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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PURIFICATION OF ENDOGENOUS TUMOUR CELL GROWTH INHIBITORY PEPTIDES

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SUMMARY

Five endogenous growth inhibitors of JB-1 ascites tumour cells have been further purified and characterized. Probably because of the high biological activity of most of the inhibitors which are low molecular weight peptides, these have been extremely difficult to handle using conventional purification methods. However, high-performance liquid chromatography (HPLC) turned out to be a very powerful and useful technique in the last purification steps. Although many types of HPLC packings were tried, only a few of them (Nucleosil 5C-18 and Nucleosil 5CN) behaved satisfactorily for the present purpose.

INTRODUCTION

In a series of studies it has been shown that ascites tumours in their plateau phase of growth contain substances or activity which inhibit the progression of the ascites cells through the cell cycle [1—4]. The biological activity has been tested on various tumour cells in culture using flow cytometry [5] for screening the fraction during the purification procedures. Most of the inhibitory activity turned out to be of peptidic nature [6,7], however, nearly all standard peptide purification methods had their drawbacks as described in the Discussion.

The problems with purification of the active factors were minimized by taking advantage of their good solubility in polar organic solvents [7,8]. The active factors have now been purified by means of Sephadex LH-20 and improved high-performance liquid chromatographic (HPLC) techniques.

MATERIALS AND METHODS

Preparation of tumour cell inhibitory extract

The inhibitory activity was obtained from out-grown JB-1 ascites tumours in

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their plateau phase of growth and was purified by (1) DE-52 cellulose chromatography and (2) Sephadex G-15 chromatography of the methanol-soluble material as recently described [7,8]. The active fractions were further purified by Sephadex LH-20 chromatography and HPLC as described below.

Chemicals and reagents

The water used in all operations was deionized laboratory water purified by the Milli-Q reagent-grade water system (Millipore, Bedford, MA, U.S.A.). Methanol and acetonitrile were of HPLC grade (Rathburn Chemicals, Peeblesshire, Great Britain), and acetic acid was of sequencer grade (Rathburn). Trifluoroacetic acid, 99% pure, was obtained from Pierce Chemical Company, Rockford, IL, U.S.A. Ethanol, 96% or 99% (De Danske Spritfabrikker, Copenhagen, Denmark) was distilled from a mixture with charcoal before use.

Sephadex LH-20 chromatography

Twenty to fifty milligrams of freeze-dried active fraction from Sephadex G-15 were dissolved in 1.5 ml of the mixture ethanol—0.1% trifluoroacetic acid in water (4:1) and applied to a Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column and eluted with the above-mentioned solvent mixture. Five milligrams of glycerol were added to pooled fractions which were evaporated in vacuo at 40°C in a Rotavapor R-110 (Büchi Laboratoriums-Technik, Flawil, Switzerland) and lyophilized to dryness. Aliquots were taken for biological testing.

High-performance liquid chromatography

Two types of columns packed with Nucleosil® 5 μm C_{18} and Nucleosil® 5 μm CN (Macherey-Nagel & Co., Düren, G.F.R.) were used. The columns were packed as described in detail elsewhere [9]. All chromatograms run in the isocratic mode and at room temperature were done using a Waters HPLC apparatus (Waters, Milford, MA, U.S.A.) consisting of two Model 6000A pumps, a Model 660 solvent flow programmer, a Model U6K injector, a Model 440 fixed-wavelength UV detector, having an 8-μl flow-through cell, and an Omniscribe® dual-channel chart recorder (Houston Instruments, Austin, TX, U.S.A.). Samples were injected via the U6K injector with a 25-μl syringe (Hamilton, Reno, NV, U.S.A.) into the column.

For the chromatograms performed with acetonitrile gradients, a Hewlett-Packard 1084B liquid chromatograph (Hewlett-Packard, Geneva, Switzerland) equipped with a processor-controlled sampling and gradient elution system was used. The column was operated in an accessible temperature-controlled compartment. The absorbance of the column eluate was measured with a Cecil CE 588 UV Scanning Spectrophotometer (Cecil Instruments, Cambridge, Great Britain). Samples were injected into the column with the automatic sampling system.

Prior to use, all solvents used for HPLC were filtered under suction through 0.5-μm FH Millipore filters to remove solid particles and to degas solutions.

Fractions were taken from the chromatographic runs by hand as judged from the absorbance profiles of the eluates. After completion of a chromatogram, aliquots were taken for biological testing, the organic modifier was removed by

flushing the fractions at 40°C with a nitrogen stream and, finally, they were freeze-dried.

