PREPARATION OF BIOLOGICALLY ACTIVE RADIOIODINATED THYMOPENTIN

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SUMMARY

Thymopentin (TP5, Arg-Lys-Asp-Val-Tyr), a synthetic pentapeptide corresponding to amino acids 32-36 of the thymic hormone thymopoietin, has the biological activity of the parent compound. The tyrosyl residue was iodinated using the chloramine-T method and the reaction mixture separated by HPLC into peaks corresponding to free TP5, mono-iodo TP5 and di-iodo TP5, identified by mass spectrometry. Biological activity was retained in the iodinated peptides but, surprisingly, free TP5 had lost biological activity during the separation process. The biologically active monoiodinated TP5 had a specific activity of 28 μCi/μg and will be useful in studies of receptor binding.

Key Words: Thymopoietin, thymopentin, radioiodination, cGMP.

INTRODUCTION

Thymopoietin is a polypeptide hormone of the thymus that induces selective differentiation of prothymocytes to thymocytes (1), affects peripheral lymphocytes (2-4), and also affects neuromuscular transmission (5-7). The entire 49 amino acid sequence of thymopoietin has been determined (8), and the synthetic pentapeptide corresponding to thymopoietin$_{32\text{-}36}$ (TP5) was shown to have the biological activity of native thymopoietin (9). Native thymopoietin is difficult to iodinate using conventional methods. Iodination by the chloramine-T method destroyed biological and antigenic properties of the peptide (10), and it was presumed that iodination of Tyr_{36} at the active site might have altered its conformation, leading to loss of binding to specific cell surface receptors. Iodination by the Bolton-Hunter method (10) produced material with 50% immunoreactivity, but biological activity was not retained. Thymopoietin can be radioiodinated using a specialized technique

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involving side chain protection, post-proline cleaving enzyme, and N-terminal substitution (11), but this method is complex and difficult to perform reproducibly. TP5 has been labelled synthetically with tritium or ^{14}C, but both these approaches yield product of low specific activity, with the tritium labelled TP5 proving very unstable. We now report an additional technique for radioiodination of TP5 using the chloramine-T iodination method. A critical step was separation and identification of the reaction products, using an HPLC-MS system, as free TP5, mono-iodo TP5 (I-TP5) and di-iodo TP5 (I_2-TP5).

MATERIALS AND METHODS

TP5 was synthesized by classical solution phase techniques. Radioactive iodine (^{125}I) was obtained from Amersham (Arlington Heights, IL). Sodium iodide, chloramine-T and sodium m-bisulfite were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents used were of analytical grade.

Cell culture and cGMP measurement. CEM, a human T-lymphoblastoid cell line was obtained from ATCC (Rockville, MD). Cells were grown in RPMI-1640 with heat inactivated fetal calf serum, 3 mM L-glutamine and 50 μg/ml gentamycin. Day 4 cells were used for cGMP studies as described previously (11). The assay kit for cGMP was from NEN (Boston, MA).

Iodination of TP5. All reagents were made in 0.1M phosphate buffer, pH 7.2. In a typical iodination, 5-50 μg of TP5 in 20 μl (2.5 mg/ml stock) was mixed with 1 mCi carrier free $Na^{125}I$ or 2.5 - 25 μg of NaI in 10 μl, then 10 μg of chloramine T (20 μl) as an oxidizing agent. The reaction was stopped after 15 seconds with 20 μg of sodium-m-bisulfite (20 μl).

