDS-PAGE following active site labelling with [^{14}C]diisopropyl fluorophosphate. The results of the purification are summarized in Table III and Fig. 3.

ESP4 was shown to be completely different from the extracellular protease of *H. halobium* (see Table 1V), especially with regard to monovalent ionic concentration. Whereas the *H. halobium* protease is fully and irreversibly inactivated at concentrations below 2 *M* NaCl [9], ESP4 is active at concentrations up to 0.3 *M* NaCl. Also, the molecular weights of the two proteases are different: ESP4 exhibits a molecular weight of 60 000 dalton in SDS gel systems, whereas that of the protease isolated from *H. halobium* is 56 000 dalton, so ESP4 is obviously larger.

In some experiments, gel filtration on a Superose 12 HR 10/30 column (300 mm × 10 mm I.D.) in 1 *M* NaCl–10 m*M* $CaCl_2$–20 m*M* $MgCl_2$–50 m*M* Tris–HCl (pH 9.0) was used as a third step after acetone precipitation (Fig. 4). This step was not very effective because only small amounts of protein could be applied to the gel filtration column and the separation was not satisfactory. In order to purify large amounts of protein in a short time, the anion-exchange column Q-Sepharose HiLoad was tested (Fig. 5). We found that the protease activity separated into three different peaks which were very unstable (after 24 h at −20°C the activity was irreversibly lost)

so that further purification of the protease activities eluted from this column was not possible.

ACKNOWLEDGEMENTS

We thank L. Phillips and B. Pillay for helpful discussions and M. Wuenscher and L. Phillips for critical reading of the manuscript. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Rd 1/4-5).

REFERENCES

1 C. R. Woese, *Zentralbl. Bakteriol. Hyg., Abt. 1: Orig., Reihe C*, 3 (1982) 1.
2 P. Norberg and B. v. Hofsten, *J. Gen. Microbiol.*, 55 (1969) 251.
3 D. Oesterhelt and W. Stoeckenius, *Methods Enzymol.*, 31A (1974) 667.
4 B. Surek, B. Pillay, U. Rdest, K. Beyreuther and W. Goebel, *J. Bacteriol.*, 70 (1988) 1746.
5 M. F. Mescher and J. L. Strominger, *J. Biol. Chem.*, 251 (1976) 2005.
6 M. Shoham, J. Dijk, R. Reinhardt and B. Wittmann-Liebold, *FEBS Lett.*, 204 (1986) 323.
7 W. Leicht and W. Pundak, *Anal. Biochem.*, 114 (1981) 186.
8 L. I. Hochstein and B. P. Dalton, *Biochim. Biophys. Acta*, 302 (1973) 216.
9 L. S. Izotova, A. Y. Strongin, L. N. Chekulaeva, V. E. Sterkin, V. I. Ostoslavskaya, L. A. Lyublinskaya, E. A. Timokhina and V. M. Stepanov, *J. Bacteriol.*, 155 (1983) 826.
10 R. Tsuboi, T. Sanada, K. Takamori and H. Ogawa, *J. Bacteriol.*, 169 (1987) 4104.
11 *Ion-Exchange Chromatography: Principles and Methods*, Pharmacia-LKB, Uppsala, 1979.
12 M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
13 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
14 H. Blum, H. Beier and H. Gross, *Electrophoresis*, 8 (1987) 93.
15 T. Suzuki and H. Takahashi, *Methods Enzymol.*, 34 (1974) 432.
16 H. F. Hixson and A. Nishikawa, *Arch. Biochem. Biophys.*, 154 (1973) 501.
17 B. P. May and P. P. Dennis, *J. Bacteriol.*, 169 (1987) 1417.