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SINGLE-STEP HIGH-YIELD PURIFICATION OF A SYNTHETIC PEPTIDE FRAGMENT OF HUMAN INTERLEUKIN 1 BY PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

It was recently shown that fragment 163–171 of human interleukin 1 beta (hIL1β) possesses interesting biological properties, as it presents immunostimulatory activity both *in vitro* and *in vivo*, whilst being apparently devoid of the strong inflammatory properties that prevent the possible therapeutic use of the entire interleukin 1 molecule. Therefore, this peptide may represent the fragment of the protein responsible for its immunostimulatory activity, distinct from the part involved in the inflammatory activity of the entire molecule. Large amounts of highly purified peptide are needed for an adequate pharmacological characterization, in view of its possible therapeutic applications. A suitable method for the preparation of this peptide, was therefore studied paying particular attention to the purification step, which is essentially based on an efficient use of preparative high-performance liquid chromatography.

INTRODUCTION

Interleukin 1, a family of related proteins produced by macrophages and by several other cells, is a factor with multiple biological properties that vary from T lymphocyte activation and other immunomodulating activities to inflammatory interactions with different tissues[1]. In the study of human interleukin 1, two different species, alpha and beta, have been cloned and sequenced in recent years[2,3]. A first step towards an understanding of the multiple biological activities of interleukin 1 is the identification of the active sites in the molecules. As the biological activity is likely to be found in well exposed areas of the molecule, which can easily interact with other molecules of the environment, we analysed the degree of exposure of different portions of human interleukin 1 beta (hIL1β) on the basis of its primary structure. Our prediction of exposed fragments was based on the use of two different hydrophilicity scales of amino acids. One was the hydrophilicity profile, proposed by Hopp and Woods[4], which proved particulary effective in detecting antigenic determinants, *i.e.*, zones of the molecule able to interact with specific antibodies. The other was a new hydrophilicity scale, called acrophilicity, also proposed by Hopp[5], which is particularly effective in the prediction of surface portions of proteins, *i.e.*, possible active sites.

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On this basis, we synthesized peptide fragments of hIL1β that are probably exposed on the surface of the entire molecule. One of these peptides, fragment 163–171 (Val–Gln–Gly–Glu–Glu–Ser–Asn–Asp–Lys), shows high T-cell activation capacity, as judged by its ability to stimulate murine thymocyte proliferation and to induce potently interleukin 2 production in spleen cells. When injected *in vivo*, fragment 163–171 was also able to enhance the immune response to sheep red blood cells, demonstrating an immunostimulating activity of this peptide[6]. On the other hand, this nonapeptide proved devoid of the inflammatory properties of the entire interleukin 1 molecule, as it did not induce fever when injected into mice and did not stimulate the production of prostaglandin E_2 in human dermal fibroblasts. Therefore, this peptide may represent the part of hIL1β responsible for its immunostimulatory capacity, which is distinct from the structures involved in the inflammatory activities of the protein[7]. A several milligram amount of highly purified peptide is necessary for an adequate evaluation of its biological activity. The fragment was prepared by the solid-phase method of Erickson and Merrifield[8].

The purpose of this work was to improve the present techniques for the purification of peptides, in order to maximize yields and purity in a shorter time, by means of analytical high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Synthesis of hIL1β (163–171)

The 163–171 sequence of hIL1β (Val–Gln–Gly–Glu–Glu–Ser–Asn–Asp–Lys) was synthesized by Erickson and Merrifield's solid-phase method[8] on a chloromethylated polystyrene–1% divinylbenzene resin. The first amino acid was esterified to the resin as the caesium salt, giving a degree of substitution of 0.55 mmol of amino acid per gram of dry resin. The following side-chain protecting groups were used: O-benzyl for glutamic acid, aspartic acid and serine; and *o*-bromobenzyloxycarbonyl (oBrZ) for lysine. The coupling reactions were carried out with pre-formed Boc-amino acid symmetrical anhydrides, with the exception of glutamine and asparagine, which were used as *p*-nitrophenyl esters, in the presence of an equimolar amount of N-hydroxybenzotriazole. The Boc-amino acids were obtained from Novabiochem (Läufelfingen, Switzerland). Each amino acid coupling step was monitored for completeness of reaction by the ninhydrin test[9].

