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One-step chromatographic purification procedure of a His-tag recombinant carboxyl half part of the HTLV-I surface envelope glycoprotein overexpressed in *Escherichia coli* as a secreted form

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Abstract

A His-tag recombinant carboxyl half part of the HTLV-I surface envelope glycoprotein was overexpressed in *E. coli* as a secreted form in order to study its biochemical properties and to determine its three-dimensional structure by X-ray crystallography. Starting from several hundred milliliters of culture, a centrifugation was used to eliminate the cells. After solubilization and centrifugation, the protein was then purified by a one-step chromatographic purification procedure. Immobilized Metal Affinity Chromatography (IMAC) was performed by evaluating the tri-dentate iminodiacetic acid (IDA) chelating group with chelating Sepharose fast flow, and the tetra-dendate nitrilotriacetic acid (NTA) chelating group with NTA–agarose. The latter was the most suitable gel for our protein. This expression system and the use of affinity chromatography is a rapid technique to obtain a soluble protein for use in structural studies to further understand the mechanisms of HTLV-1 entry into target cells. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: His-tag recombinant carboxyl; Glycoprotein

1. Introduction

Human T-cell leukemia virus type 1 (HTLV-1) causes adult T-cell leukemia (ATL), a chronic neurological disease, tropical spastic paraparesis (TSP) or HTLV-1 associated myelopathy (HAM) [1–5] and other less severe diseases.

The virus infects 10–20 million people worldwide and about 4% of infected persons develop one of these diseases.

HTLV-1 entry into the target cell is mediated by the viral envelope glycoproteins (env). These are two non-covalently linked subunits generated by proteolytic cleavage of an env encoded glycoprotein precursor.

The glycoprotein SU (gp 46) is responsible for virus attachment to an undefined cellular receptor and the glycoprotein TM (gp 21) allows penetration of the viral core into the cytoplasm. Data on the structure of the HTLV-1 envelope glycoproteins are not so far available, even though these proteins have

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been produced in different heterologous systems [6–9]. Precious information on the three-dimensional structures and functionality of these proteins may be obtained by X-ray analysis of their crystal structure.

As information on the HTLV-1 env protein structure will further the understanding of the mechanism of infection by this virus and help in the development of a vaccine, we decided to overexpress in *E. coli* a recombinant protein containing the major immunodominant domains (amino acid 175–199 and 239–261) as well as regions that elicit neutralizing antibodies (amino acid 187–199, 213–236 and 288–317) [10]. In addition, the domain comprised between amino acid 170–233 has been shown to be involved in fusion mechanisms of viral and cellular membranes [11,12].

A low expression has already been obtained in *E. coli* from the periplasmic compartment and has been purified with a multi-step chromatographic procedure [13]. The present study presents a high expression of a secreted recombinant envelope protein, to obtain an overexpressed soluble recombinant protein corresponding to the carboxyl half part of the HTLV-1 SU using a one-step chromatographic purification procedure with Immobilized Metal Affinity Chromatography (IMAC) [15–21].

2. Experimental

2.1. Instruments

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

The electrophoresis system used was from Biorad (Ivry-sur-seine, France).

2.2. Chemicals

Chelating Sepharose® fast flow and the XK 16/20 column were purchased from Amersham Pharmacia Biotech (Saclay, France). Ni-NTA Agarose was from Qiagen (Courtaboeuf, France). Immidazole was from BDH Laboratory (Poole, UK). All metals, salts and additive were from Sigma (l'Isle d'Abeau Chesnes, France). All other salts were HPLC grade, and the buffers were filtered through a 0.22-μm membrane filter. Super Signal chemiluminescence kit was from Pierce (Rockford, IL, USA) Peroxidase-labeled goat $(Fab')_2$ fragments anti-mouse IgG were from Roche Diagnostic (Meylan, France). Culture media and ingredients for the growth of bacteria from DICO Labs (Detroit, MI, USA) The protease inhibitors "Complete mini EDTA-free" were from Roche Diagnostic (Meylan, France). IPTG (Isopropyl β-D-thio galactopyranoside) was from Euromedex (Souffelweyersheim, France).

