Journal of Chromatography, 587 (1991) 19–23
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2373

Hydrophobic interaction chromatography for the purification of a mycobacterial heat shock protein of relative molecular mass 60 000

Bernd Schoel* and Stefan H. E. Kaufmann

Department of Immunology, University of Ulm, Albert-Einstein-Allee 11, W-7900 Ulm (Germany)

ABSTRACT

A recombinant mycobacterial heat shock protein of relative molecular mass 60 000 was purified by hydrophobic interaction chromatography. Chromatographic media with ligands of medium hydrophobicity, such as phenyl-Sepharose, bound too strongly to be used for the purification of this heat shock protein. Butyl-Sepharose, with weak hydrophobicity, allowed binding and elution with decreasing concentrations of ammonium sulphate, but only alkyl-Superose allowed the separation of two similar proteins from the *Escherichia coli* clone expressing the recombinant heat shock protein (relative molecular mass 60 000) of *Mycobacterium bovis* BCG. The binding parameters of recombinant human heat shock proteins of relative molecular mass 60 000 and 70 000 indicate that phenyl-Sepharose also binds too strongly for the separation of these two heat shock proteins.

INTRODUCTION

Heat shock proteins (hsp) are induced by different types of stress in eukaryotic and prokaryotic cells. Different families of hsp exist which are grouped according to their molecular size. The hsp 60 family is ubiquitous among all species and its members have a very high degree of sequence homology [1]. The biological functions of hsp 60 cognates are now known. Hsp 60 and hsp 70 both act as chaperons, *i.e.* they are involved in the assembly of protein subunits to form multimers, the unfolding of partially denatured proteins, and in keeping the proteins in an unfolded state prior to translocation across the membrane of the endoplasmic reticulum. In mammalian cells, hsp 60 cognates are located in mitochondria and are responsible for the assembly of intruded proteins [1].

The mycobacterial hsp of relative molecular mass 60 000 is a major T-cell antigen of *Mycobacterium tuberculosis* immune mice, tuberculosis patients and normal healthy subjects [2,3]. It is identical in *M. tuberculosis* and *M. bovis* BCG, shares significant homology with mammalian hsp 60 and has been linked with autoimmunity [4,5]. T-cells from arthritic rats cross-react with hsp 60 of *M. bovis* BCG (hsp 60 BCG) [5] and cytotoxic CD8 T-cells generated *in vitro* with a tryptic digest of hsp 60 BCG lyse stressed target cells [6]. Reactivity against mycobacterial hsp of relative molecular mass 60 000 has been described for a subgroup of T-cells bearing the γ/δ T-cell receptor [7]. It has been speculated that γ/δ T-cells perform scavenger functions.

Large amounts of purified hsp are needed for immunological studies. Recombinant (r) hsp 60 BCG is routinely semi-purified by anion-exchange chromatography according to Thole *et al.* [8]. The eluted material is pure except for a double band visible on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Both bands are recognized by antibodies against hsp 60 BCG, their difference in molecular mass being 3000.

This paper describes a hydrophobic interaction chromatography (HIC) technique for the efficient separation of these highly similar proteins. The binding properties of human r-hsp 60 (r-hsp 60hu) and human r-hsp 70 (r-hsp 70hu) towards media of different hydrophobicity are also discussed.

0021-9673/91/$03.50 © 1991 Elsevier Science Publishers B.V. All rights reserved

EXPERIMENTAL

Anion-exchange chromatography of hsp 60 BCG

The protein was purified from 10-l fermenter cultures of *Escherichia coli* M1546 with plasmid pRIB1300, kindly provided by J. van Embden [8]. After sonication of the bacteria, the 20–55% ammonium sulphate fraction was dialysed against 50 m*M* piperazine–hydrochloric acid (pH 6.0) and subjected to anion-exchange chromatography on Q-Sepharose Fast Flow (Pharmacia). The hsp 60 BCG eluted within a linear gradient of sodium chloride at 0.1–0.12 *M* in 50 m*M* piperazine–hydrochloric acid (pH 6.0).

