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PURIFICATION OF HUMAN INTERLEUKIN 1 BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The utility of high-performance liquid chromatography (HPLC) in the purification of Interleukin 1 (IL 1, lymphocyte-activating factor) has been investigated Human IL 1-containing supernatants were concentrated by lyophilization and desalted using Bio-Gel P-6 DG desalting gel. Subsequently, the sample containing IL 1 activity was subjected to HPLC with a novel HPHT hydroxylapatite column. Using a sodium phosphate gradient, IL 1 was eluted as a single peak of activity separated from the major protein contaminant, yielding 90% recovery and a specific activity of $6.3 \cdot 10^4$ U/mg. Pooled fractions from Bio-Gel HPHT were concentrated and subjected either to Bio-Sil IEX 540 DEAE anion-exchange or Bio-Sil TSK 125 size exclusion chromatography. From DEAE the IL 1 activity was eluted before a linear sodium chloride gradient was started, whereas the protein contaminant was eluted at 110 m*M* NaCl. When TSK was used IL 1 activity was eluted within a molecular weight range of 20,000–10,000.

Fractions from the DEAE or TSK columns that were positive for IL 1 activity did not contain detectable protein, suggesting a good resolution. Furthermore, the recovery from DEAE was 26% whereas TSK 125 yielded 119% of the original activity. The specific activities were $6 \cdot 10^7$ and $2.5 \cdot 10^8$ U/mg, respectively. Thus, this method provides a rapid and reproducible procedure for the purification if IL 1 for further biological characterization.

INTRODUCTION

Interleukin 1 (IL 1), otherwise known as lymphocyte-activating factor (LAF), is a macrophage-derived hormone-like mediator with a multiplicity of immunological activities which have recently been reviewed[1,2]. Briefly, Interleukin 1 augments the mitogenic response of thymocytes, induces the production of lymphokines such as Interleukin 2 (T-cell growth factor) by lymphocytes and stimulates polymorphonuclear leucocytes and monocytes[2,3]. In addition IL 1 also stimulates hypothalamic cells to induce fever, induces *in vitro* fibroblast growth and prostaglandin and collagenase production[2,4].

The purification and characterization of IL 1 has been difficult, because, pro-

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duced in only minute quantities, it is biologically active in the 10^{-10}–10^{-15} *M* range and must be separated from a complex mixture of unrelated proteins. Although considerable progress has been made in the biochemical characterization of IL 1, this mediator has not yet been purified to homogeneity. The methods used are time consuming, involving procedures which can denature the protein being purified. Thus, we investigated the utility of high-performance liquid chromatography (HPLC), using aqueous elution conditions, in order to improve the speed and recovery of IL 1 activity compared to previously reported procedures.

MATERIAL AND METHODS

Production and assay of Interleukin 1

Crude human Interleukin 1 was prepared by incubation of human adherent peripheral blood lymphocytes (PBL) in the presence of 50 μg/ml silica (Sigma, St. Louis, MO, U.S.A.) as previously described[5]. Crude preparations usually contained $5.8 \cdot 10^2$ units per ml and $10.3 \cdot 10^3$ units per mg of protein. The enhancement of nitrogen-stimulated C_3H/He mouse (Versuchstieranstalt, Hannover, F.R.G.) thymocyte proliferation was used for the detection of IL 1 activity as described elsewhere[6]. Briefly, thymocytes in suspension culture were incubated for 3 days at $1.5 \cdot 10^6$ cells per well in microculture plates in the presence of 1 μg/ml concanavalin A (Con A; Calbiochem.-Behring, La Jolla, CA, U.S.A.) and various concentrations of IL 1. In order to quantitate the degree of DNA synthesis, cultures were pulsed with tritiated thymidine (^{3}H-TdR, 0.5 μCi per well) for the final 6 h of incubation. Cells were collected and counted in an automatic scintillation counter. In order to have a more reliable index of IL 1 activity, units of IL 1 activity were established as described previously[7].

Concentration and purification of Interleukin 1

In order to concentrate supernatants, the samples were freeze-dried using a lyophilizer (Edwards, Crawley, U.K.). Subsequently, the samples were resolved in distilled water and desalted by gel filtration using Bio-Gel P-6 DG (Bio-Rad, Richmond, CA, U.S.A.) desalting gel. Ten millilitres of 36.5-fold concentrated crude IL 1 were applied to a 2.5 $\times$ 10 cm column. The column was equilibrated in distilled water using a flow-rate of 300 ml/h. Eluents were monitored by UV absorbance at 254 nm and the protein-containing fractions (25 ml) were collected. This procedure was repeated three times and the resulting 75 ml were lyophilized and rehydrated in 200 μl distilled water.