RESULTS

Peak 1 from the LH-20 column (Fig. 1) had no affinity for the C_{18} columns when using methanol—acetic acid—water (50:8:42), being eluted in the front volume. However, the activity could be purified by eluting the C_{18} column with acetic acid—water (4:96). The activity splits into two activity peaks (I_7 and I_6 in Fig. 2) showing weak retardation in the columns (Fig. 3, left part). These peaks could be further purified with 0.1% trifluoroacetic acid in water developed with an acetonitrile gradient (Fig. 4). The first eluting activity peak (I_7) in Fig. 3, left part, was eluted shortly after the front (after 3.8 min) and before the start of the gradient (Fig. 4, left part). The second activity peak (I_6) in Fig. 3, left part, was slightly more retarded (eluting after 4.4 min) but also appeared before the start of the gradient (Fig. 4, right part). Both activity peaks (I_7 and I_6) appeared chemically homogeneous as evidenced by the accordance of absorbance at 218 nm and the biological activity. After hydrolysis, the following ratios of moles of amino acids were found: I_7: 1 Asx : 1 Ser : 1 Glx : 2

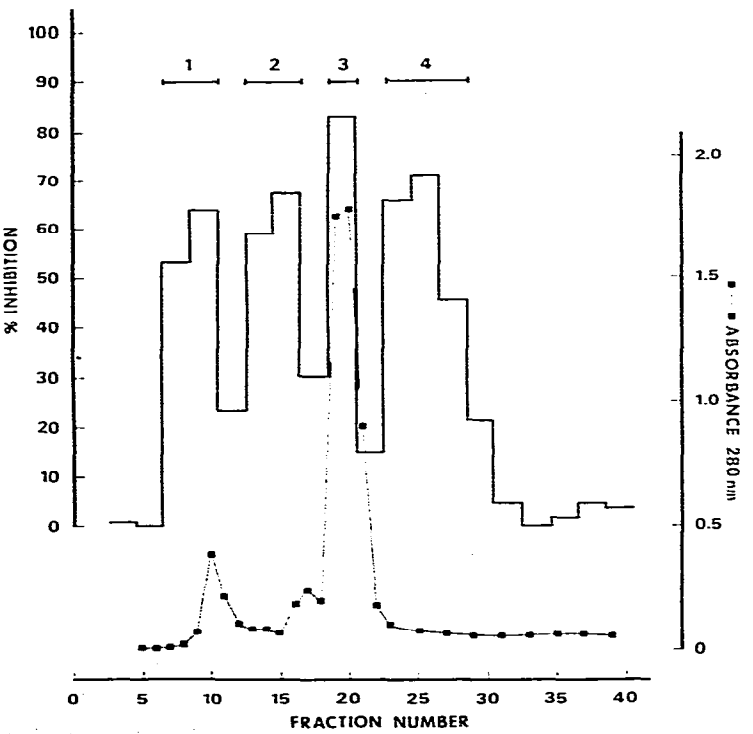

Fig. 1. Purification of tumour cell growth inhibitors by Sephadex LH-20 column (95 × 2.0 cm) eluted with 80% ethanol + 20% water containing 0.1% trifluoroacetic acid using a flow-rate of 36 ml/h and collecting 15 ml per fraction. The active peaks were pooled into four fractions as indicated in the figure by the horizontal bars.

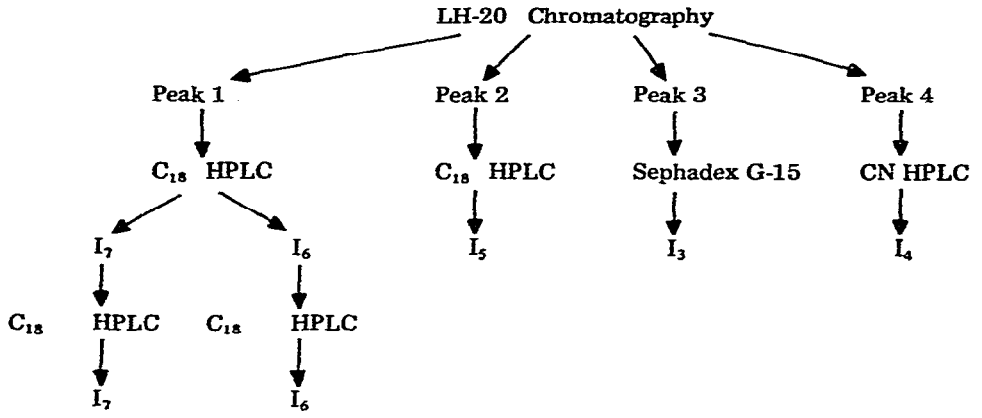

Fig. 2. Scheme of purification.

