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Note

Analysis of contaminants in commercial preparations of the tetrapeptide tuftsin by high-performance liquid chromatography

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Tuftsin, a hormone-like tetrapeptide of the sequence L-Thr-L-Lys-L-Pro-L-Arg, has been isolated from the leukophilic immunoglobulin G (IgG)[1,2]. This peptide binds to macrophages and granulocytes[3,4] and stimulates their phagocytic and bactericidal activities[5,6]. Tuftsin has also been shown to stimulate the tumoricidal activity of both cell types[7,8]. The degree and reproducibility of these biological responses may vary with the source of tuftsin and is usually a reflection on its purity. Its ability to stimulate the migration of polymorphonuclear leukocytes, however, has encountered some discrepancy. Nishioka *et al.*[1,9] have shown a stimulatory effect at concentrations of 2.5 and 12.5 μg/ml using a non-commercial preparation of tuftsin. In contrast, Horsmanheimo *et al.*[10] found no increase in response using a commercial preparation of tuftsin at concentrations 10- to 100-fold greater than those reported by Nishioka *et al.*[1,9]. The ability of tuftsin to enhance the migration of mononuclear cells has also encountered similar discrepancies. Goetzl[11] found no increase in response in the concentration range of 10^{-4}–10^{-1} μg/ml (source of tuftsin not reported) whereas Nishioka[12] has shown a stimulatory effect at concentrations of 10^{-2}–10 μg/ml. Similarly, Horsmanheimo *et al.*[10] have shown a stimulatory effect at concentrations of 10–100 μg/ml. It must be noted that most of the published work on tuftsin has emanated from laboratories where it was synthesized and purified. It has been our experience that cells

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stimulated with commercially available tuftsin exhibit a lower than normal phagocytic response and very often are unresponsive to tuftsin's phagocytic, bactericidal and tumoricidal stimulatory activity. Peptides of similar structure have been documented as potent inhibitors of tuftsin's stimulatory activities[13]. These and other contaminants could indeed lead to discrepancies in target cell response and may, in certain cases, require higher concentrations to elicit biological activities. Therefore, it is imperative that the purity of commercial preparations of tuftsin be verified before their use in future investigations. Consequently, we undertook a detailed study of a biologically inactive commercial preparation of tuftsin using reversed-phase high-performance liquid chromatography (HPLC).

Over the past several years, reversed-phase HPLC in conjunction with ion-pairing reagents, has been employed in the analysis of peptides and proteins with great success[14–16]. It offers several advantages over the purification procedures currently employed in tuftsin syntheses[17–22], including a relatively rapid analysis time, the ability to distinguish racemized forms and the relative ease of adapting it to a preparative scale. The chromatographic system in this report demonstrates the heterogeneity of a commercial preparation of tuftsin with subsequent enhancement of biological activity of the purified product above that of the starting sample.

EXPERIMENTAL

HPLC

The apparatus consisted of a Model HPLPS-1 liquid pumping system (Glenco, Houston, TX, U.S.A.) coupled to a Hitachi Model 100-20 spectrophotometer equipped with an Altex Model 155 20-μl flow cell, an LKB Model 2550 single-channel chart recorder and an LKB Model 7000 fraction collector. The column used was a Whatman C_{18} ODS-2 (10 μm, 25 cm $\times$ 4.6 mm I.D.). Sample injections were made with a Glenco Model VIS 50-μl syringe, in conjunction with a Glenco Model SV-3 sample injection valve. Filtration of solvents was carried out using a 0.2-μm filter (Fisher Scientific, Pittsburgh, U.S.A.) and the pH adjusted with ammonium hydroxide. Water was glass distilled and deionized. Organic solvents were spectrophotometric grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). All other chemicals were reagent grade (Fisher). Gradient elution was performed with a Glenco gradient mixer. A flow-rate of 0.5 ml/min was maintained by a pressure of 500 p.s.i. All tests were made at room temperature. Detection was at 210 nm. Samples of commercially available tuftsin, Lot number 701375 (Calbiochem, Los Angeles, CA, U.S.A.), were taken up in starting solvent prior to injection. All injections consisted of 20-μl volumes. Solvents were degassed for 30 min and the column was equilibrated with starting solvent for at least 30 min.

