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RAPID SEPARATION OF BIOLOGICALLY ACTIVE SYRIAN HAMSTER LYMPHOTOXIN IN HIGH YIELD BY SIZE-EXCLUSION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, simple and reproducible high-performance liquid chromatography (HPLC) procedure is described for the partial purification of biologically active Syrian hamster lymphotoxin. Lymphotoxin, a series of lymphocyte derived immunological glycoprotein hormones with molecular weights of 20,000 to 56,000, has been partially purified by a single-step HPLC procedure or by a two-step isoelectric focusing–HPLC procedure. The HPLC separation method uses silica-based protein separation columns eluted with a 0.1 *M* sodium phosphate, pH 7.4, 0.1% polyethylene glycol 4000 buffer at a flow-rate of 0.5 ml/min at room temperature. Nearly complete recoveries of biologically active lymphotoxin can be obtained with as much as a 13-fold purification in less than 1 h.

INTRODUCTION

Lymphokines are immunological hormones which are released by stimulated lymphocytes (see ref. 1 for review). Lymphotoxin, a cytotoxic lymphokine secreted by mitogen- or antigen-stimulated lymphocytes, inhibits tumor cell growth *in vitro* and *in vivo*[2] and recently has been shown to inhibit carcinogenesis, the process of the conversion of a normal cell to a tumor cell[3]. Lymphotoxin is a multicomponent family of glycoproteins with isoelectric points that occur frequently in the range of pH 4.5 to 5.5 and with molecular weights distributed from 12,000 to greater than 200,000 daltons[4,5].

Current interest in lymphokines, especially those that exhibit antitumor activity, requires highly efficient isolation techniques. Lymphotoxin is present in extremely low concentration in biological fluids and has been difficult to purify to high specific activity without extensive loss of biological activity. Methods for the purification of lymphotoxins have utilized standard techniques including ammonium sulfate precipitation, gel filtration chromatography, ion exchange chromatography, lectin affinity chromatography, hydrophobic chromatography, and gel electrophoresis[6–9]. While gel filtration has typically afforded good recoveries of lymphotoxin,

especially with the use of gelatin as a stabilizer[10], the time required for this type of chromatography can be as long as 24 h. Lymphotoxins of most species tend to lose activity upon storage, during freezing and thawing and during lengthy or vigorous purification protcols[6,8,9,11]. Because of its increased speed relative to other methods, high-performance liquid chromatography (HPLC) was evaluated as an isolation method which might substantially reduce the time required for the purification of a labile hormone such as lymphotoxin.

The separation of proteins by HPLC has been described[12] utilizing size-exclusion, ion-exchange, affinity, and reversed-phase chromatography. Recent studies also have shown that low-molecular-weight biologically active peptides[13] can be analyzed and preparatively isolated by HPLC. Ion-exchange HPLC methods have been employed for separation of biologically active isoenzymes with impressive recoveries of 85% or more[14] and reversed-phase liquid chromatography is being utilized increasingly for separations of polypeptides and other biologically active materials[15]. Ion-exchange methods require the use of salt gradients, which can destroy biological activity. Reversed-phase applications, on the other hand, require the use of organic solvents and low pH, and frequently are associated with low recoveries of higher-molecular-weight proteins[13,16]. Either method would severely complicate the [^{3}H]thymidine release biological assay required for the determination of lymphotoxin activity in the column effluent. Size-exclusion chromatography and low ionic strength, near neutral pH sodium phosphate buffers determined to be compatible with both the lymphotoxin assay and column performance, therefore, were evaluated in this study to determine if separations would be sufficient to remove unrelated proteins and other macromolecules while retaining biological activity of the isolated lymphotoxin equal to or in excess of non-HPLC methods for which recoveries of 10 to 80% of the original biological activity are reported[6–8].

EXPERIMENTAL

Lymphotoxin preparation

Lymphotoxin was prepared by *in vitro* lectin stimulation of Syrian hamster leukocytes as described previously[3]. Briefly, mineral oil induced peritoneal leukocytes from Syrian golden NIH/N hamsters were cultured with phaseolus vulgaris phytohemagglutinin (PHA) (Burroughs Wellcome, Research Triangle Park, NC, U.S.A.) for 24 h in serum-free RPMI-1640 medium. The culture medium containing secreted lymphotoxin was centrifuged and concentrated 10-fold in a diafiltration cell on a 10,000 nominal molecular weight (MW) exclusion limit YM10 membrane (Amicon, Lexington, MA, U.S.A.) against 0.01 *M* sodium phosphate buffered saline pH 7.4 (PBS) containing 0.1% (w/v) 4000 MW polyethylene glycol (PEG 4000) (Sigma, St. Louis, MO, U.S.A.)[17]. Lymphotoxin activity was assayed by release of [^{3}H]thymidine from αL929 cells as described in detail elsewhere[2]. Protein concentration was determined by the method of Lowry *et al.*[18].

