ELSEVIER

Journal of Chromatography B, 737 (2000) 143–150

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Three-step chromatographic purification procedure for the production of a His-tag recombinant kinesin overexpressed in *E. coli*

S. Gibert[a], N. Bakalara[a], X. Santarelli[b,*]

[a]*Laboratoire de Biologie Moléculaire et Immunologie des Protozoaires Parasites, Université Victor Ségalen Bordeaux 2, 146 Rue Léo Saignat, 33076 Bordeaux Cédex, France*

[b]*Ecole Supérieur de Technologie des Biomolécules de Bordeaux (ESTBB), Université Victor Segalen Bordeaux 2, 146 Rue Léo Saignat, 33076 Bordeaux Cédex, France*

Abstract

A kinesin gene has been cloned by RT-PCR (reverse transcription polymerase chain reaction) from *Trypanosoma brucei* and the corresponding protein overexpressed as a recombinant His-tag (histidine-tag) kinesin in *E. coli* in order to study its biochemical properties and to determine its three-dimensional structure by X-ray crystallography. Starting from several liters of culture, an ultrasonic homogenizer was used for cell disruption and an unclarified feedstock was obtained. From this homogenate, a protein was then purified by immobilized metal affinity chromatography (IMAC) using expanded bed adsorption (EBA) technology (Streamline chelating). For this capture step, 100% of the recombinant protein was purified with more than 90% of purity. This step was followed by ion-exchange chromatography (Q Sepharose Fast Flow) for intermediate purification (96% purity, 53% recovery) and by size-exclusion chromatography with Superdex 75 as a polishing step (99% purity, 93% recovery). We then separated two forms of kinesin, a dimer (70%) and a monomer (30%). It was then possible to purify His-tag recombinant protein directly from feedstock in a rapid and efficient way and to isolate two forms of kinesin.

Keywords: Expanded bed adsorption; Purification; Kinesin; Proteins

1. Introduction

Flagellar movement, vesicle transport, organelle placement, subcellular protein sorting, mitosis and meiosis are dependent on microtubule movements [1].

Three classes of microtubule-associated motor proteins have been characterized to power these movements. They include dynein, kinesin and dynamin [2]. The kinesin family is characterized by a motor domain of 350 amino acids located either at the N- or C-terminal part of the protein [3]. This motor domain hydrolysis ATP generates the movement force [4]. At present, more than 80 members of the kinesin superfamily have been sequenced and reported.

We recently purified and cloned a kinesin from the parasitic protozoa *Trypanosoma brucei*.

To study the biochemical properties of the kinesin protein and its three-dimensional structure, we overexpressed the corresponding motor domain in *E. coli*

*Corresponding author. Tel.: +33-05-5757-1299; fax: +33-05-5757-1044.

E-mail address: xavier.santarelli@estbb.u-bordeaux2.fr (X. Santarelli)

0378-4347/00/$ – see front matter
PII: S0378-4347(99)00524-1

as a His-tag protein. In this paper we propose a three-step purification process of the recombinant His-tag protein, and show its ATPase activity. A capture step using immobilized metal affinity chromatography (IMAC) [5–9] was performed. Expanded bed technology was preferred as the capture step because it was an alternative to traditional clarification (centrifugation, tangential micro and ultrafiltration and the first chromatography step) [10–12] and allowed us to process large volume of crude feedstock directly after ultrasonic homogenization with high throughput. The low processing time at the capture step just after fermentation was essential because the fast adsorption of the target molecule early in the process, prevents its degradation. This capture step was followed by anion-exchange chromatography as an intermediate purification step and size-exclusion chromatography as a polishing step.

The objective of this process is to obtain a highly purified protein for further crystallography and to separate the monomer and dimer molecules.

2. Experimental

2.1. Instruments

The chromatographic systems used throughout this study were the fast protein liquid chromatography (FPLC) system and Biopilot workstation from Amersham Pharmacia Biotech (Saclay, France). The data were collected and evaluated using the FPLC director and Unicorn Data system.

The electrophoresis apparatus used was the Mini-Protean II from Bio-Rad (Ivry-sur-Seine, France).

For recovery studies, we used a Uvikon 930 spectrophotometer (Kontron, Montigny Lebretonneux, France) to measure absorbance at 595 nm.