At the end of the synthesis, the peptide was deprotected and detached from the resin by treatment with liquid hydrofluoric acid in the presence of 10% anisole and 10% pyridine in order to prevent anisylation of glutamic acid, for 30 min at -10°C and 30 min at 0°C.

Analytical and preparative HPLC

Both analytical and preparative runs were performed on a Perkin-Elmer Series 400 liquid chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.), provided with a Rheodyne 7125 injector (Rheodyne, Cotati, CA, U.S.A.) with a 175-μl injector loop for analytical runs and a 2-ml injector loop for preparative runs. Detection was performed with a Perkin-Elmer LC95 spectrophotometer, set at 210 nm, and data were collected and recorded by a Perkin-Elmer LCI-100 computing integrator.

We used a Waters μBondapack C_{18} analytical column (30 cm $\times$ 3.9 mm I.D.)

(Waters Assoc., Milford, MA, U.S.A.) and a Perkin-Elmer C_{18} (25 cm × 2.2 cm I.D.) preparative column, provided with a pre-column filter Guard Pack C_{18} (Waters Assoc.). The eluents were trifluoroacetic acid (TFA) (HPLC grade) (Pierce, Rockford, IL, U.S.A.), 0.1% solution in water, Milli-Q grade, passed through a LiChroprep RP-8 column (Merck, Darmstadt, F.R.G.), and methanol (HPLC grade) (INALCO, Milan, Italy).

Amino acid analysis

The amino acid composition was determined after acid hydrolysis in 6 *M* hydrochloric acid at 110°C for 24 h. Amino acid analyses were conducted using a Kontron Chromakon 500 analyser (Kontron, Zürich, Switzerland).

RESULTS AND DISCUSSION

After cleavage of the peptide from the resin, hydrofluoric acid was removed under vacuum, the crude product was washed with anhydrous diethyl ether and the peptide was extracted from the resin by treatment with 1 *M* acetic acid. A considerable amount of crude peptide was also recovered from the ethereal washings by treatment with 1 *M* acetic acid. Extracts were concentrated to dryness under vacuum; the crude peptide was then solubilized in 12 ml of 0.1% TFA and filtered through a 0.22-μm membrane.

To assess the degree of purity, the crude peptide was first analysed on the analytical column with a linear gradient of methanol in 0.1% TFA (Fig. 1). Then, to determine the optimal conditions for purification, we tested different gradients until we obtained that which gave the best resolution among the peaks (Fig. 2).

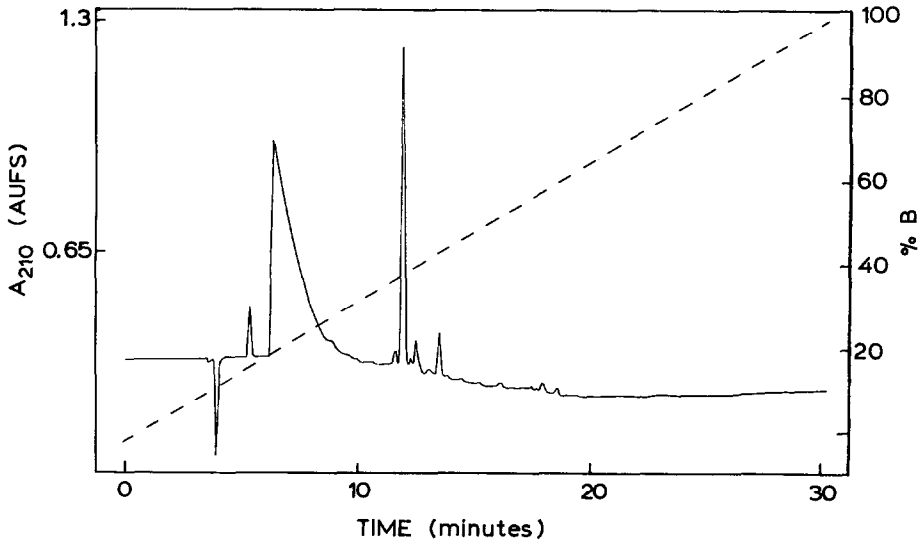

Fig. 1. Analytical chromatogram of the crude peptide after cleavage with hydrofluoric acid. Load, 1.5 μl (*ca.* 150 μg); buffers, A = 0.1% TFA, B = methanol; gradient (dotted line), 0 to 100% B in 30 min; flow-rate, 0.8 ml/min, chart speed, 8 mm/min.