Isoelectric focusing

Diafiltered lymphotoxin was further purified by preparative isoelectric focusing (IEF) on a pH 4–6 ampholine gradient. IEF was performed in a 110-ml preparative IEF column (LKB, Bromma, Sweden) on a 5–50% (w/v) sucrose gradient containing

1.0% ampholines. The gradient was formed by mixing a 54-ml solution containing 5% sucrose, 0.7 ml pH 4–6 ampholines (LKB), 0.1% PEG and 0.25–0.5 ml lymphotoxin with a second 54-ml solution containing 50% sucrose, 2 ml pH 4–6 ampholines, 0.1% PEG and 0.75–1.5 ml lymphotoxin[17]. The anode electrolyte at the bottom of the column was 0.16 *M* orthophosphoric acid and 60% sucrose, the cathode electrolyte was 0.25 *M* sodium hydroxide solution. Focusing was performed for 16 h at 4°C at a constsnt power of 15 W, 1.6 kV (max.). The 3-ml fractions, collected from the bottom of the column, were diluted 1:2 with PBS, sterile filtered through 0.22 μm Millex-GV filters (Millipore, Bedford, MA, U.S.A.), and assayed for lymphotoxin activity.

HPLC separations

Separations were accomplished on two I-125 or two I-125 and one I-60 30 × 0.78 cm silica-based protein separation columns (Waters Assoc., Milford, MA, U.S.A.) connected in series to a Waters Model 6000 pump. Columns were equilibrated and eluted isocratically with 0.1 *M* sodium phosphate, pH 7.4, containing 0.1% (w/v) PEG at room temperature. Eluate was monitored with a Waters Model 440 absorbance detector at 280 nm. Samples were injected in 250-μl volumes via a Waters Model U6K injector. Fractions of 0.5 ml were collected, sterile filtered and assayed for lymphotoxin activity (see above) and protein concentration.

The molecular weights of lymphotoxin components were estimated by comparing the HPLC retention times of lymphotoxin components with those of proteins with well-characterized molecular weights. Protein standards and their molecular weights were bovine serum albumin (BSA), 68,000 (Miles Labs, Elkhart, IN, U.S.A.), ovalbumin (OVA), 45,000, soybean trypsin inhibitor (STI), 21,500, and ribonuclease I (RNASE), 13,700 (Sigma) and bovine pancreas insulin, 6000 (Calbiochem, San Diego, CA, U.S.A.). Blue dextran (BD), 2,000,000 (Pharmacia, Uppsala, Sweden), was used as a void volume marker.

RESULTS

Protein standards were eluted from two series-connected I-125 protein separation columns with good separations at a flow-rate of 0.5 ml/min. Resolution of high-MW protein standards was insufficient at flow-rates of 1 or 2 ml/min. Lower flow-rates increased the separation of standard proteins particularly between the void volume and the 40,000 MW region. Similar observations have been reported by Regnier and Gooding[12]. A flow-rate of 0.3 ml/min gave the best separation of BSA (68,000 MW) and OVA (45,000 MW) but increased development time to nearly 2 h. At 0.5 ml/min, BSA and OVA peaks remained separable with peak retention times differing by approximately 1.5 min. At this flow-rate, a total development time of less than 1 h was achieved for proteins ranging from greater than 100,000 to 6000 daltons. The effects of varying solvent ionic strength and pH on protein standard retention times were also investigated. A 0.1 *M* sodium phosphate, pH 7.4, buffer, which provided conditions favorable for the maintenance of lymphotoxin activity, produced protein separations that were adequate for the partial purification of lymphotoxin components without risking excessive column degradation due to elevated pH.