2.3. Induction, expression and culture medium isolation

An overnight culture of BL21DF3 containing the PET 2240 plasmid in Luria–Bertani (LB) medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) was diluted (1:100) in LB medium supplemented with 0.4 *M* sucrose, and incubation was continued at 37°C with vigorous shakincubing until an absorbance value of 0.3 at 600 nm was obtained. Then, synthesis of the HTLV-1 envelope 6-histidines tagged protein was induced by IPTG at a final concentration of 0.4 m*M*. After 16 h of incubation at 25°C, the cells were harvested by centrifugation (Sorvall GSA rotor, 5500 rpm, 15 min, 4°C) and the supernatant corresponding to the culture medium was supplemented with protease inhibitors and used as a source of recombinant protein in the purification procedure.

2.4. Monoclonal antibody

The monoclonal antibody MF2 recognizes an epitope corresponding to the amino acid stretch 190–197 and was produced according to previously reported procedures [14].

2.5. Construction of the env expression vector

The construction of the vector was performed according to techniques reported elsewhere [13].

2.6. Preparation of supports

2.6.1. Package of gels

The gels were packed in an XK16/20 column, a slurry was prepared with binding buffer in a ratio of 75% settled gel to 25% buffer and was de-gassed.

The column was filled through the outlet with a few centimeters of binding buffer and was closed. The slurry was poured into the column in one continuous motion. The remainder of the column was filled with buffer and the top 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 (1 ml/min). The packing flow-rate was maintained for three bed volumes after a constant bed height was reached.

2.7. Evaluation of chelating groups and purification procedure

The selection of immobilized metal chromatographic supports was performed in an XK16/20 column with two types of gels (chelating Sepharose fast flow, Ni–NTA–agarose). Two metals were tested: Ni^{2+}, Cu^{2+} with chelating Sepharose fast flow and only Ni^{2+} with NTA–agarose (this gel was supplied with the metal by the manufacturer). For chelating sepharose fast flow, the adsorption of metal was performed with 0.3 *M* metal solution in distilled water at 5 ml/min (150 cm/h). The columns were equilibrated with 0.02 *M* Tris–HCl pH 8, 0.5% *N*-octyl glucoside, 2 *M* guanidine, 0.01 *M* β mercaptoethanol. After the through flow and until the UV absorbance return to base line, the column was washed with 0.02 *M* Tris–HCl, 0.5% *N*-octyl glucoside, 0.5 *M* NaCl, 0.01 *M* β mercaptoethanol. The elution was performed with linear gradient up to 1 *M* Imidazole.

2.8. Analytical procedures

2.8.1. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [22] using a Mini-protean II apparatus and a Tris–glycin buffer were used to monitor the purification during the chromatographic procedures.

Electrophoresis was performed for 45 min at 200 V using 12.5% polyacrylamide gels. Detection was done by silver staining or western-blot analysis.

2.8.2. Silver staining

The polyacrylamide gel was incubated three times for 1 h in methanol–water–acetic acid (5:5:1) with gentle shaking. The gel was then incubated for 15 min in 50% methanol, 12% trichloroacetic acid (TCA), 2% $CuCl_2$; 15 min in 10% isopropanol, 5% acetic acid; 15 min in a freshly prepared solution of 0.01% $KMnO_4$; 10 min in 10% isopropanol, 5% acetic acid. After two washes with water (10 min each), the gel was incubated in 0.1% $AgNO_3$ for 10 min and washed with water. Finally, the gel was placed in a solution containing 2% Na_2CO_3, 1 ml/l formaldehyde for 4–6 min. Staining was stopped with 5% acetic acid.