2.2. Chemicals

PET (plasmid expression tag) 23a and *E. coli* BL21 (bacteria lysogenic for bacteriophage DE3) were from Novagen (Madison, WI, USA). pBlue Script was purchased from Stratagene (La Jolla, CA, USA). Anti-mouse IgG1 conjugated to horse radish peroxidase was from Sanofi-Pasteur (Marnes la Coquettes, France).

Streamline chelating, Q Sepharose Fast Flow, Superdex 75 prep grade gel and XK16/20, XK16/70, Streamline 25 columns were from Amersham Pharmacia Biotech.

Anti-polyhistidine antibody and all salts were from Sigma (L'Isle d'Abeau Chesnes, France), and the buffers were filtered through a 0.22-μm membrane filter.

Immobilon P was from Millipore (Saint-Quentin-en-Yvelines, France).

2.3. Preparation of the cellular extract

2.3.1. Cloning and expression

A 1-kb fragment comprising the motor domain was generated by polymerase chain reaction (PCR). A 5.5 kb Banm H1 (*Bacillus amyloliquefaciens H*)/Eco (*Escherichia coli*) R1 genomic fragment from *T. brucei* ANTat 1 was subcloned in pBlue Script and used as a template in the PCR reaction. The 5′ primer contained a 12 nucleotide linker with a Nhe 1 (*Neisseria mucosa heidelbergensis*) restriction site to facilitate subcloning, codons for six histidine tag residues to allow binding to immobilized metal affinity chromatography column and codons for the N-terminal residues. The 3′ primer contained Bam H1 site, a stop codon and six codons for the C-terminal extremity. The PCR product was inserted into the Nhe 1/Bam H1 linearized pEt 23a plasmid to create the kinesin His-tag construction. The resulting recombinant kinesin His-tag protein was expressed in *E. coli* BL21(DE3) according to the manufacturer's instructions.

2.3.2. Cellular culture

E. coli BL21 transfected with the plasmid pet-23a was cultured in M9 medium (6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.5 g/l NaCl, 2 g/l glucose, 0.0005 g/l vitamin B_1, 1 g/l casamino acids) with ampicillin (100 μg/ml) and with an inoculate of cell culture ($A_{600\ nm}=0.4$) containing the fusion plasmid, so that the volume of the culture medium was 200-fold bigger than the inoculated volume.

The culture was grown at 37°C to $2\cdot10^8$ cells/ml ($A_{600\ nm}=0.7$). Then IPTG (isopropyl β-D-thiogalactopyranoside) was added to a final concentration of 0.5 m*M* and the cells were incubated at 37°C for 2 h.

After induction, cells were placed in an ice-water

bath and sonicated with four 1-min pulses with 2 min in ice between each pulse.

2.4. Chromatographic procedures

2.4.1. Expanded bed adsorption and immobilized metal affinity chromatography (EBA–IMAC): capture

An IMAC system using EBA technology (Streamline chelating) was performed with the Streamline 25 column containing 93 ml of Streamline chelating corresponding to a sedimented bed height of 19 cm. The Streamline 25 column was linked to a Biopilot workstation. The evaluation of bed stability was performed by visual inspection, by measuring the degree of expansion and by the number of theoretical plates according to the manufacturer's instructions.

The bed expansion/equilibration, feed application and washing were performed at an upward flow velocity of 390 cm/h. The buffer used during expansion/equilibration was 5 m*M* imidazol, 0.5 *M* NaCl, 20 m*M* Tris–HCl, pH 8. The crude and unclarified feedstock was applied directly onto the expanded bed followed by washing with the equilibration buffer until UV baseline was reached. Then the pump was turned off and the bed sedimented. Next, the adaptor was moved down towards the sedimented bed surface. After a run of two volumes (sedimented gel) of equilibration buffer, the second wash was performed with 60 m*M* imidazol, 0.5 *M* NaCl, 20 m*M* Tris–HCl, pH 8 at a flow velocity of 180 cm/h using a downward flow in the sedimented bed mode. The elution buffer, 1 *M* imidazol, 0.5 *M* NaCl, 20 m*M* Tris–HCl, pH 8, was run at 180 cm/h.