Gel filtration chromatography

Gel filtration for desalting was performed on Sephadex G-15 (Pharmacia, Uppsala, Sweden). The mobile phase consisted of 2.5 *M* acetic acid or 0.1 *N* HCl (Ultrex, J. T. Baker, Phillipsburg, NJ, U.S.A.). The latter was monitored at 210 nm using the flow cell described earlier. The former was analyzed at 570 nm using the standard ninhydrin reaction[23]. The presence of inorganic phosphorus was determined by the method of Chen *et al.*[24] and Lowry *et al.*[25] and quantitated at 820 nm. Fifty-

microliter samples from even-numbered fractions were used for ninhydrin and phosphorus determinations.

Amino acid analysis

Samples for analysis were lyophilized, dissolved in 500 μl of constant boiling HCl (Pierce, Rockford, Ill., U.S.A.) and hydrolyzed *in vacuo* at 110°C for 20 h. Samples were then lyophilized three times with deionized-distilled water. They were finally dissolved in 500 μl of sodium citrate buffer (pH 2.2, Pierce) prior to analysis. One-hundred-microliter samples were used for injection. Samples were analyzed on a Glenco MM-60 single column amino acid analyzer, supplied with a cation-exchange resin (HP-C; Bio-Rad Labs., Richmond, CA, U.S.A.) and eluted with sodium citrate buffers (Pico Buffer System II, Pierce). Eluted samples were monitored simultaneously at 440 and 570 nm using ninhydrin (Pierce) as the detecting reagent.

Phagocytosis and bactericidal assay

Human neutrophils were prepared by the method of Chee *et al.*[26]. The assay was that described by Phillips *et al.*[27]. From the assay results, the following values were calculated: phagocytosis index = NTt = 0/NCt = 0; bactericidal index = (NTt = 3/NTt = 0)/(NCt = 3/NCt = 0) where NTt = 0 and NTt = 3 are the numbers of viable bacteria ingested by tuftsin-treated neutrophils after 10 min and 3 h, respectively, and NCt = 0 and NCt = 3 are the numbers of viable bacteria ingested by non-tuftsin treated neutrophils after 10 min and 3 h respectively.

RESULTS AND DISCUSSION

Analysis of this commercial preparation of tuftsin by reversed-phase HPLC proved it to be heterogeneous (Fig. 1). No peaks were present upon injection of 20 μl of starting solvent alone (not shown). Peak b_2, acetate, is common to all tuftsin samples supplied in the form of an acetate salt, and can be seen upon injection of 0.02 *M* acetic acid alone (not shown). Twenty samples were chromatographed and the respective peaks pooled. Peak c, tuftsin, was collected as shown in Fig. 1 since it was not known whether the tailing portion was due to an unresolved peak or band spreading. Any attempt to reduce retention times and tailing by the addition of organic solvent resulted in a rapid co-elution of tuftsin and contaminants (not shown). This result is ascribed to the high degree of hydrophilicity of the molecule, dictated by its constituent amino acids. Since peak c, tuftsin (Fig. 1) would subsequently be tested for its phagocytic and bactericidal activity in its acetate form, it was desalted using 2.5 *M* acetic acid as the mobile phase as shown in Fig. 2. The ninhydrin-positive, phosphate-negative peak was collected and lyophilized three times with a 1% acetic acid solution. To minimize non-specific loss of the remaining peaks in Fig. 1 upon desalting, a smaller column (60 × 1.5 cm) was used. Since these would be processed directly for amino acid analysis, 0.01 *N* HCl was used as the mobile phase, which allowed monitoring at 210 nm. Elution profiles were similar to that shown in Fig. 2, except for peak j, which resulted in two peaks eluting before the ammonium phosphate salt. The earlier and later eluting peaks will be referred to as j-I and j-II, respectively.

Amino acid analysis ratios are listed in Table I. With exception of peaks g

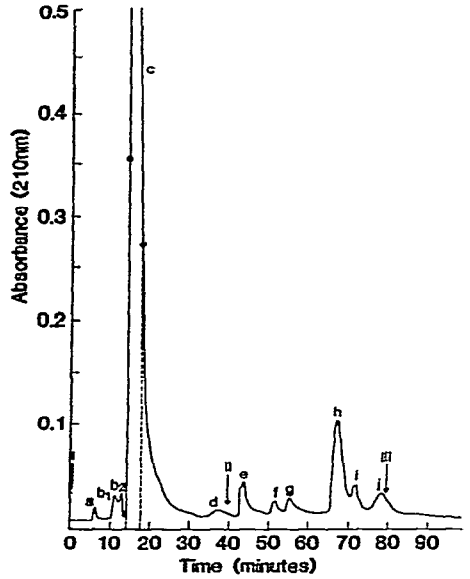

Fig. 1. Elution profile of a commercial preparation of tuftsin (136 μg) on a C_{18} ODS-2 column. The mobile phase (0.5 ml/min) consisted of the following as indicated by the arrows: I, 0.1 *M* $NH_4H_2PO_4$, pH 4.5, 40 min; II, 0.1 *M* $NH_4H_2PO_4$, pH 4.5 containing a linear gradient of 0–5% acetonitrile (20 ml total volume), 40 min; III, 0.1 *M* $NH_4H_2PO_4$, pH 4.5, 5% acetonitrile. One-milliliter fractions were collected.