2.8.3. Western blotting

The proteins contained in the culture medium and in the chromatographic fractions were separated by electrophoresis as above and transferred onto a nitrocellulose membrane. This membrane was incubated overnight at room temperature in 2% non-fat dry milk and 0.5% BSA in PBS (phosphate-buffered saline), 20 m*M* Tris–HCl pH 7.4 and 0.05% Tween 20. The membranes were then incubated in the same buffer containing 20 μg/ml of MF2 monoclonal antibody specific for the HTLV-I gp 46 for 2 h at 37°C and washed in PBS containing 0.1% Tween 20. The membrane was then incubated with peroxidase-labeled goat $(Fab')_2$ fragments anti-mouse IgG for 1 h at room temperature. After five washes, the bound antibodies were revealed using the super Signal chemilunescence kit according to the manufacturer's instructions.

3. Results and discussion

3.1. Solubilization of PET 2240 overexpressed in *E. coli*

To estimate the amount of soluble recombinant HTLV-1 envelope protein in the culture medium of BL21DE3 transformed by PET 2240, the proteins isolated were submitted to centrifugation (Sorvall, GSA rotor, 16 000 rpm, 30 min, 4°C), fractionated

by SDS–PAGE and analyzed by western blotting using the MF2 monoclonal antibody specific for the HTLV-1 SU envelope (Fig. 1). In the fraction corresponding to PET 2240 transformed bacteria (lane 1), this antibody revealed the env protein of the expected size (22 K).

No protein was revealed in the supernatant corresponding to the centrifugation at 16 000 rpm while all the recombinant protein was recovered in the pellet (lane 2).

These results indicated that the secreted PET 2240 env protein was aggregated or bound to cell debris. Therefore, we decided to perform centrifugation (Sorval SS34, 16 000 rpm, 30 min, 4°C) in order to reduce the culture volume and the pellet was submitted to a solubilization procedure. The proteins were resuspended in 0.02 *M* Tris–HCl pH 8, 0.5% *N*-octyl glucoside, 2 *M* guanidine, 0.01 *M* β-mercaptoethanol, 1 h at 4°C and submitted to final centrifugation (Sorvall SS 34, 16000 rpm, 30 min, 4°C). The solubilized protein was used as a source of recombinant protein in the subsequent chromatographic step.

3.2. Evaluation of chelating groups

The tri-dentate iminodiacetic acid (IDA) chelating group was tested (chelating Sepharose fast flow) with

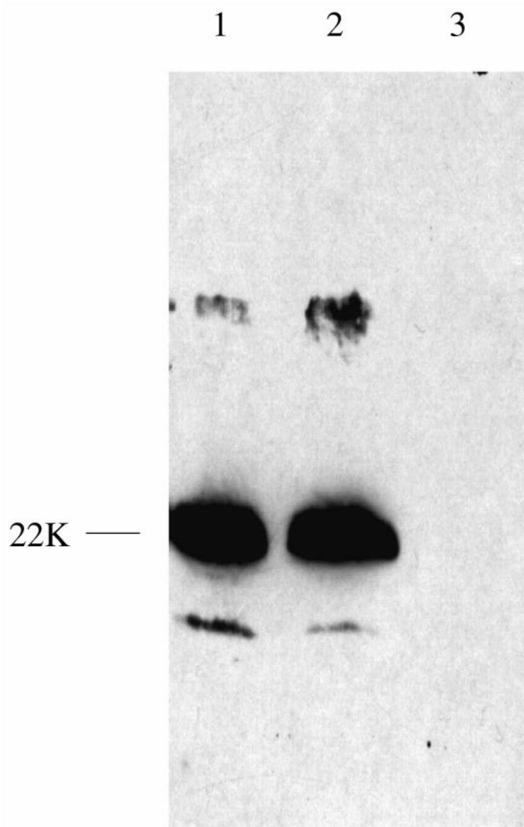

Fig. 1. SDS–PAGE analysis of expression of the recombinant HTLV-1 envelope protein. A 15-μl volume fraction of culture medium of BL21DE3 transformed by PET 2240 was analyzed by western-blotting (lane 1). Lane 2 represents the proteins contained in the pellet corresponding to centrifugation at 16 000 rpm while lane 3 represents the supernatant corresponding to the centrifugation at 16 000 rpm.