High-performance liquid chromatography

All HPLC procedures were carried out at room temperature using two Kontron LC 410 pumps, a Kontron System Controller Model 200 and a Rheodyne 7010 Injector (Rheodyne, Berkely, CA, U.S.A.). Column eluents were monitored on a Uvicon 720 LC ultraviolet visible variable-wavelength detector (Kontron). The results were plotted on a Shimadzu C-R1B integrator (Kontron) and fractions were collected using a Pharmacia Frac 100 fraction collector (Pharmacia, Uppsala, Sweden).

The columns used were a Bio-Gel HPHT Hydroxylapatite column (100 $\times$ 7.8

mm) with an attached guard column (50 × 4 mm) packed with a spherical hydrophilic polymer matric (Bio-Rad), an anion-exchange column (Bio-Sil TSK IEX 540 DEAE, 300 × 4 mm; Bio-Rad) and size exclusion columns (Bio-Sil TSK 125, 300 × 7.5 mm and Bio-Sil TSK guard column, 75 × 7.5 mm; Bio-Rad).

Concentrated sample (100 μl) was applied to the HPHT column, which was equilibrated with 0.01 *M* phosphate buffer containing 0.3 m*M* $CaCl_2$. Elution was carried out by applying a continuous gradient ranging from 0.01 *M* sodium phosphate and 0.3 m*M* $CaCl_2$, to 0.5 *M* sodium phosphate and 0.01 m*M* $CaCl_2$ (pH 7.4), at a flow-rate of 1 ml/min. The gradient applied was linear from 7 to 11 min, isocratic from 11 to 14 min (0.25 *M* phosphate) and linear again from 14 to 18 min. Subsequently the column was regenerated with 0.01 *M* phosphate buffer, pH 6.5. Eluents were monitored by UV absorbance at 280 nm and 0.5-ml fractions were collected, dialysed against RPMI 1640 medium (Grand Island Biological, Grand Island, NY, U.S.A.) before being tested.

Subsequently the fractions containing IL 1 activity were pooled (3 ml), lyophilized, desalted as described above, rehydrated in 200 μl distilled water and 100-μl samples were subjected to either DEAE anion exchange or Bio-Sil TSK 125 size exclusion chromatography. The DEAE column was equilibrated with 50 m*M* Tris buffer (pH 7.2) and elution was carried out using a linear sodium chloride gradient (0–0.5 *M* NaCl) at a flow-rate of 1.5 ml/min from 4 to 18 min. Between 18 and 20 min the gradient was isocratic. Subsequently the column was regenerated with 0.05 *M* Tris buffer. The optical density of the eluate was measured at 280 nm. When a TSK 125 column was used, elution was carried out isocratically with phosphate-buffered saline (PBS) pH 7.2, at a flow-rate of 1 ml/min. The column effluent was monitored by UV absorbance at 210 nm.

Protein determination

Protein concentration were determined by the method of Lowry *et al.*[8] with bovine serum albumin as standard.

RESULTS

Human Interleukin 1 was produced by induction of adherent cells derived from buffy coats with silica in RPMI 1640 medium. After removal of the cells, the supernatants were lyophilized, desalted using Bio-Gel P-6 DG desalting gel and rehydrated with distilled water. Using this procedure, the recovery was still 95% and the specific activity was $0.99 \cdot 10^3$ U/mg (Table I). Although lyophilization and desalting of large amounts of supernatants is time-consuming, the only slight loss of activity justifies the use of this method.

In attempts to utilize the speed of HPLC in the purification of human IL 1 we first investigated a new Bio-Gel hydroxylapatite column (HPHT). Using a linear sodium phosphate gradient, IL 1 activity was eluted with the major protein peak (data not shown). In order to improve the separation, a continuous sodium phosphate gradient was used and IL 1 activity was eluted as a single peak between 0.25 and 0.28 *M* sodium phosphate (Fig. 1). In contrast, the major bulk of proteins, as determined by UV absorption, was eluted between 0.1 and 0.2 *M* sodium phosphate. Because of its good resolution and high capacity, the Bio-Gel HPHT is suitable for