Hydrophobic interaction chromatography of hsp 60 BCG

Alkyl-Superose, butyl-Sepharose and phenyl-Sepharose were from Pharmacia. Samples were adjusted to 35% ammonium sulphate in 20 m*M* sodium phosphate (pH 6.5). Up to 5 mg of prepurified protein from the anion exchanger were loaded onto alkyl-Superose HR5/5. Elution was performed with a decreasing linear gradient of ammonium sulphate in 20 m*M* sodium phosphate (pH 6.5).

Identification of hsp

Samples (1 μl) were dotted on reinforced nitrocellulose (Schleicher and Schuell) and developed as Western blots using monoclonal antibodies (mab) IIC8 for hsp 60 BCG, ML30 for hsp 60hu and N27 for hsp 70hu. Hsp 60hu and hsp 70hu were kindly supplied by Dr. R. A. Young, mab IIC8 by Dr. T. Gillis, ML30 by Dr. J. Ivanyi and N27 by Dr. W. J. Welch.

SDS-PAGE and Western blotting

Electrophoresis was performed under reducing conditions in a 10% polyacrylamide gel according to standard procedures [9] in a mini-electrophoresis system (Bio-Rad) and the blots were silver-stained [10]. For the immunological detection of specific bands, proteins were transferred to nitrocellulose in a semi-dry blotting system. After blocking the membrane in 1% casein–20 m*M* Tris (pH 8.0), mab IIC8 against hsp 60 BCG was used as the first antibody (1 μg/ml). Further incubation steps were performed with goat anti-mouse $(Fab')_2$ (H + L) biotin conjugate (1:20 000) and streptavidin alkaline phosphatase conjugate (1:10 000) (both from Dianova). The blots were then developed with 0.8 μg/ml 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt and 1.6 μg/ml 4-nitrotetrazolium blue chloride (both Fluka) in 1 *M* diethanolamine, 0.5 m*M* magnesium chloride (pH 9.8) [11].

RESULTS AND DISCUSSION

Lysates of a recombinant *E. coli* clone expressing r-hsp 60 BCG were first purified by anion-exchange chromatography. The material obtained contained two similar proteins, both recognized by mab against hsp 60 BCG, and differing by 3000 in molecular mass. Previous attempts to separate these two similar proteins on metal chelate chromatographic columns had failed, although r-hsp 60 BCG could be bound to copper ions in a sodium phosphate buffer at pH 8.0. Elution occurred as a single peak at 20 m*M* glycine. Other methods were not feasible because r-hsp 60 BCG has a p*I* of 4.6, which excludes cation-exchange chromatography and chromatofocusing, and both proteins are too similar in size to allow their separation by gel permeation chromatography. Therefore, HIC appeared to be a promising means to further purify r-hsp 60 BCG.

The binding and elution properties of proteins from HIC resins are essentially controlled by four parameters: (1) the hydrophobic strength of the ligands bound to the matrix; (2) the general solvent effects of ions in the solvent system; (3) pH; and (4)

TABLE I

CONDITIONS USED FOR THE PRECIPITATION OF HSP

Aliquots of hsp were incubated for 1 h under different conditions and, after centrifugation, the supernatants were tested for residual, soluble hsp with a dot-blot assay.

Condition	r-hsp 60 BCG	r-hsp 60hu	r-hsp 70hu
pH[a]	5	6	< 4
AS at pH 8 (%)[b]	40	25	30
AS at pH 6 (%)[b]	30	20	25

[a] Highest pH at which the hsp precipitated when assayed at pH 7, 6, 5 and 4 using 100 m*M* citric acid–sodium hydroxide.