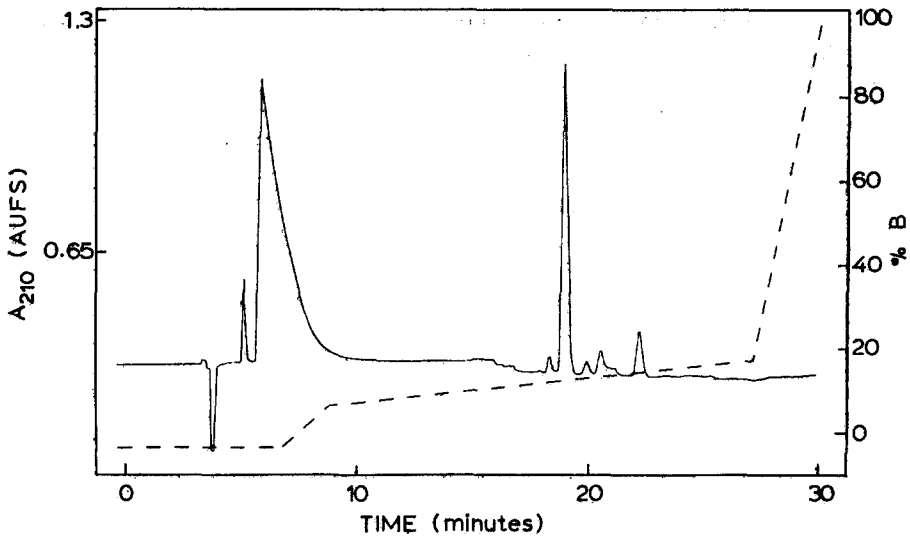

Fig. 2. Analytical chromatogram of the crude peptide after cleavage with hydrofluoric acid. Load, 1.5 μl (*ca.* 150 μg); buffers, A = 0.1% TFA, B = methanol; gradient (dotted line), 0% (7 min) to 10% B in 2 min, to 20% B in 18 min, to 100% B in 3 min; flow-rate, 0.8 ml/min; chart speed, 8 mm/min.

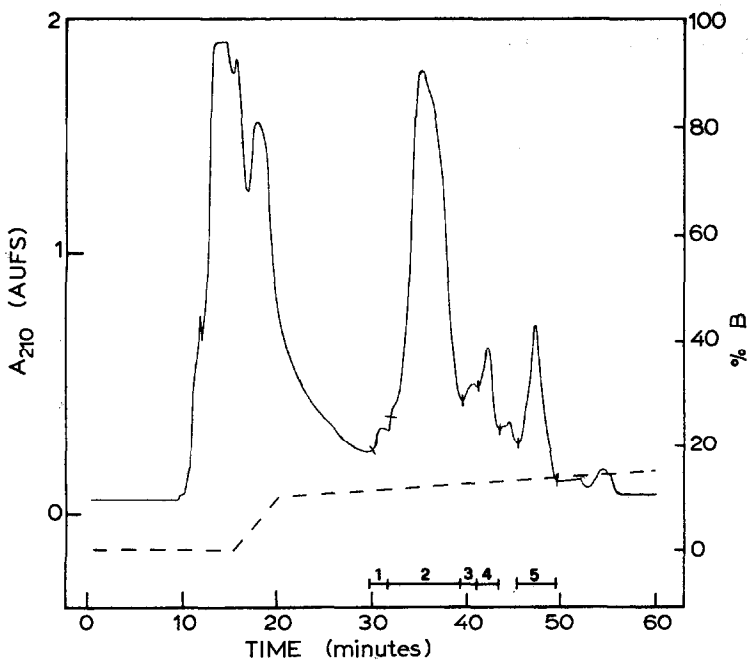

Fig. 3. Preparative chromatogram of the crude peptide after cleavage with hydrofluoric acid. Load, 1.5 ml (*ca.* 150 mg); buffers, A = 0.1% TFA, B = methanol; gradient (dotted line), 0% (15 min) to 10% B in 5 min, to 16% B in 40 min; flow-rate, 7 ml/min; chart speed, 3 mm/min.