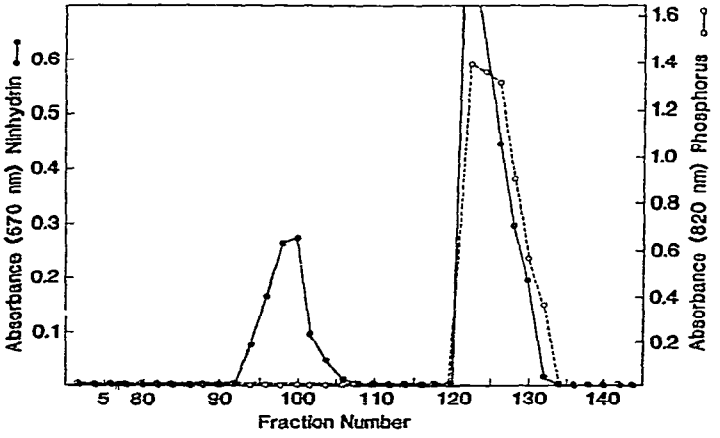

Fig. 2. Desalting of peak c from reversed-phase HPLC on a Sephadex G-15 column (110 × 2.4 cm). The mobile phase (9 ml/h) consisted of 2.5 *M* acetic acid. One-milliliter fractions were collected. Fifty-microliter aliquots from even-numbered fractions were assayed for the presence of peptide and inorganic phosphorus as described under Experimental.

and j-II, all yielded approximate unimolecular ratios, suggestive of racemized forms. In addition, unimolecular ratios may possibly be a result of incomplete deprotection of the peptide upon completion of synthesis. The low ratio of Thr, Lys in j-II may have been a result of contamination by peak j-I upon desalting due to incomplete

TABLE I
AMINO ACID ANALYSIS RATIOS FROM REVERSED-PHASE HPLC OF TUFTSIN

Peak	*Threonine*	*Lysine*	*Proline*	*Arginine*
a	1.0	1	1.1	1.0
b_1	0.8	1	0.9	0.9
b_2 (acetate)				
c (tuftsin)	0.9	1	1.0	0.9
d	1.1	1	1.0	1.0
e	1.0	1	0.9	1.1
f	1.0	1	1.0	1.0
g	0.9	2	0.9	1.9
h	0.9	1	0.9	0.8
i	1.0	1	1.0	0.9
j-I	1.2	1	1.2	1.1
j-II	0.3	0.3	0.9	1.2

baseline separation. It would implicate j-II as the dipeptide, Pro-Arg. The amino acid analysis ratio of peak g may have been due to the presence of unprotected amino acids in minor quantities in the starting protected amino acid preparations.

In assessing the biological activity of the unfractionated and fractionated tuftsin, a concentration of 1 μg/ml was used at a final volume of 1 ml. The phagocytic index for the unfractionated samples was 0.98, indicating a control level phagocytic response. Its bactericidal index of 2.25 indicates little killing. The phagocytic and bactericidal index for peak c was 1.69 and 0.10 respectively, which clearly indicates an enhancement of biological activity. The degree to which each of the contaminants contributed to the suppression of biological activity could not be determined on the basis of a limited amount of sample. However, it was estimated that the contaminants represented 16% of the total sample by optical density measurements.

Degrees of racemization in peptide synthesis are dependent upon the type of synthesis chosen, the coupling method used and the optical purity of the protected amino acids. Thus, the number of racemized forms implied by Table I is not surprising for a commercial sample that was assessed by the supplier as homogeneous for purity using thin-layer chromatography. Thin-layer chromatography's questionable ability to separate racemized forms and its requirement for substantial quantities for detection of contaminants may have indeed led to a conclusion of homogeneity.

The chromatographic system presented in this report allows a sensitive and relatively rapid analysis of tuftsin. The possibility of adapting it to preparative systems presently available appears promising. Since there would be no need for gradient elution on a preparative scale, the purification scheme becomes rapid and applicable to individual laboratory syntheses. However, if commercially available preparations of tuftsin are to be used in future investigations, we strongly urge that the users verify its purity by some biological or more sensitive biochemical criteria.

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