2.4.2. Intermediate purification

Q Sepharose Fast Flow was packed in an XK26/20 column (2.5 ml). A slurry was prepared with equilibration buffer in a ratio of 75% settled gel to 25% buffer and was degassed.

The column was filled with a few centimeters of equilibration buffer and then closed. The gel slurry was poured into the column in one continuous motion. The remainder of the column was filled up with buffer and the column top piece mounted and connected to a pump. The bottom outlet of the column was opened and the pump set at 133% of the flow-rate to be used during chromatography (300 cm/h). The packing flow-rate was maintained for three bed volumes after a constant bed height was reached.

For chromatography, the column was equilibrated at 10 ml/min (300 cm/h) with three column volumes of 50 m*M* Tris–HCl, pH 8 buffer.

The eluate of the EBA–IMAC was diluted and injected onto the column. The column was washed with four column volumes of equilibration buffer and the protein was eluted with a step salt gradient at 0.3 *M* NaCl with 1 *M* NaCl, 50 m*M* Tris–HCl, pH 8 buffer. The eluate was concentrated for size-exclusion chromatography.

2.4.3. Size-exclusion chromatography: polishing

Preparing the gel: the Superdex 75 prep grade gel was washed with 10 column volumes of distilled water on a glass filter and suspended with 325 ml of distilled water.

Packing the gel: packing preparation and operations were performed according to manufacturer's instructions. The gel (120 ml) was packed in an XK 16/70 column. The quality of the column packing was checked with an efficiency test.

Chromatography: the column was equilibrated with 0.5 *M* NaCl, 50 m*M* Tris–HCl, pH 8 and the chromatography run at 1 ml/min (30 cm/h) on a FPLC workstation. The fractions were then collected.

2.5. Analytical procedures

2.5.1. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [13] using a Mini-Protean II apparatus and a Tris–glycine buffer system were used to monitor the purification during the chromatographic steps.

Electrophoresis was performed for 45 min at 200 V using 12% polyacrylamide gels. Detection was done by Coomassie brilliant blue R250.

2.5.2. Western blotting

Purified proteins in sample buffer (0.1% SDS, 10% glycerol, 50 m*M* dithiothreitol, 90 m*M* Tris–HCl, pH 6.8) were boiled for 5 min and subjected to 10% polyacrylamide gel electrophoresis. Proteins were transferred to polyvinyllidone difluoride mem-

brane (Immobilon P) by semi-dry blotting [14]. Filters were blocked for 15 min with PBS (phosphate-buffered saline)–Tween–milk (137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4, 0.005% Tween 20, 5% milk), and incubated overnight at 4°C with a solution of a monoclonal antibody against the poly-His-tag in PBS–Tween–milk, then incubated for 2 h with either a 1/100 goat anti mouse IgG1 conjugated to horseradish peroxidase in PBS–Tween–milk. Immunoreactive bands were revealed by washing in 20 m*M* NaCl, 50 m*M* Tris–HCl, pH 7.5 and a solution containing 0.05% H_2O_2 and 2.8 m*M* 4-chloro-1-naphthol for the peroxidase conjugate.

The mid-range molecular mass marker from Novagen has been used for size estimation.

2.5.3. Protein concentration

The protein concentration was estimated by Coomassie blue [15] using bovine serum albumin as standard.

2.5.4. ATPase activity

Steady-state ATPase activity was measured according to the amount of ^{32}P released from 1 m*M* [γ-^{32}P] ATP ($0.5 \cdot 10^8$ cpm/μmol) [16]. Kinesin (0 to 10 μg) was incubated at 22°C for 15 min in 80 ml of PEM buffer (1 m*M* $MgCl_2$, 1 m*M* EGTA, 0.1 *M* Pipes, pH 6.9) containing 2 m*M* dithiothreitol, 10 m*M* taxol and 1 m*M* GTP. After incubation the released phosphate was quantified.