TABLE I

PURIFICATION OF HUMAN INTERLEUKIN 1

	Amount (ml)	*Total activity (units)*	*Specific activity (units/mg)*	*Purification factor*	*Recovery (%)*
Crude IL 1	365	$2.1 \cdot 10^5$	$10.3 \cdot 10^3$	–	100
Concentration (lyophilization)	30	$1.9 \cdot 10^5$	$0.94 \cdot 10^3$	–	90
Bio-Gel P-6 DG	75	$2.0 \cdot 10^5$	$0.99 \cdot 10^3$	–	95
Concentration (lyophilization)	0.2	$2.0 \cdot 10^5$	$0.99 \cdot 10^3$	–	95
HPHT	4.0	$1.9 \cdot 10^5$	$6.3 \cdot 10^4$	6.1	90
Concentration (lyophilization)	100	$1.7 \cdot 10^5$	$6.1 \cdot 10^4$	–	81
DEAE	2	$6.0 \cdot 10^4$	$>6.0 \cdot 10^7$*	5820	26
or					
TSK 125	4.5	$2.5 \cdot 10^5$	$>2.5 \cdot 10^8$	24,270	119

* Only the major peak of IL 1 activity was used.

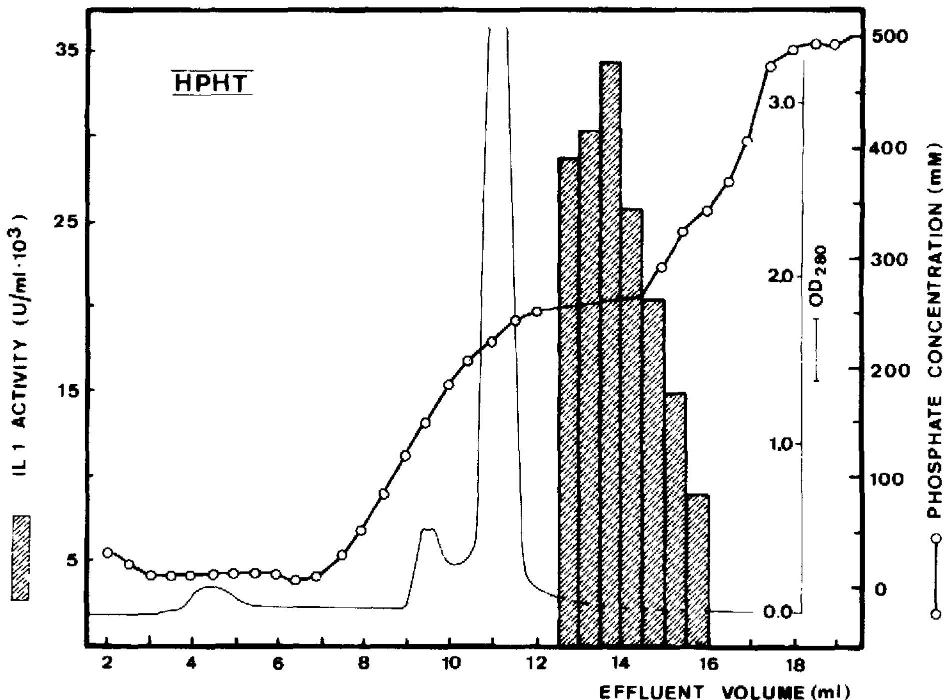

Fig. 1. HPLC of human Interleukin 1 on a Bio-Gel HPHT hydroxylapatite column. Applying a phosphate, $CaCl_2$ gradient at a flow-rate of 1 ml/min (O—O), IL 1 activity (▨) was eluted between 0.23 and 0.28 *M* sodium phosphate, whereas the protein contaminant (|—|) was eluted between 0.1 and 0.2 *M* sodium phosphate.

an early step in the purification of Interleukin 1. Additionally, Bio-Gel HPHT separation of IL 1 yielded 90% of the activity and a 6.1-fold purification (Table I).

Pooled fractions from Bio-Gel HPHT were concentrated by lyophilization, desalted and subjected to either Bio-Sil TSK IEX 540 DEAE anion-exchange chromatography or Bio-Sil TSK 125 size exclusion chromatography. Using the DEAE anion exchanger and applying a linear sodium chloride gradient, the protein contaminant was eluted between 0.1 and 0.15 *M* sodium chloride, whereas the major peak of IL 1 activity was eluted before the gradient was started (Fig. 2). However, some of the IL 1 activity (11%) was eluted at a higher salt concentration. The highly purified fraction containing the major peak of IL 1 activity contained no detectable amounts of protein, but only 26.0% of the original IL 1 activity, had a specific activity of $6 \cdot 10^7$ U/mg and represented a 5820-fold purification (Table I).