[b] Lowest concentration of ammonium sulphate (AS) at pH 8 or 6 at which the hsp precipitated. Assayed at concentrations from 15 to 50% in steps of 5% in 50 m*M* Tris–hydrochloric acid (pH 8) or 100 m*M* citric acid–sodium hydroxide (pH 6).

temperature. In an attempt to establish the conditions for the chromatographic separation of three different hsp, the pH and ammonium sulphate concentration at which these proteins begin to precipitate were determined. A semi-purified fraction of r-hsp 60 BCG after anion-exchange chromatography and crude extracts of r-hsp 60hu and r-hsp 70hu were subjected to different pH conditions ranging from pH 7 to 4 at intervals of one pH unit. Different concentrations of ammonium sulphate ranging from 15 to 50% in 5% steps at pH 8 and 6, respectively, were used. After high-speed centrifugation, the supernatants were assayed by blotting onto nitrocellulose and development with specific mab.

Table I summarizes the results. With decreasing pH, r-hsp 60 BCG remained soluble until pH 5, r-hsp 60hu until pH 6 and r-hsp 70hu until pH 4. Precipitation with ammonium sulphate started at 40% for r-hsp 60 BCG, 25% for r-hsp 60hu and 30% for r-hsp 70hu, when tested at pH 8. At pH 6, precipitation occurred at lower concentrations of ammonium sulphate.

The binding properties of these proteins to two different HIC media, butyl-Sepharose and phenyl-Sepharose, were assessed. Aliquots of each hsp were added to 100 μl of an equilibrated suspension containing one volume of packed HIC resin and one volume of buffer. The supernatants were analysed by dotblot assay for residual, non-bound hsp. The results shown in Table II indicate that all the hsp bound to phenyl-Sepharose at 20% ammonium sulphate. At a 0% concentration of ammonium sulphate, phenyl-Sepharose still bound r-hsp 60hu and r-hsp 70hu. r-hsp 60 BCG did not bind at pH 8, but binding occurred at pH 6. Butyl-Sepharose bound r-hsp 60 BCG at a concentration of 30%, and r-hsp 70hu at 20% ammonium sulphate. At 0% ammonium sulphate butyl-Sepharose did not bind any of these hsp; butyl-Sepharose is therefore a suitable chromatographic medium for the HIC of hsp. Although it was valuable for prepurification and concentration, the resolving power of butyl-Sepharose was not sufficient to separate prepurified r-hsp 60 BCG. To improve the separation alkyl-Superose was used, which has a similar hydrophobicity as butyl-Sepharose but a higher resolving power.

Attempts to separate these hsp were finally successful using alkyl-Superose. Fig. 1 shows an elution profile and Fig. 2 the purity of the separated proteins. The protein with the higher molecular mass of 65 000 eluted at higher concentration of ammonium sulphate. By comparing the binding and elution properties of r-hsp 60 BCG on butyl-Sepharose and alkyl-Superose it is concluded that alkyl-Superose has a less hydrophobic character. At 16% ammonium sulphate and pH 8.0 the protein could not be bound to alkyl-Superose, whereas under the same conditions it just eluted from butyl-Sepharose. The binding of the protein to alkyl-Superose was enhanced at higher concentrations of ammonium sulphate (35%) and pH 6.5. Elution then occurred at 26% ammonium sulphate.

The hsp 60 cognates from different species could have similar chromatographic properties because of

TABLE II

BINDING PROPERTIES OF hsp TO HIC MEDIA

Medium	pH	AS (%)[a]	r-hsp 60 BCG	r-hsp 60hu	r-hsp 70hu
Butyl-Sepharose	8	None	–[b]	–	–
		High	+	–	+
	6	None	–	–	–
		High	+	–	+
Phenyl-Sepharose	8	None	–	+	+
		High	+	+	+
	6	None	+	+	+
		High	+	+	+

[a] Concentration of ammonium sulphate (AS) at which binding of hsp was assayed. For r-hsp 60 BCG, 30% was used as high concentration and for r-hsp 60hu and r-hsp 70hu 20% AS was used.

[b] (–) No binding; (+) binding to HIC medium.