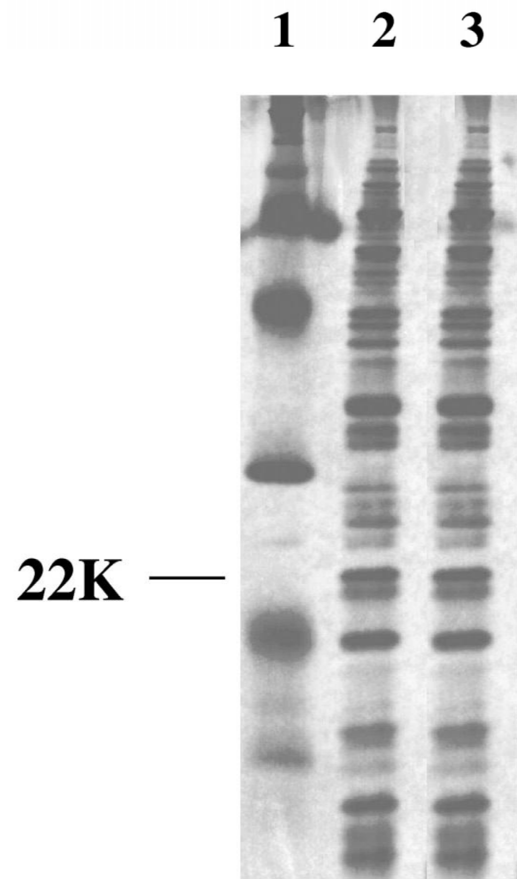

Fig. 2. SDS–PAGE analysis of IDA chelating group. A 15-μl volume fraction of culture medium of BL21DE3 transformed by PET 2240 was analyzed by silver staining (lane 2). Lane 3 represents the proteins contained in the flow through fraction while lane 1 represents the weight molecular marker.

two metals, Cu^{2+} and Ni^{2+}. The elution and the flow through fraction of the purification procedure were analyzed by SDS–PAGE followed by silver staining. The results indicated that the recombinant protein was only recovered in the flow through fraction (Fig. 2) while the elution fraction presented none proteins (data not shown).

These results showed that the His tag recombinant carboxyl half part of the HTLV-1 surface envelope glycoprotein was not retained on the support suggested that this protein stripped the metal from the gel (these data were confirmed by the fact that the flow through fraction was colored by Cu^{2+} or Ni^{2+}. Therefore, we have tested a tetra-dentate nitrilotriacetic acid chelating group (NTA–agarose). With this gel, the chelation of metal was stable and allowed the purification of our protein.

3.3. Purification of the His-tag recombinant carboxyl half part of the HTLV-1 surface envelope glycoprotein

The solubilized culture medium containing the recombinant His-tag protein was loaded onto a NTA–agarose column (1 ml, Ni^{2+}) equilibrated in 0.02 *M* Tris–HCl pH 8, 2*M* guanidine, 0.5% *N*-octyl glucoside, 0.01 *M* β mercaptoethanol, 0.02 *M* imidazole. Chromatography was performed at 1 ml/min. After adsorption of the protein of interest, the gel was washed with in 0.02 *M* Tris–HCl pH 8, 0.5 *M* NaCl, 0.5% *N*-octyl glucoside, 0.01 *M* β mercaptoethanol, 0.02 *M* imidazole. Desorption of the protein was performed by linear gradient up to 1 *M* imidazole (Fig. 3). The fraction was eluted at 0.17 *M* imidazole and the contaminants passed into the through flow. The fraction containing the His-tag recombinant carboxyl half part of the HTLV-1 surface envelope glycoprotein was analyzed by western blotting (Fig. 4A) and by SDS–PAGE (Fig. 4B). This showed that the purified protein really corresponded to the His-tag protein, since the antibody recognized the purified protein at a relative molecular mass of 22 000.