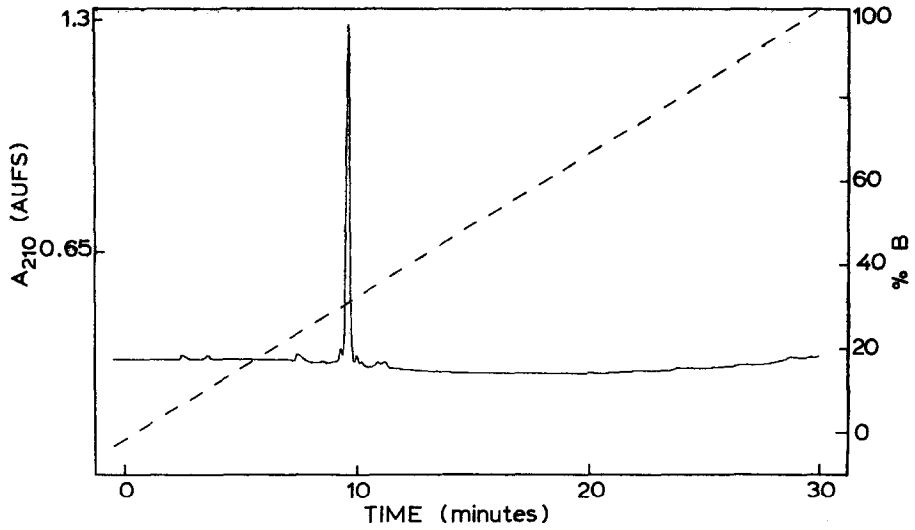

Fig. 4. Analytical chromatogram of the purified peptide (peak 2 in the preparative chromatogram). Load, 100 μl (*ca.* 100 μg); buffers, A = 0.1% TFA, B = methanol; gradient (dotted line), 0 to 100% B in 30 min; flow-rate, 1 ml/min; chart speed, 8 mm/min.

Purification was then performed on the preparative column (Fig. 3), using the same gradient as in the best analytical separation. Eight runs were needed to complete the purification of the material produced in the synthesis, starting from 1.1 g of dry Boc-Lys (oBrZ) resin.

The fractions obtained from each run (numbered 1–5 in Fig. 3) were pooled with similar fractions from other runs, concentrated, filtered through a 0.22-μm membrane and lyophilized. We analysed the amino acid composition of each fraction. The material present in fraction 2 shows the correct analytical data for the 163–171 sequence of hIL1β: Val (1) 1.0, Glu (3) 2.9, Gly (1) 1.0, Ser (1) 0.9, Asp (2) 2.0, Lys (1) 1.1. The large initial peak is probably composed of smaller by-products such as benzyl alcohol, *tert.*-butanol and anisole, as this peak is absent in chromatograms of the same peptide obtained after preliminary gel chromatography; the amino acid analyses of the other secondary peaks show that they represent incomplete peptides.

Starting with 1.1 g of Boc-Lys (oBrZ) resin, we obtained 520 mg of over 90% pure peptide, as assessed by the final analytical HPLC (Fig. 4) and amino acid analysis, with an overall yield of 70% (calculating the peptide as bistrifluoroacetate).

We have therefore shown that if relatively large amounts of a highly purified peptide are required, we can utilize the same HPLC apparatus for both analytical and preparative separations, obtaining a good and fast resolution of the components. Moreover, with this procedure, we can avoid the preliminary gel chromatography that is often used after deprotection and detachment of peptides to eliminate the smaller by-products.

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