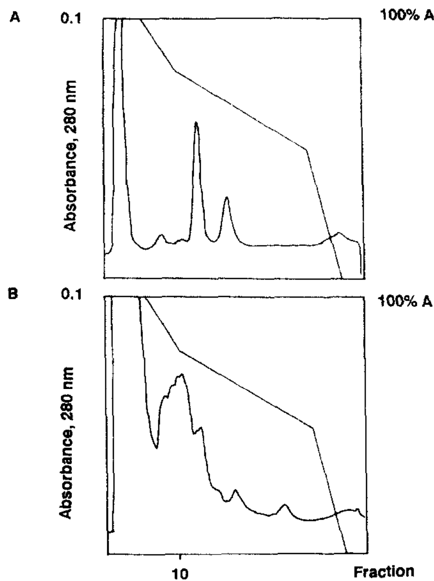

Fig. 1. Separation of r-hsp 60 BCG on alkyl-Superose HR5/5. (A) Prepurified r-hsp 60 BCG (200 μg) was loaded onto the column in 50 m*M* sodium phosphate–35% ammonium sulphate (pH 6.5) and eluted with a linear gradient of ammonium sulphate down to 0%. Each fraction contained 1 ml of eluent. (B) *E. coli* lysate (500 μg) separated as in (A).

the high sequence homology. Another hsp of the relative molecular mass 60 000 family and one of the 70 000 family were included. Hsp of the 70 000 family are usually purified by ion exchangers and affinity chromatography on columns with adenosine 5′-triphosphate as the ligand [12]. Owing to their similar biological function and involvement in the folding and unfolding of proteins, these two families might have similar properties on HIC columns. r-hsp 60hu could not be bound to HIC media of low hydrophobicity owing to the precipitation of this protein at too low concentrations of ammonium sulphate, but r-hsp 70hu could be bound and eluted from alkyl-Superose. Phenyl-Sepharose was too strongly hydrophobic for these two hsp.

Only a few proteins of *E. coli*, the host of these recombinant genes, bound to alkyl-Superose (Fig. 2A). If a hsp can be bound to a weak hydrophobic ligand, then this kind of media is valuable as an early hsp purification step, because few non-membrane-bound proteins bind to HIC media of low hydrophobicity.