Chromatography of lymphotoxin utilizing the conditions optimized for sepa-

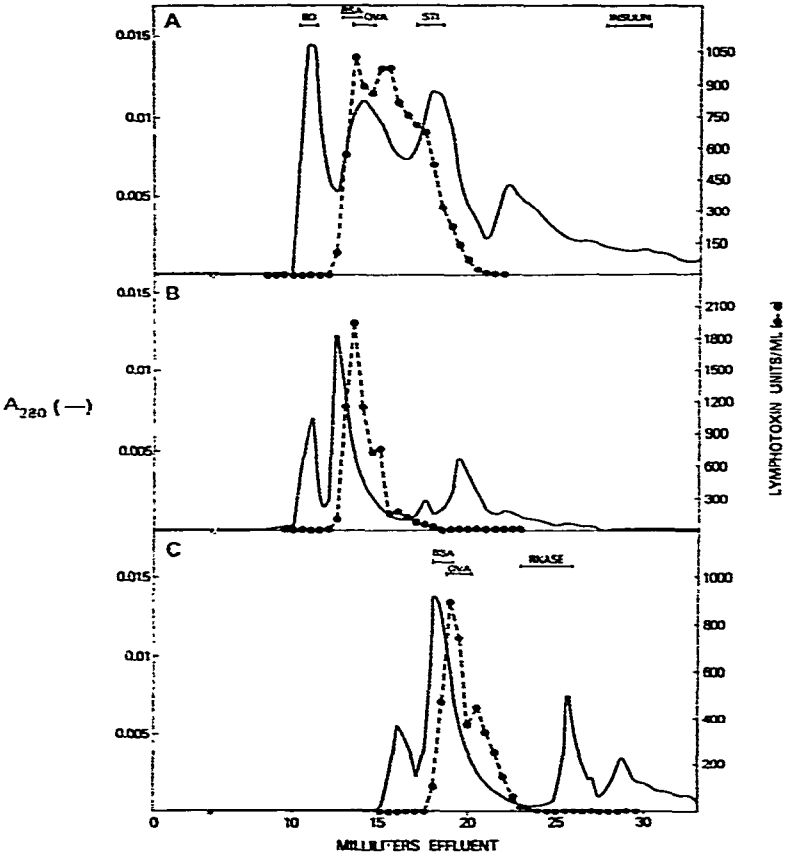

Fig. 1. Separation of hamster lymphotoxin on rigid, hydrophilic, silica gel columns by HPLC. Diafiltered (A) or IEF purified (B) lymphotoxin separated on two Waters I-125 protein separation columns connected in series. (C) IEF purified lymphotoxin separated on two Waters I-125 and one Waters I-60 protein separation columns connected in series. Columns were eluted at 0.5 ml/min with 0.1 *M* sodium phosphate, pH 7.4 containing 0.1% PEG as a stabilizer for lymphotoxin activity. Fractions of 0.5 ml were collected and assayed for lymphotoxin activity. Protein standards were: BSA (68,000 MW), OVA (45,000 MW), STI (21,500 MW), RNASE (13,500 MW), and insulin (6000 MW). Positions of protein standards in (B) are as indicated in (A).

ration of protein standards produced a multicomponent pattern of 280 nm absorbing material, yet, failed to yield eluted material with any residual biological activity. These results suggested that a protective agent which would stabilize lymphotoxin activity was needed. Gelatin has been reported to stabilize lymphotoxin during chromatographic procedures[10]. Low concentrations of PEG have been used to stabilize the activity of interleukins[19], to reduce free radical damage[20] and recently at a higher concentration of 0.1%, to protect lymphotoxin from loss of biological activity during IEF[17]. Since gelatin is a mixture of variously sized, charged species, PEG, a small unchanged molecule, was selected for use in HPLC. To determine its effectiveness as a stabilizer during HPLC, PEG 4000 was added to the elution buffer and the effect of its presence was evaluated on the separation of protein standards. At a concentration of 0.1%, PEG caused a slight broadening of protein standard peaks and decreased the

TABLE I

PURIFICATION OF BIOLOGICALLY ACTIVE SYRIAN HAMSTER LYMPHOTOXIN

ND = Not determined.

*HPLC separation**	*Columns*	*Specific activity applied to column*		*Specific activity recovered*		*Purification factor*		*Biological activity recovered (%)*
		LT Units A_{280}	*LT Units mg Protein*	*LT Units A_{280}*	*LT Units mg Protein*	*(A_{280})*	*(Protein)*	
Diafiltered	Two							
Peak	I-125							
1 (13–14.5)**			ND	9929	ND	2.1	ND	
2 (15–15.5)			ND	16,491	ND	3.5	ND	
3 (16–18)			ND	8955	ND	1.9	ND	
Total (13–18)		4691	ND	14,022	ND	3.0	ND	87.2
IEF Purified	Two							
Peak	I-125							
1 (13–14.5)				99,269	375,265	4.1	4.6	
2 (15–15.5)				73,443	110,617	3.0	1.3	
3 (16–17)				48,876	145,000	2.0	1.8	
Total (13–17)		24,544	82,506	89,986	262,922	3.7	3.2	85.1
IEF Purified	Two							
Peak	I-125							
1 (18.5–20)	+			60,973	430,522	5.1	9.1	
2 (20.5–21.5)	one			96,939	625,000	8.1	13.2	
3 (22–23)	I-60			25,938	26,211	2.2	0.6	
Total (18.5–23)		11,905	47,524	67,628	313,871	5.7	6.6	145.6

* Data are derived from HPLC separations presented in Fig. 1.

** Numbers in parentheses are milliliters effluent of HPLC separations in Fig. 1.

resolution between BSA and OVA by only a small amount.