4. Conclusion

The process described in this paper allows the expression of a His-tag recombinant carboxyl half

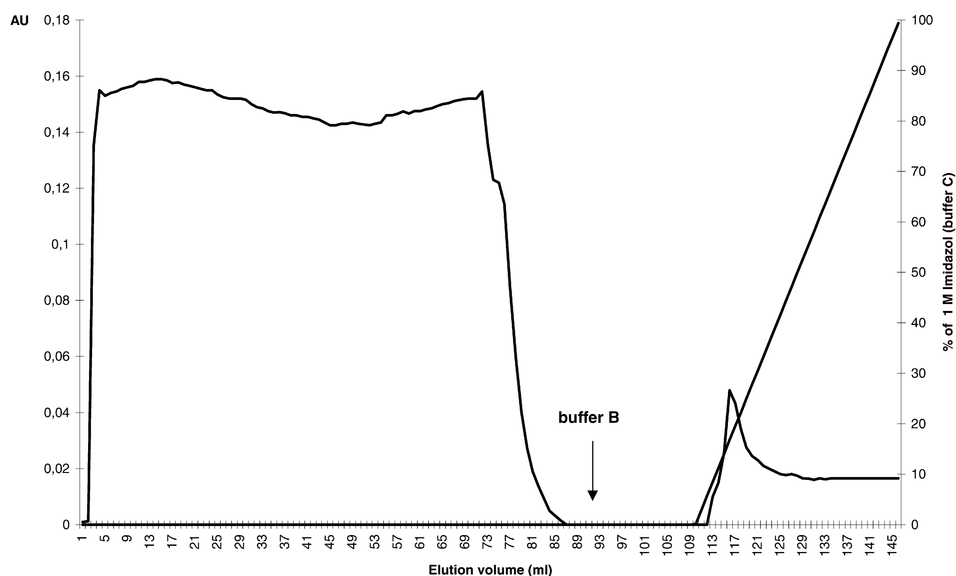

Fig. 3. Immobilized Metal Affinity Chromatography (IMAC). Column: NTA–agarose (1 ml of gel). Sample: 80 ml of clarified supernatant; buffer A: 0.02 *M* Tris–HCl pH 8, 0.5% *N*-octyl glucoside, 2 *M* guanidine, 0.01 *M* β mercaptoethanol, buffer B: 0.02 *M* Tris–HCL, 0.5% *N*-octyl glucoside, 0.5 *M* NaCL, 0.01 *M* β mercaptoethanol, buffer C: 1 *M* imidazole in buffer B. Detection at 280 nm; flow-rate: 1 ml/min.

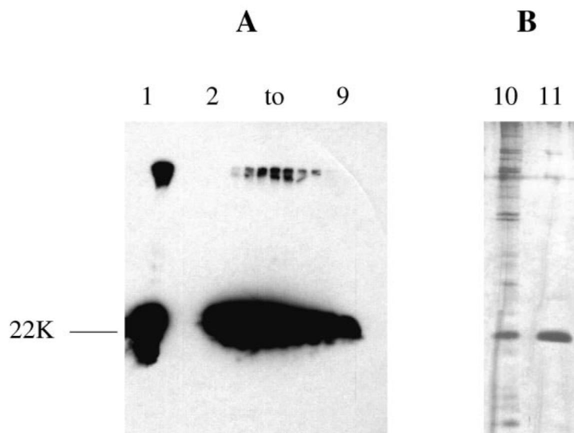

Fig. 4. Analysis of fractions eluted from IMAC by western-blotting (A) or silver staining (B). (A) The total proteins (15 μl) in the culture medium of bacteria transformed by PET 2240 (lane 1) and a 15-μl volume of each eluted fraction (lanes 2–9) were analyzed by western-blotting. (B) Lane 10 represents 15 μl of the total proteins contained in the culture medium of bacteria transformed by PET 2240. Pooled fractions (5 μl) from IMAC were shown on lane 11.

part of the HTVL-1 surface envelope glycoprotein in a soluble secreted form.

