Journal of Chromatography, 421 (1987) 355–359
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3838

Note

Purification of murine macrophage colony-stimulating factor using hydroxyapatite high-performance liquid chromatography in the presence of sodium dodecyl sulphate

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(Received May 18th, 1987)

Macrophage colony-stimulating factor (M-CSF, also called CSF-1) is one of a family of haemopoietic regulators stimulating proliferation and differentiation of haemopoietic progenitor cells. It stimulates the proliferation in vitro of murine precursor cells to form colonies of mature macrophages [1]. Murine M-CSF is produced by mouse L cells, and some studies for the purification and characterization of murine M-CSF have been reported [1, 2].

This paper reports our study of the purification of mouse L cell CSF on a large scale. In the purification, we found that the adsorption of CSF on hydroxyapatite (HA) gel depended on the presence of sodium dodecyl sulphate (SDS) resulting in effective separation from the contaminating proteins. SDS-HA high-performance liquid chromatography (HPLC) is a new approach to the purification of proteins.

EXPERIMENTAL

Conditioned media (CM) preparation

Mouse L929 cells were propagated in DM-160AU media (Kyokuto Pharmaceutical, Tokyo, Japan) supplemented with 5% fetal bovine serum (FBS) in multi-tray culture systems [3]. After reaching confluency, growth media were replaced with maintenance media, DM-160AU supplemented with 0.7% FBS. Cells were incubated for three days at 37°C, then the CM were harvested and pooled up to 300 l.

0378-4347/87/$03.50

Purification

The 300 l of mouse L cell CM were concentrated to 4.9 l (61-fold concentration) with a hollow-fibre concentrator (HFAK B-1, Toray Industries, Tokyo, Japan). Concentrated CM were applied to a DEAE-Toyopearl 650M (Toyo Soda, Tokyo, Japan) column (12×7.4 cm I.D.). CSF activity was eluted with 50 m*M* Tris–HCl (pH 7.5) containing 0.3–0.4 *M* sodium chloride. CSF-active fractions were pooled and, after adjustment to pH 3.5, applied to a column of silica beads (500 Å, 8.5×2.6 cm I.D., 30–60 mesh, Fuji-Devison Chemical, Tokyo, Japan). This column was washed with 10 m*M* formic acid (pH 3.5) and with 0.1 *M* Tris–HCl (pH 7.0). SDS was added to eluate with 0.1 *M* Tris–HCl (pH 7.0) to a final concentration of 0.1%, and the eluate was applied to an HA column (HCA A-7610, 100×7.6 mm I.D., Mitsui Toatsu, Tokyo, Japan). CSF activity was adsorbed and eluted with a linear gradient of sodium phosphate (0.05–0.5 *M*) in the presence of 0.1% SDS on the HA HPLC column. The eluate from the HA column was successively applied to a C_4 column (Cosmosil 5C4-300, 250×4.6 mm I.D., Nakarai Chemical, Kyoto, Japan) and a C_{18} column (Cosmosil 5C18-300, 250×4.6 mm I.D., Nakarai Chemical). Elution from both reversed-phase columns was performed with a linear gradient of acetonitrile (0–70%, v/v) containing 0.1% (v/v) trifluoroacetic acid. CSF-active fractions from the C_{18} column were neutralized to pH 7.5 with 1 *M* ammonium carbonate (pH 8.0). The purified protein was further analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli [4]. The gel was then sliced into 2-mm sections and immersed in 0.1% SDS solution overnight at 4 °C. The extracted protein was assayed and CSF-active fractions were subjected to N-terminal amino acid sequecne analysis on an Applied Biosystems protein sequencer, Model 470A. During the purification, CSF activity was measured as described by Stanley and Heard [1], and protein was determined by the method of Bradford [5] using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The purification of mouse L cell CSF is summarized in Table I. With the SDS-PAGE analysis, CSF activity was detected only in a non-reducing preparation at fractions 2–6, corresponding to molecular mass of ca. 100 000 (Fig. 1a). As shown in Table II, the N-terminal sequence of purified mouse L cell CSF was determined up to thirteen residues; this sequence was identical with that of murine M-CSF reported previously [2, 6]. Under reducing conditions, neither CSF activity nor any amino acid sequence was detectable in the corresponding zone, and the N-terminal sequence of the M-CSF was detected at the position corresponding to a molecular mass of ca. 50 000 (fractions 10–12) (data not shown). These results confirm that murine M-CSF forms an active dimer linked by disulphide bonds. The relative molecular mass of 100 000 of the M-CSF was higher than that reported previously [1], possibly owing to the inhibition of migration by ammonium trifluoroacetate on SDS-PAGE in our system. We have confirmed that the molecular mass of murine M-CSF is 55 000–70 000 (mainly 60 000) on SDS-PAGE employing the ammonium trifluoroacetate-free preparation (Fig. 1b), as