When diafiltered lymphotoxin was subjected to HPLC on two protein separation columns equilibrated and eluted with 0.1 *M* sodium phosphate containing 0.1% PEG 4000, lymphotoxin activity was eluted in two distinct peaks with molecular weights of approximately 45,000–56,000 and 30,000–35,000 daltons and in a shoulder indicative of a third peak of approximately 20,000–25,000 daltons (Fig. 1A). The separation of active lymphotoxin required only 40 min. Recovery of lymphotoxin activity was 87%. Material absorbing at 280 nm was eluted throughout the whole MW range, however, the ratio of 280 nm absorbing material eluting with lymphotoxin activity to total 280 nm absorbing material was less than 30%. Specific activities calculated as units of lymphotoxin activity per quantity of 280 nm absorbing material, estimated by integration of the absorbance peak areas, were increased by a factor of 3 (seee Table I).

To assess further the purification of lymphotoxin, HPLC separations of diafiltered lymphotoxin were compared with HPLC separations of lymphotoxin purified by diafiltration and preparative IEF. Diafiltered lymphotoxin focused as a single peak between pH 4.4 and pH 5.0 (Fig. 2). Fractions between pH 4.0 and 5.5 containing lymphotoxin activity were pooled, concentrated by diafiltration and separated by HPLC as described above. Lymphotoxin activity was found in two peaks (Fig. 1B)

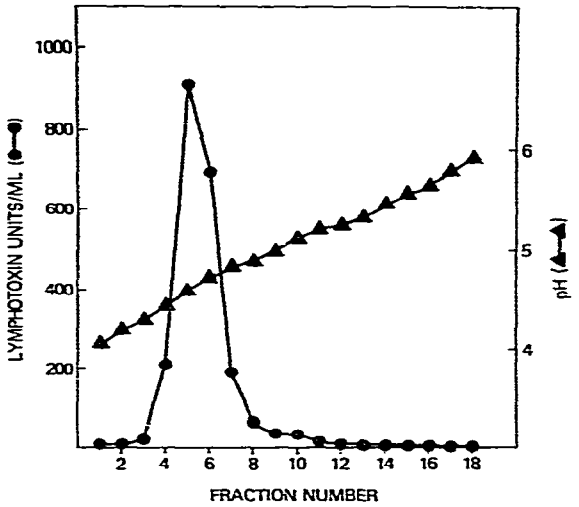

Fig. 2. Partial purification of hamster lymphotoxin by preparative IEF in the presence of PEG. IEF was conducted on a pH 4–6 ampholine gradient, 3 ml fractions were collected, and aliquots assayed for lymphotoxin activity.

with retention times identical to those of the two major 45,000–56,000 and 30,000–35,000 dalton lymphotoxin peaks seen in the separation of diafiltered non-focused lymphotoxin (Fig. 1A). The 20,000–25,000 MW peak of non-focused lymphotoxin activity, however, was absent from the IEF purified material and the quantity of lymphotoxin in the 30,000–35,000 dalton range was significantly reduced. Similar results were obtained when IEF purified lymphotoxin was chromatographed on two Waters I-125 protein separation columns connected in tandem with a lower MW-separating I-60 protein column (Fig. 1C). Lymphotoxin specific activities were increased during these separations 3.7- and 5.7-fold, respectively and up to 13-fold for individual components (see Table I).

DISCUSSION

These results demonstrate that lymphotoxin can be purified with high recovery of biological activity and can be resolved into several molecular components by rapid, non-denaturing chromatography on silica-based size exclusion HPLC columns. HPLC separation of diafiltered lymphotoxin activity into three distinct molecular weight regions (Fig. 1A) has not been reported previously for hamster lymphotoxin. HPLC separation of lymphotoxin following purification by preparative IEF resulted in the elution of two peaks (Fig. 1B) corresponding in molecular weight to peaks 1 and 2 of the HPLC separation of diafiltered lymphotoxin (Fig. 1A). The absence of a third peak in the 20,000–25,000 MW region and the reduction in relative quantity of the peak 2 material have not been investigated in these studies but may be due to variations in lymphotoxin preparations or to differential loss of activity by the lower-molecular-weight species of lymphotoxin during the combined IEF–HPLC procedure. These results and others (not shown) in which peak 1 is the predominant form of lymphotoxin detected suggest that the 45,000–56,000 MW component may be the

most stable form of hamster lymphotoxin.

The lymphotoxin purification procedure reported here is rapid, simple, and highly reproducible which should make it useful as a preparative method in which recently available high-volume preparative HPLC protein separation columns can be utilized. Using similar HPLC methods, biologically active interluken-1, another immunological hormone, has been purified 300-fold[21,22]. Further investigation of the nature of the interaction of lymphotoxin with the hydrophilic silica stationary phase should permit improved separation of the molecular forms of lymphotoxin as well as increase the overall efficiency of the procedure.

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