The tag allow easy purification using a one-step chromatography by immobilized Metal Affinity Chromatography (IMAC).

These conditions allowed us to obtain more than 90% pure 22-K envelope protein which could be utilized in structural studies to further understand the mechanisms of HTLV-1 entry into the cell.

References

[1] A. Gessain, F. Barin, J.C. Vernant, O. Gout, L. Maurs, A. Calender, G. De The, Lancet 11 (1985) 407.

[2] Y. Hinuma, K. Nagata, M. Hanaoka, M. Nakai, T. Matsumoto, K.I. Kinoshita, S. Shirakawa, I. Miyoshi, Proc. Natl. Acad. Sci. USA 78 (1981) 6476.

[3] M. Osame, K. Usuku, S. Izumo, N. Ijichi, H. Amitani, A. Igata, M. Matsumoto, M. Tara, Lancet 1 (1986) 1031.

[4] B.J. Poiesz, F.W. Ruscetti, A.F. Gazdar, P.A. Bunn, J.D. Minna, R.C. Gallo, Proc. Natl. Acad. Sci. USA 77 (1980) 7415.

[5] M. Yoshida, I. Miyoshi, Y. Hinuma, Proc. Natl. Acad. Sci. USA 79 (1982) 2031.

[6] T. Kiyokawa, H. Yoshikura, S. Hattori, M. Seiki, M. Yoshida, Proc. Natl. Acad. Sci. USA 81 (1984) 1202.

[7] T. Kuga, S. Hattori, M. Yoshida, T. Tanigushi, Gene 44 (1986) 337.

[8] H. Shida, T. Tochikura, T. Sato, T. Konno, K. Hirayoshi, M. Seiki, Y. Ito, M. Hatanaka, Y. Hinuma, M. Sugimoto, F. Takahashi-Nishinaki, T. Maruyama, K. Miki, K. Suzuki, M. Morita, H. Sashiyama, M. Hayami, EMBO J. 6 (1987) 3379.

[9] J. Arp, C.M. Ford, T.J. Palker, E.E. King, G.A. Dekaban, J. Gen. Virol. 74 (1993) 211.

[10] M.K. Hart, T.J. Palker, B.F. Haynes, in: M.F. Powel, M.J. Newman (Eds.), Vaccine Design, Plenum Press, New York, 1995, p. 821.

[11] L. Delamare, A. Rosenberg, C. Pique, D. Pham, M.-C. Dokhelar, J. Virol. 71 (1997) 259.

[12] C. Pique, D. Pham, T. Tursz, M.-C. Dokhelar, EMBO J. 9 (1990) 4243.

[13] B. Tallet, T. Astier-Gin, D. Londos-Gagliardi, B. Guillemain, J. Chromatogr. B 737 (2000) 8595.

[14] D. Londos-Gagliardi, V. Jauvin, M.H. Armengaut, T. Astier-Gin, M. Goetz, S. Huet, B. Guillemain, AIDS Res. Hum. Retrovirus 15 (1999) 909.

[15] J. Porath, J. Carlsson, I. Olsson, G. Belfrage, Nature 258 (1975) 598.

[16] J. Porath, M. Belew, in: I.M. Chaiken, M. Wilchek, I. Parikh (Eds.), Affinty Chromatography and Biological Recognition, Academic Press, San Diego, 1983, p. 173.

[17] J. Porath, B. Olin, B. Granstrand, Arch. Biochem. Biophys. 225 (1983) 543.

[18] B. Lönnerdal, C.L. Keen, J. Appl. Biochem. 4 (1982) 203.

[19] E. Sulkowski, Trends Biotechnol. 3 (1985) 1.

[20] J. Porath, B. Olin, Biochemistry 2 (1983) 1621.

[21] J. Porath, Protein Expr. Purif. 3 (1992) 263.

[22] U.K. Laemmli, Nature 222 (1970) 680.