TABLE I

PURIFICATION OF MOUSE L CELL CSF

Step	Volume (ml)	CSF ($\times 10^6$ U)	Protein (mg)	Specific activity (U/mg)	Yield (step/overall) (%)	Purification (fold)
L cell CM concentration	4900	417	49 490	$8.4 \cdot 10^3$	–/100	1
DEAE-Toyopearl	2750	137	251	$5.0 \cdot 10^5$	33/33	60
Silica beads	50	32	4.5	$7.1 \cdot 10^6$	23/7.7	850
SDS-HA HPLC	24	19	N.D.*	N.D.*	59/4.6	N.D.*
C_4 HPLC	14.5	6.3	0.076	$8.3 \cdot 10^7$	33/1.5	9900
C_{18} HPLC	7.4	4.4	N.D.*	N.D.*	70/1.1	N.D.*
SDS-PAGE	0.7	3.6	0.032**	$1.1 \cdot 10^8$	81/0.86	13 000

*N.D. = not determined.
**Determined by amino acid analysis.

previously reported [2]. In the purification, it was noted that almost all of the proteins and CSF activity were found in a breakthrough fraction on HA HPLC without SDS, as shown in Fig. 2a. In contrast, in the presence of 0.1% SDS, CSF activity was retained and eluted with a linear gradient of sodium phosphate on HA HPLC, as shown in Fig. 2b, where CSF fractions were purified ca. eight-fold, by estimation from the chromatographic area.

Although SDS-HA chromatography was first described by Moss and Rosenblum [7] for the analytical separation of protein subunits in a denaturing condition using SDS and dithiothreitol, its mechanism has not yet been fully elucidated. In order to prevent denaturation during the purification of biologically active proteins, it is preferable to use only a low concentration (0.05–0.1%) of SDS in the chromatography.

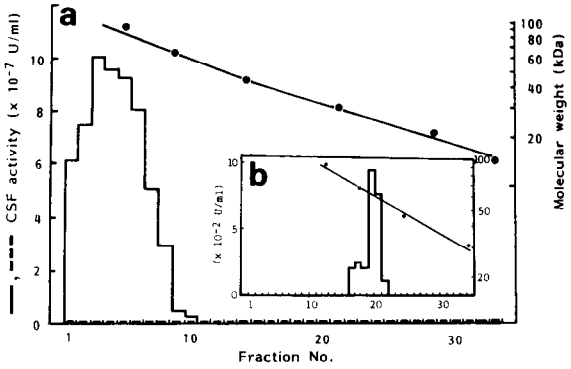

Fig. 1. SDS-PAGE patterns of purified mouse L cell CSF activity. (a) CSF preparation containing ca. 100 μmol of ammonium trifluoroacetate was applied to 12.5% acrylamide gel with (broken line) and without (solid line) 10% 2-mercaptoethanol. (b) Salt-free CSF preparation was applied to 11% acrylamide gel without 2-mercaptoethanol. Molecular markers were phosphorylase b (92 500), bovine serum albumin (66 000), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500) and lysozyme (14 400).