The major protein contaminant of the concentrated partially purified (HPHT) IL 1 preparation exhibited a molecular weight of approximately 70,000 on the TSK

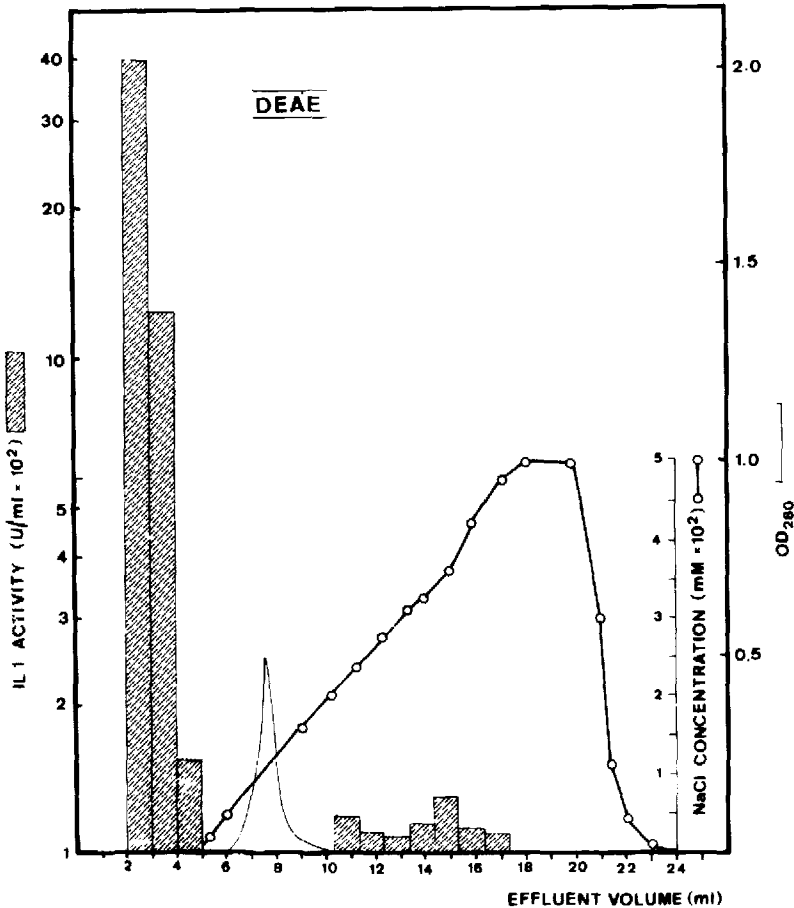

Fig. 2. HPLC of human Interleukin 1 on a Bio-Sil IEX 540 DEAE anion-exchange column. Applying a linear NaCl gradient at a flow-rate of 1.5 ml/min (O—O), IL 1 activity (▨) was eluted before the gradient was started. The major protein peak (|—|) was eluted between 0.1 and 0.12 *M* NaCl.

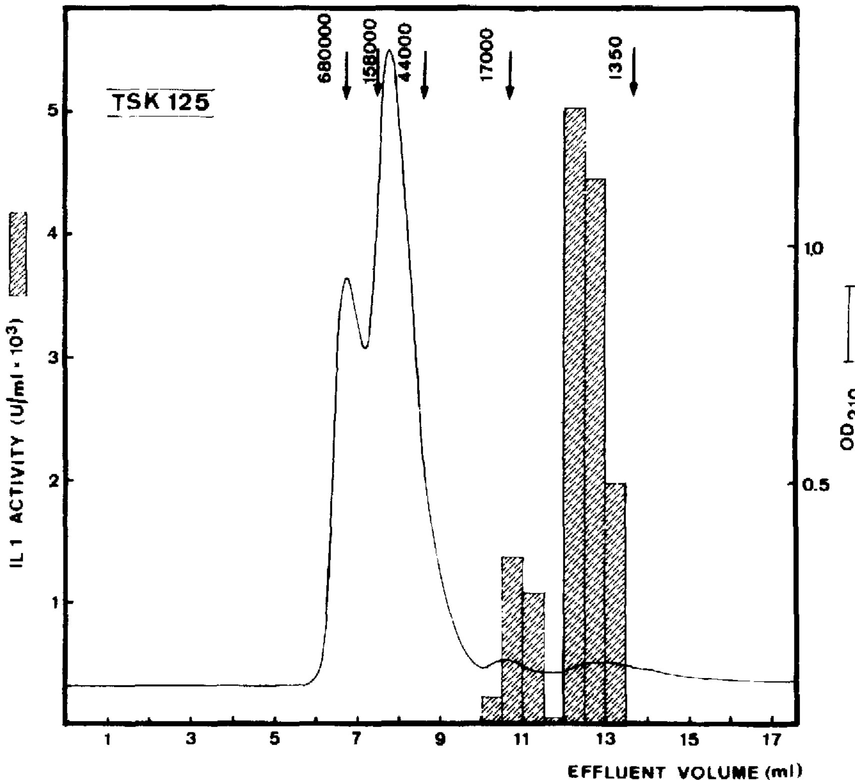

Fig. 3. HPLC of human Interleukin 1 on a Bio-Sil TSK 125 size exclusion column. Elution was carried out with PBS, at a flow-rate of 1 ml/min. IL 1 activity (▨) was eluted within a molecular weight range of 20,000–10,000, whereas the contaminant protein (|—|) exhibited a MW of approximately 70,000.