3. Results and discussion

3.1. Immobilized metal affinity chromatography–expanded bed adsorption

After optimization of the IMAC system with chelating Sepharose Fast Flow (2 ml of gel), we run the protocol with 2 ml of Streamline chelating in packed bed (2 ml). Similar results were obtained, so

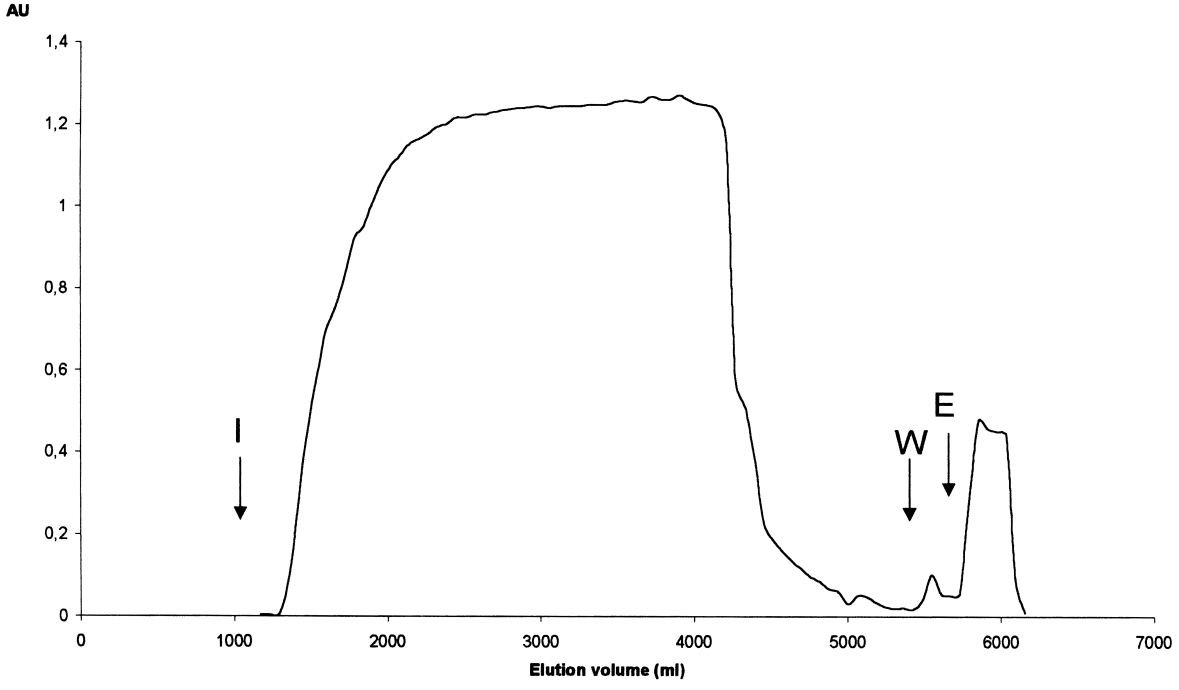

Fig. 1. Capture of recombinant kinesin with chelating Streamline. Column: Streamline 25 (93 ml of gel). Sample: Unclarified and ultrasonic homogenate of cell culture: 2000 ml (I); buffer A: 5 m*M* imidazol, 0.5 *M* NaCl, 20 m*M* Tris–HCl, pH 8; buffer B: 60 m*M* imidazol, 0.5 *M* NaCl, 20 m*M* Tris–HCl, pH 8 (W); buffer C: 1 *M* imidazol, 0.5 *M* NaCl, 20 m*M* Tris–HCl, pH 8 (E). Detection at 280 nm; flow-rate: 390 cm/h in expansion/equilibration and feed application and 180 cm/h in sedimented bed mode for washing and elution.

we performed an easy scale-up with a Streamline 25 column.

Expansion, equilibration and washing were performed using a flow velocity of 390 cm/h, which resulted in a degree of expansion of around 3.2. The application of the unclarified feedstock was performed at the same flow-rate with a lower degree of expansion (2.5). This can be explained by the increased density of the beads due to fouling.

After the injection of all the crude feedstock (I in Fig. 1) and washing with the equilibration buffer, two volumes of equilibration buffer were passed through the column(sedimented gel) and a second wash (W in Fig. 1) was performed with 60 m*M* imidazol to eliminate the contaminants. Desorption of the enzyme from the adsorbent was performed with 1 *M* imidazol (E in Fig. 1).