TABLE II

N-TERMINAL AMINO ACID SEQUENCE ANALYSIS OF PURIFIED MOUSE L CELL CSF

Cycle No.	Fractions 2–6 (pmol)			L cell CSF-1 (see ref. 6)
	Without 2-mercaptoethanol		With 2-mercaptoethanol	
1	(Lys)*	167	Not detected	Lys
2	Glu	421		Glu
3	Val	529		Val
4	Ser	86		Ser
5	Glu	286		Glu
6	His	69		His
7	X**	-		Cys
8	Ser	47		Ser
9	His	44		His
10	Met	211		Met
11	(Ile)*	104		Ile
12	(Gly)*	189		Gly
13	Asn	109		Asn

*Residue not clearly identified.
**Not identified owing to malfunction of HPLC.

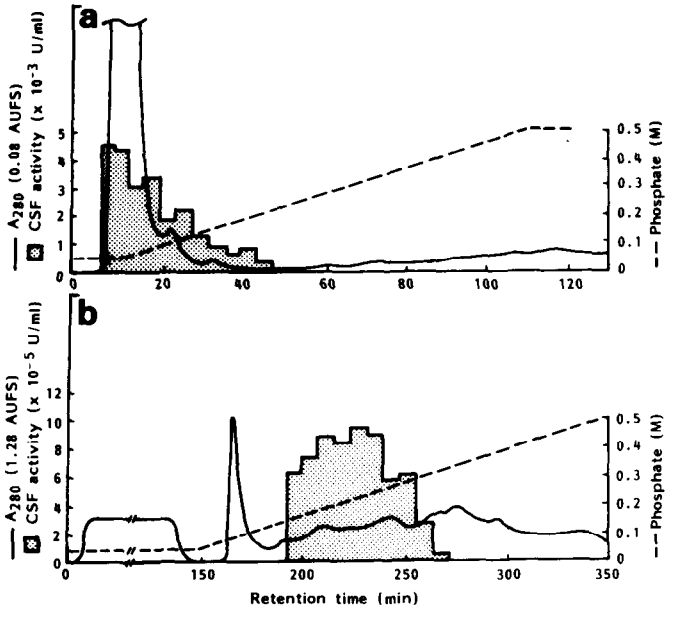

Fig. 2. HA HPLC of mouse L cell CSF. (a) About 60 μg of partially purified CSF eluted from a silica bead column was applied to HA HPLC in the absence of SDS. (b) About 4.5 mg of partially purified CSF eluted from a silica bead column was applied to HA HPLC in the presence of 0.1% SDS.

It is considered that proteins are adsorbed at calcium sites (positive charge) and/or phosphate sites (negative charge) on HA gels [8]. Eluted proteins containing CSF from a DEAE column were mainly acidic proteins so that they would interact with calcium sites on HA gels. As the interaction between calcium ions and carboxyl groups of the protein might be weaker than that between calcium ions and phosphate ions, almost all of acidic proteins would readily break through on the HA column. Conversely, SDS-bound proteins could be adsorbed at calcium sites on HA gels. Furthermore, because the binding amount of SDS would depend on the intrinsic hydrophobicity of a protein and the length of its polypeptides, the resolution of SDS-HA HPLC might partially reflect hydrophobic and molecular sieve chromatographies.

ACKNOWLEDGEMENTS

We thank Mr. T. Kamikouchi for the preliminary chromatographic study of the purification. We also thank Dr. H. Anaguchi, Dr. M. Kajitani, Mr. M. Kihara and Mr. S. Sone for the preparation of mouse L cell CM; Mrs. S. Sasano for the bioassay; Mr. T. Shimazu for the N-terminal sequence analysis; and Drs. S. Yamazaki and Y. Ota for their helpful discussions.

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