125 column. This peak was tentatively identified as serum albumin present in the cell culture media. The main IL 1-containing peak was clearly resolved from the contaminant and was eluted within a MW of 25,000–12,000 (Fig. 3). As is the case after DEAE anion-exchange chromatography, the fraction containing IL 1 had an activity of $2.5 \cdot 10^8$ U/mg and represented a 24,270-fold purification (Table I).

DISCUSSION

Interleukin 1 has previously been purified to apparent homogeneity using conventional time-consuming multiple concentration, chromatographic and electrophoretic procedures[9]. The recovery of this highly purified IL 1 was usually very low. Thus, the purpose of this study was to increase the recovery of IL 1 by employing HPLC as a rapid purification technique. In addition, we used a different method to concentrate and desalt samples, including lyophilization and the Bio-Gel P-6 DG desalting gel. Although this method is somewhat time-consuming, the recovery was improved to 95%, compared to a conventional Amicon ultrafiltration cell with UM 10 membranes which usually yielded 80% of the originally activity[5].

The Bio-Gel HPHT hydroxylapatite column has recently been introduced for

protein and nucleic acid separation. Hydroxylapatite separates macromolecules by surface binding to phosphate binding sites and not by size, charge or biological activity, and thus results in high resolution[10,11]. Because of the capacity of 100 mg protein, the high speed and high resolution, we felt this might be a suitable first step for the purification of IL 1. Indeed, applying a continuous sodium phosphate gradient, IL 1 was separated from the major proteins and the recovery (90%) was very good. Additionally, the HPHT column was also used for the separation of an IL 1-like mediator, produced by epidermal cells, which has recently been described as epidermal-cell thymocyte-activating factor (ETAF)[5]. ETAF, like IL 1, is eluted as one major peak of activity between 0.23 *M* and 0.28 *M* sodium phosphate[12]. Thus, HPHT also is suitable for the purification of ETAF, and the similar elution profiles of IL 1 and ETAF further supports our hypothesis that the mediators, although produced by different cells, are identical.

HPLC with a DEAE anion-exchange column for the separation of partially purified (HPHT) IL 1 also yielded a high resolution together with a reasonable recovery (26%). However, it was somewhat surprising that IL 1 under the conditions described above mainly was eluted before the sodium chloride gradient was started. In contrast, using DEAE anion-exchange chromatography, IL 1 or ETAF activity was eluted as a single peak at 0.15 *M* sodium chloride[7]. In these experiments, elution was carried out using 0.05 *M* Tris and 0.1 *M* NaCl as starting buffer at pH 10.0. Thus, the different buffer conditions as well as the different pH values may account for the different elution profiles. However, DEAE anion-exchange chromatography provides an additional purification step since the IL 1-containing fractions were not contaminated with detectable amounts of protein.

TSK 125 size exclusion chromatography constitutes another improvement in purification. This method also yielded no contaminating serum proteins within the major IL 1 peak. In addition, a 24,270-fold concentration could be achieved and the recovery (119%) was clearly better than on DEAE. These results indicate that HPLC using TSK 125 is a good step for further purification of IL 1 and also may be used in sequence after DEAE anion-exchange chromatography. Furthermore, the use of TSK 125 columns also yielded a very good separation of ETAF. ETAF, like IL 1 was eluted as a major peak exhibiting a MW of approximately 15,000[12].

The data reported here demonstrate that it is possible to obtain significant purification of human IL 1 within a few days using lyophilization, Bio-Gel HPHT hydroxylapatite and DEAE anion exchange or TSK 125 size exclusion chromatography. The high recovery in part may be due to the high speed of HPLC and in part to the use of mild elution conditions. The relatively high quantitative recovery of IL 1 activity is not unusual in IL 1 purification[5] and may be due to the removal of IL 1-inhibiting and or thymocyte growth-blocking components in the crude preparations. Thus, the method described here is a rapid and reproducible technique for the purification of IL 1, which can be purified to a specific activity over $2.6 \cdot 10^8$ U/mg. This protocol is useful for rapid production of human IL 1 for further characterization.

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