Most of the contaminant was eliminated at this step (Fig. 2A). The Western blot (Fig. 2B) indicates that the purified protein really corresponded to the His-tag kinesin protein, since the anti-poly-His antibody recognised the purified protein at a relative molecular mass of 38 000. ATP hydrolysis was shown by incubating ^{32}P[ATP] with different amounts of purified His-tag protein. Fig. 3 shows a dose-dependent hydrolysis of ATP, thus indicating the presence of an intrinsic ATPase activity.

3.2. Intermediate purification

After equilibration of the Q Sepharose Fast Flow column, the eluate of the EBA–IMAC was diluted (1/10) and 300 ml were injected (Fig. 4). The column was washed with equilibration buffer and the protein was eluted with step gradient at 0.3 *M* NaCl. At this step, the imidazol and the remaining contaminants were eliminated. The eluate (10 ml) was concentrated to 1 ml.

The low level of recovery of the C1 His-tag

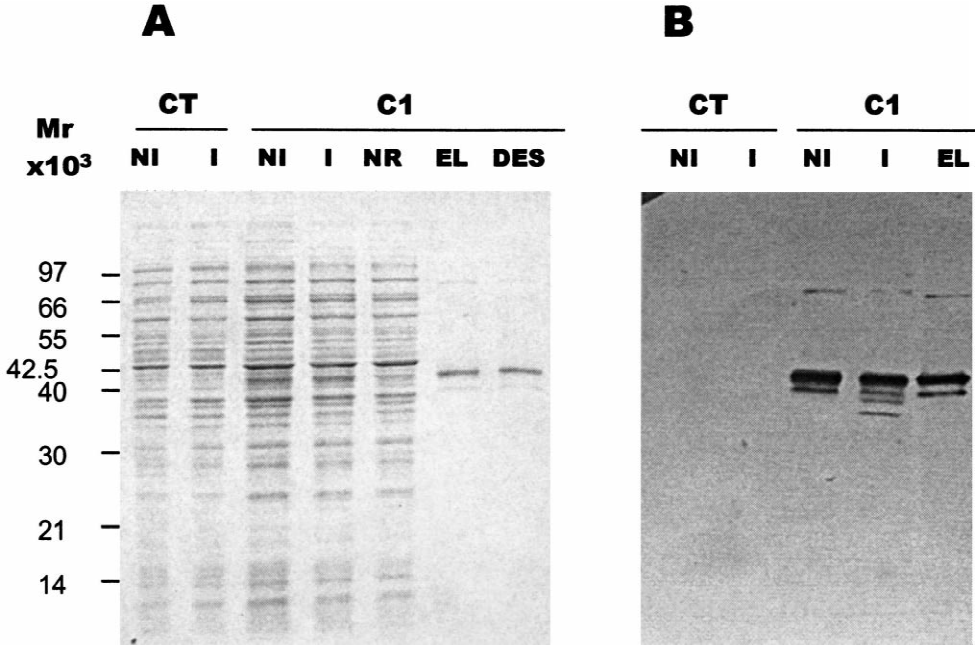

Fig. 2. (A) C1 kinesin expression and purification analysis: 1 μg of soluble protein was loaded on a 12% SDS–PAGE system and Coomassie-stained. Ct: Control expression of a non-soluble His-tag peroxidase, C1: His-tag kinesin, I: IPTG induced culture, NI: non-induced culture, NR: not retained by IMAC, EL: eluate of IMAC, DES: EL after desalting. (B) Western blot analysis of the recombinant His-tag protein. C1 kinesin protein was expressed in *E. coli* BL21 and purified from a EBA–IMAC. One μg of protein was loaded on a 12% SDS–PAGE system and transferred to an Immobilon P membrane. A monoclonal antibody, anti-polyhistidine, was used as first antibody. Revelation was performed using a goat antimouse peroxidase conjugated antibody.