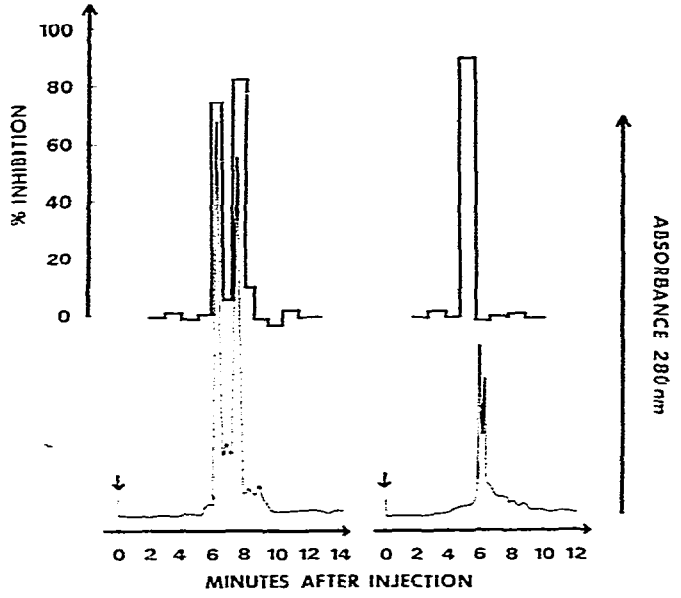

Fig. 3. Reversed-phase HPLC of pool 1 (left part) and pool 2 (right part) from LH-20 column. Column: Nucleosil 5 μm C_{18}, 250 x 4.6 mm I.D. Column temperature: room temperature. Mobile phase: 96% water + 4% acetic acid; flow-rate: 0.5 ml/min. Detection: 1.0 a.u.f.s. at 280 nm. Chart speed: 0.5 cm/min. Sample volume: 25 μl. Upper part of the curves: biological activity in individual fractions.

Gly, and I_6 : 1 Asx : 1 Thr : 1 Ser : 1 Glx : 2 Gly : 1 Ala, as described recently [7].

The activity peak 2 from the LH-20 column was even more hydrophilic, being eluted very near the front of the C_{18} column (Fig. 3, right part). Despite this non-optimal elution behaviour, the compound (I_5) seemed chemically pure, consisting of 1 mole of Asx and at least 4 moles of glucosamine, thus suggesting a very small glycopeptide with an extensive carbohydrate structure [7].

Peak 3 (I_3) could be further purified by Sephadex G-15 chromatography

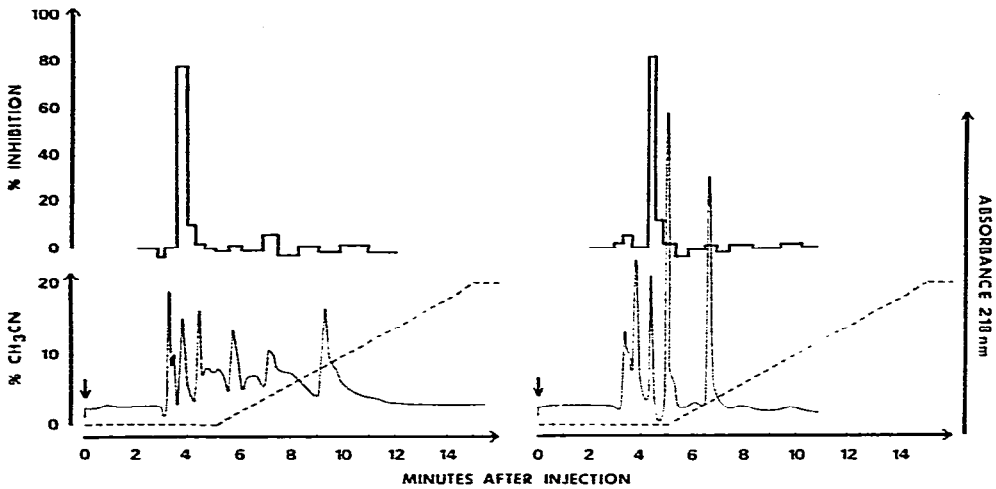

Fig. 4. Reversed-phase HPLC of inhibitors purified as described in Fig. 3, left part. Column: as in Fig. 3: Nucleosil 5C-18. Column temperature: 40°C. Elution conditions: 0.1% trifluoroacetic acid in water isocratic for 2 min followed by a gradient of 2% acetonitrile per min at 1.0 ml/min up to 20% acetonitrile. Detection: 0.2 a.u.f.s. at 218 nm. Chart speed: 1.0 cm/min. Sample volume: 20 μl. Left part: HPLC of first activity peak in Fig. 3, left part (I_7). Right part: HPLC of second activity peak in Fig. 3, left part (I_6). Upper part: As in Fig. 3.