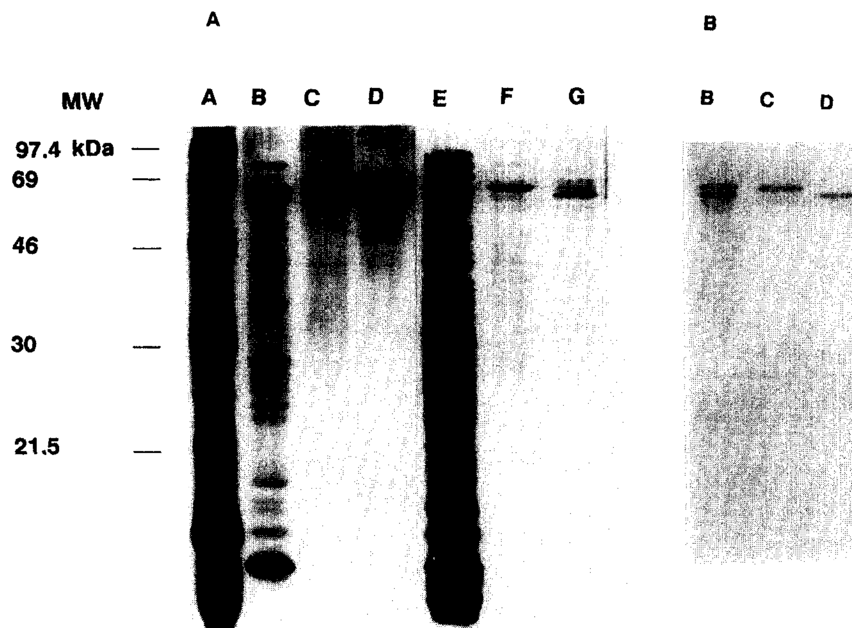

Fig. 2. SDS-PAGE of hsp: (A) silver-stained and (B) Western blot. (A) Molecular weight markers; (B) partially purified r-hsp 60 BCG of an *E. coli* lysate expressing r-hsp 60 BCG, after chromatographic separation on the anion-exchanger Q-Sepharose Fast Flow (1 μg of protein); (C) and (D) first and second peaks of eluted proteins from alkyl-Superose HR5/5 loaded with partially purified r-hsp 60 BCG from (B) (0.1 and 0.05 μg of protein, respectively); (E) 2 μg of protein from a crude lysate of *E. coli* expressing r-hsp 60 BCG; (F) and (G) similar peaks to (C) and (D), from alkyl-Superose HR5/5 loaded with crude *E. coli* lysate from (E) (0.2 and 0.05 μg of protein, respectively).

ACKNOWLEDGEMENTS

The authors thank Mrs. R. Mahmoudi for typing the manuscript. The work described was supported by the following grants to S.H.E.K: UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases, EC-India Science and Technology Cooperation Program, A. Krupp Award for Young Professors, and the SFB 322. We thank T. Gillis, J. Ivanyi and W. J. Welch for mab, J.D.A. v. Embden and R. van der Zee for recombinant *E. coli* clones, and R. A. Young and S. Jindal for recombinant hsp. K. Schilling and T. Schechinger provided much helpful technical information for the establishment of the FPLC system.

NOTE ADDED IN PROOF

Sequence analysis of the separated proteins confirmed that the larger protein (Fig. 2, lane C) was r-hsp 60 BCG. This protein was sequenced up to 30 amino acids resulting in an identical sequence. The smaller protein (Fig. 2, lane D) was sequenced up to 20 amino acids and identified as the truncated r-hsp 60 BCG, the aminoterminus starting with WGAPT (amino acid single letter code) at position 42 from the whole protein. Sequence analysis was done by Dr. Thomas Ruppert at the Department of Virology, University of Ulm, with an Applied Biosystems 471A Protein Sequencer.

REFERENCES

1 T. Langer and W. Neupert, *Curr. Top. Microbiol. Immunol.*, 167 (1991) 3.
2 F. Emmerich, J. Thole, J. van Embden and S. H. E. Kaufmann, *J. Exp. Med.*, 163 (1986) 1024.
3 S. H. E. Kaufmann, U. Väth, J. E. R. Thole, J. D. A. van Embden and F. Emmrich, *Eur. J. Immunol.*, 17 (1987) 351.
4 D. Elias, D. Markovits, T. Reshef, R. van der Zee and I. R. Cohen, *Proc. Natl. Acad. Sci. USA*, 87 (1990) 1576.
5 W. van Eden, J. E. Thole, R. van der Zee, A. Noordzij, J. D. van Embden, E. J. Hensen and I. R. Cohen, *Nature (London)*, 331 (1988) 171.
6 T. Koga, A. Wand-Württenberger, J. DeBruyn, M. E. Munk, B. Schoel and S. H. E. Kaufmann, *Science*, 245 (1989) 1112.
7 S. H. E. Kaufmann and D. Kabelitz, *Curr. Top. Microbiol. Immunol.*, 167 (1991) 191.
8 J. E. R. Thole, W. J. Keulen, A. H. J. Kolk, D. G. Groothuis, L. G. Berwald, R. H. Tiesjema and J. D. A. van Embden, *Infect. Immun.*, 55 (1987) 1466.
9 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
10 C. R. Merril, D. Goldmann and M. L. van Keuven, *Methods Enzymol.*, 104 (1984) 441.
11 D. A. Knecht and R. L. Dimond, *Anal. Biochem.*, 136 (1984) 180.
12 W. J. Welch and J. R. Feramisco, *Mol. Cell. Biol.*, 5 (1985) 1229.