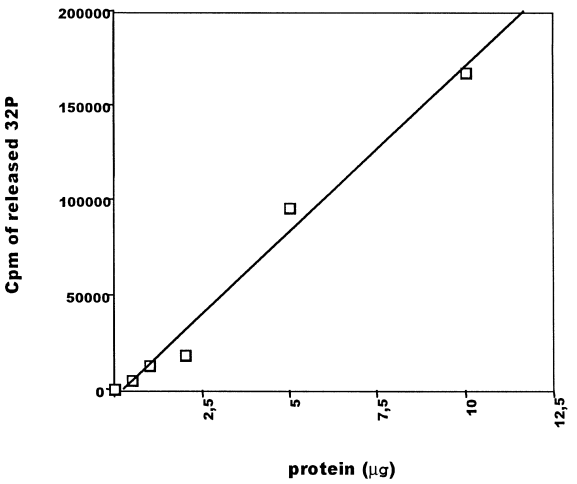

Fig. 3. Steady-state ATPase activity was measured by the amount of ^{32}P released from 1 m*M* [γ-^{32}P] ATP ($0.5 \cdot 10^8$ cpm/μmol). Kinesin (0 to 10 μg) was incubated at 22°C for 15 min in 80 ml of PEM buffer (1 m*M* $MgCl_2$, 1 m*M* EGTA, 0.1 *M* Pipes, pH 6.9) containing 2 m*M* dithiothreitol, 10 m*M* taxol and 1 m*M* GTP. After incubation the released phosphate was quantified [16].

protein after this step was due to a non-specific adsorption on the matrix. Complete elution was obtained only in drastic conditions (data not shown).

3.3. Size-exclusion chromatography: polishing

The concentrated prepared above was injected and the chromatography was performed at 1 ml/min (30 cm/h) and the fractions collected (Fig. 5). Two peaks may be seen in Fig. 5. The first (87%) corresponds to the dead volume of the column and the second (13%) to a relative molecular mass of 40 000. When we observed the electrophoretic gel (Fig. 6), we noticed that the two peaks revealed in SDS–PAGE a protein of relative molecular mass 38 000. Therefore, the first peak corresponded to the homodimer of kinesin and the second peak to the monomer. The results are summarized in Table 1.