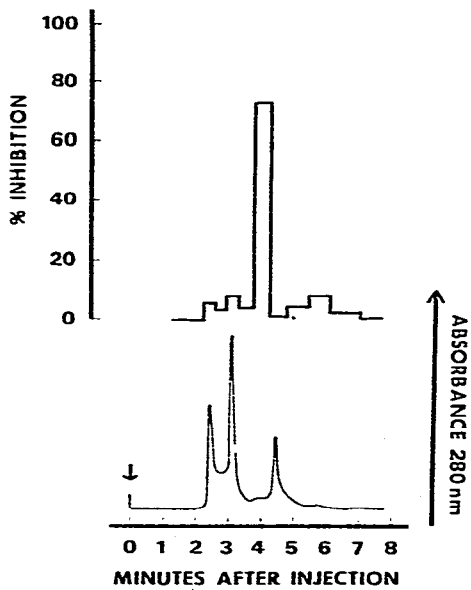

Fig. 5. HPLC of pool 4 from LH-20 column. Column: Nucleosil 5 μm CN, 250 × 4.6 mm I.D. Column temperature: room temperature. Mobile phase: acetonitrile—0.1% trifluoroacetic acid in water (1:1); flow-rate 1.0 ml/min. Detection: 0.5 a.u.f.s. at 280 nm. Chart speed: 0.5 cm/min. Sample volume: 25 μl. Upper part: as in Fig. 3.

eluted with 1.0 *M* acetic acid. As judged from its UV spectrum, showing a maximum absorption at 260 nm, it could be a nucleotide derivative.

The last eluting activity peak from the LH-20 column (peak 4) could be purified on a CN-cyanopropyl column (CN Nucleosil) (Fig. 5). The inhibitory peak

(I_4) appeared homogeneous after analysis, which showed 1 Asx : 1 Ser : 1 Glx : 2 Gly [7].

For more biochemical and cell biological characterization of the purified peptides see ref. 7.

DISCUSSION

The results obtained earlier with purification of endogenous growth inhibitors from the JB-1 ascites tumour indicated problems due to aggregation with other components in the extract, poor recovery and accumulation of toxic substances during the various purification steps [8]. The aggregation problems have been solved by extracting the active substances into methanol and primarily partitioning the activity in organic solvents in the subsequent purification steps, as described in the present paper. The poor recoveries were mainly associated with ion-exchange chromatography and high-voltage paper electrophoresis [6,8]. The bad recovery obtained by high-voltage paper electrophoresis may have been caused by ineffective elution of the factors from the electrophoresis paper. An important drawback of high-voltage paper electrophoresis was the coelution of toxic substance from the paper, possibly generated during the electrophoresis or picked up from the cooling tanks. All these problems have been greatly diminished by using the above-mentioned strategy.

The active extract has been purified recently [7] by HPLC without the first LH-20 step using a Nucleosil 5-C_{18} column eluted with methanol—acetic acid—water (50:8:42). This yielded inhibitors I_5 + I_6 + I_7 eluting together in the front fractions while I_3 and I_4 were more retarded. This procedure could not be used on a preparative scale due to the dominant occurrence of inhibitor I_3, which required a preparative purification step (LH-20) to separate I_3 from the other inhibitors. Besides, the most hydrophobic inhibitor I_4 appeared as a smeared peak in some HPLC experiments.

Other types of column packing material were tried for HPLC purifications. μBondapak C_{18} (Waters Assoc.) gave results similar to Nucleosil C_{18}, but LiChrosorb RP-18 (Altex Scientific, Berkeley, CA, U.S.A.) gave satisfactory separation of only the hydrophilic inhibitors (I_5, I_6 and I_7) while the more hydrophobic inhibitor I_3 produced a smeared peak; in addition, I_4 was totally lost and could not be eluted, even when the concentration of the organic modifier was increased. Spherisorb 5S ODS (Phase Separations, Queensferry, Great Britain) was totally unusable for the purification of any of the inhibitory peptides, all being irreversibly bound to the column. Although it produced satisfactory separation during LH-20 chromatography, Sephasorb™ HP Ultrafine (Pharmacia), which is the HPLC version of Sephadex LH-20, gave no separation of the various peptides: they were all eluted in the front volume although the same solvent was used as in LH-20 chromatography. Lowering the concentration of organic modifier did not improve the resolution since the various inhibitory peaks broadened and smeared into each other.

After LH-20 chromatography inhibitor I_4 could be purified by CN-Nucleosil HPLC, eluting isocratically with acetonitrile—0.1% trifluoroacetic acid in water (1:1) (Fig. 5). Lowering the acetonitrile concentration to 40% gave a better

resolution of the chromatogram. However, the recovery of activity decreased from 95% in the first system to approximately 50%, probably caused by poorer solubility or irreversible binding to the column of some of the active material.

Although very soluble in methanol—water (9:1), inhibitors I_5, I_6 and I_7 seemed rather hydrophilic, showing low affinity for the C_{18} column used. After HPLC purification these inhibitors seemed relatively pure as judged from amino acid analysis. However, a more powerful purification step could perhaps have been obtained by ion-exchange HPLC with Aminex A-28 or similar materials.

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