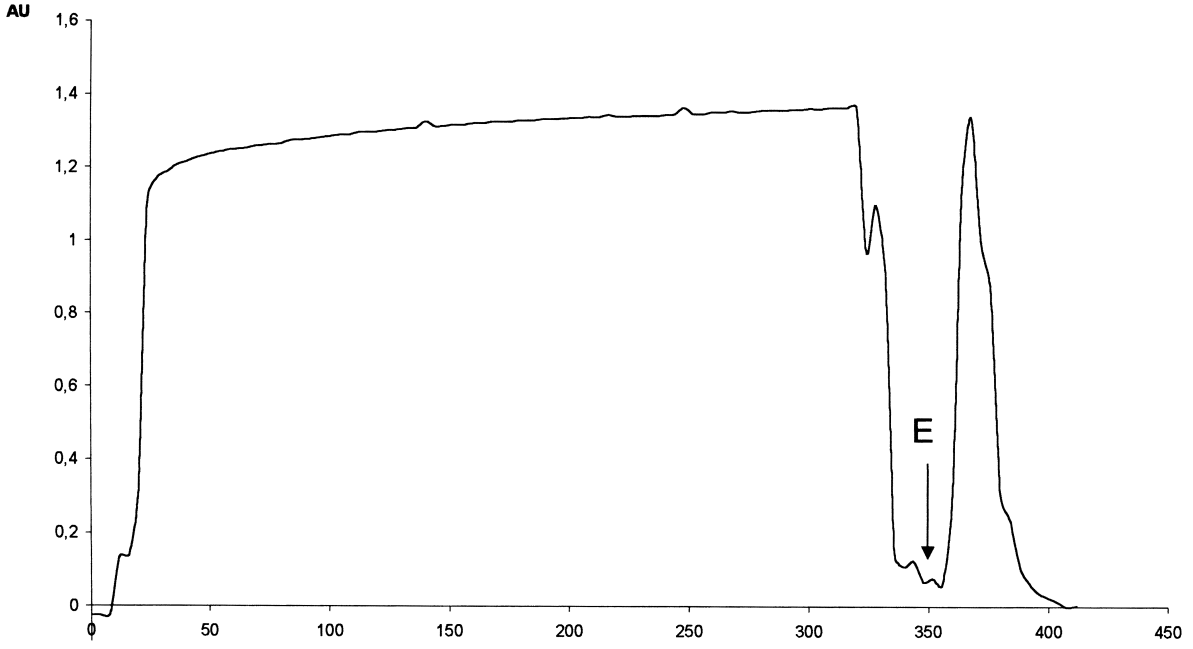

Fig. 4. Intermediate purification with Q Sepharose Fast Column: XK16/20 (2.5 ml of gel). Sample: Eluate of EBA–IMAC diluted to 300 ml; buffer A: 50 m*M* Tris–HCl, pH 7.5; buffer B: 1 *M* NaCl in buffer A, elution (E) 30% of buffer B. Detection at 280 nm; flow-rate: 300 cm/h.

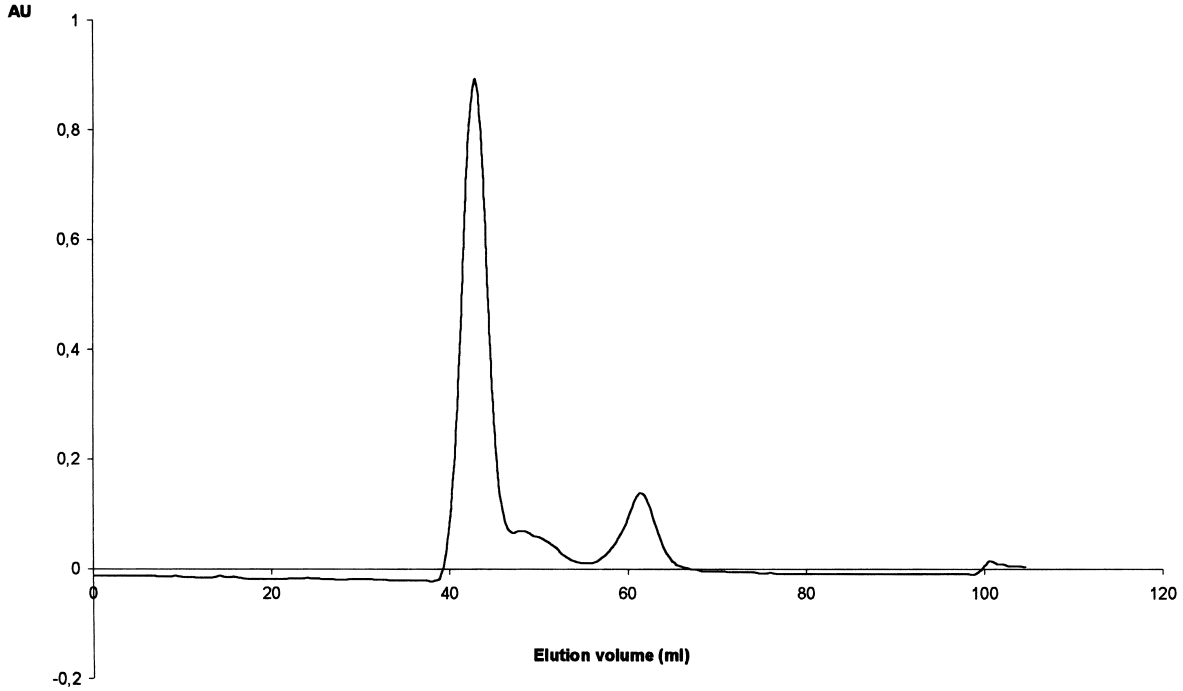

Fig. 5. Polishing step with Superdex 75 prep grad. Column: XK16/70 (120 ml of gel). Sample: Eluate of Q Sepharose Fast Flow concentrated to 1 ml. Buffer: 0.5 *M* NaCl, 50 m*M* Tris–HCl, pH 8. Detection at 280 nm; flow-rate: 30 cm/h.

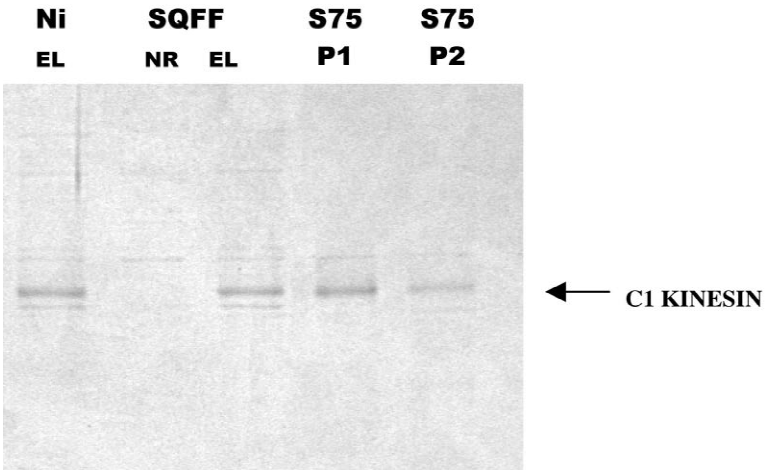

Fig. 6. SDS–PAGE (12%) analysis of the three-step purification process of the C1 kinesin. A 500-ng amount of protein was loaded in each lane. NiEl: eluted fraction from the EBA–IMAC; SQFF: NR and El Q Sepharose Fast Flow non-retained and eluted fraction; S75: Superdex 75 P1 (peak 1), P2 (peak 2).

Table 1
Purification of one liter of culture medium of recombinant His-tag kinesin

	Volume (ml)	[Protein] (μg/ml)	Total protein (mg)	Step recovery (%)
Starting material	1000	107	107	
IMAC n.r.[a]	2000	50	100	
IMAC wash	280	8	2.2	
IMAC eluate	40	70	2.8	100
Ion exchange eluate	10	150	1.5	53
Size-exclusion peak 1	2	500	1	67
Size-exclusion peak 2	2	200	0.4	27

[a] Non-retained fraction.

4. Conclusion

The process described in this paper allows the purification of an His-tag recombinant kinesin over-expressed in *E. coli* directly from several liters of an unclarified feedstock just after ultrasonic homogenization.

As shown in Fig. 2 the level of kinesin expression in BL21 is low. Therefore, a large amount of culture is needed to produce a high level of purified kinesin protein.

An easy scale-up was performed with EBA–IMAC. A high flow-rate processed a large volume of feedstock. Thus, the kinesin was adsorbed immediately and degradation by protease was prevented.. Hence the eluted purified protein still harbors its catalytic activity (Fig. 3).

Anion-exchange chromatography concentrated the sample and eliminated imidazol and any minor contaminants. Size-exclusion chromatography enabled two forms of kinesin, a dimer (70%) and a monomer (30%), to be separated.

Acknowledgements

We thank R. Cooke for linguistic help.

References

[1] R.D. Vale, Annu. Rev. Cell Biol. 3 (1987) 347–378.
[2] R.B. Vallee, H.S. Slapetner, Annu. Rev. Biochem. 59 (1990) 909–932.
[3] L.S. Golstein, Annu. Rev. Genet. 27 (1993) 319–351.
[4] S.P. Gilbert, M.R. Webb, M. Brune, K. Johnson, Nature 373 (1995) 671–676.
[5] J. Porath, J. Carlson, I. Olsson, G. Belfrage, Nature 258 (1975) 598–599.
[6] E. Sulkowski, Trends Biotechnol. 3 (1985) 1–7.
[7] T.W. Hutchens, T.-T. Yip, Anal. Biochem. 191 (1990) 160–168.
[8] E. Hochuli, W. Bannwarth, H. Dodeli, R. Gentz, D. Stuber, Bio/Technology 6 (1988) 1321–1325.
[9] J. Porath, Protein Express. Pur. 3 (1992) 263–281.
[10] M.N. Draeger, H.A. Chase, Bioseparation 2 (1991) 67–80.
[11] H.A. Chase, M.N. Draeger, J. Chromatogr. 597 (1992) 129–145.
[12] H.A. Chase, M.N. Draeger, Separation Sci. Technol. 27 (1992) 22021–22039.
[13] U.K. Laemmli, Nature 277 (1970) 680–685.
[14] J. Kyshe-Andersen, J. Biochem. Biophys. Methods 10 (1984) 203–209.
[15] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[16] H. Mitsui, K. Yamagushi-Shinozaki, K. Shinozaki, K. Nishikawa, H. Takahashi, Plant Molecular Biol. 25 (1994) 865–876.