Separation and identification of iodinated peptides. Separation of the iodinated TP5 mixture was accomplished by HPLC using a 4.6 mm X 25 cm YMC-basic column (YMC-Inc., Morris Plains, NJ). The mobile phase was 22% CH_3CN with 0.1% trifluoroacetic acid, at a flow rate of 1.0 ml/min. HPLC instrumentation consisted of a Perkin-Elmer (Norwalk, CT) Series 4/410 LC pump, a Rheodyne (Cotati, CA) 7125 injection valve (20 μl), and a Perkin-Elmer LC-95 detector (220 nm) and LCI-100 integrator. On-line HPLC/MS was

accomplished using a Sciex TAGA 6000E MS/MS equipped with an atmospheric pressure ion source and "ionspray" interface (14). Chromatographic effluent into the mass spectrometer was limited to 40 μl/min by reducing the column diameter to 2.0 mm (400 μl/min) and splitting the flow into the MS 1:10. After identifying the reaction products, I-TP5 was separated and purified routinely with a Waters HPLC system (Milford, MA) connected to a Radiomatic (Tampa, FL) flow detector.

RESULTS AND DISCUSSION

Pilot experiments were performed with nonradioactive iodine. Following iodination by the chloramine-T method, the mixture was separated by HPLC as described above. The outlet of the UV detector was connected directly to the mass spectrometer using an "ionspray" interface and the identity of the components determined to be unreacted TP5, I-TP5 and I_2-TP5 (Fig. 1A). The ionspray/electrospray interface gently protonates the basic residues of the peptide without fragmenting it. If the peptide contains more than one basic site, as in the case of TP5, multiple protonation can occur. In the mass spectrum of TP5

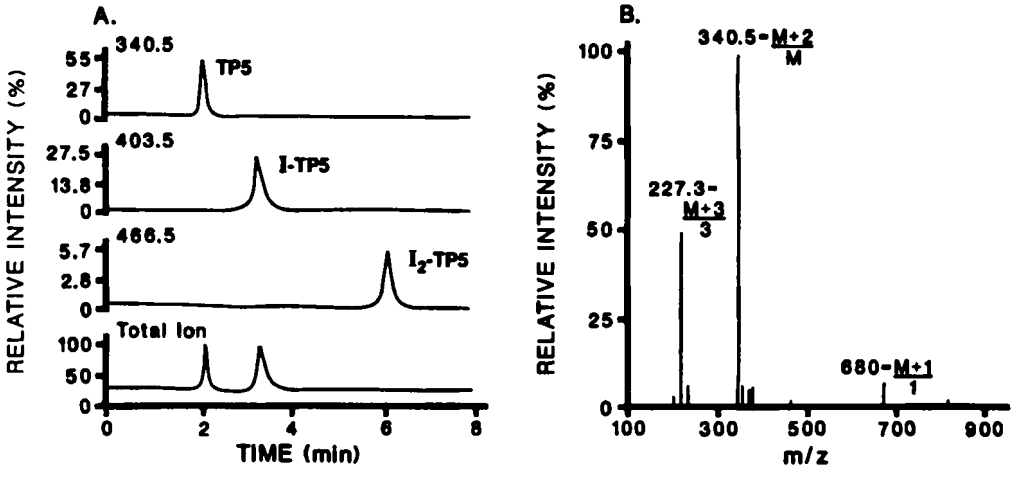

Figure 1. (A) Determination of the identity of products of the cold TP5 iodination reaction. Doubly protonated peptides were the predominant ion in the electrospray mass spectrum. (B) Ionspray/electrospray mass spectrum of TP5. The doubly charged species (340.5) was the predominant ion.

(679 MW) the predominant peak was the doubly protonated peptide at mass 340.5 (Fig. 1B).

Upon identification of the component mixture, a radiolabeled sample was analyzed on a similar HPLC system with a radioactive flow detector. Three radioactive peaks were obtained by HPLC (Fig. 2A), the first corresponding to iodine, the second to ^{125}I-TP5, and the third to $^{125}I_2$-TP5. The yields of ^{125}I-TP5 and $^{125}I_2$-TP5 in a typical HPLC run were 32% and 2% respectively. Rechromatography of the ^{125}I-TP5 peak yielded a product of about 90% purity (Fig. 2B). The specific activity of the purified ^{125}I-TP5 was calculated to be 28 μCi/μg.

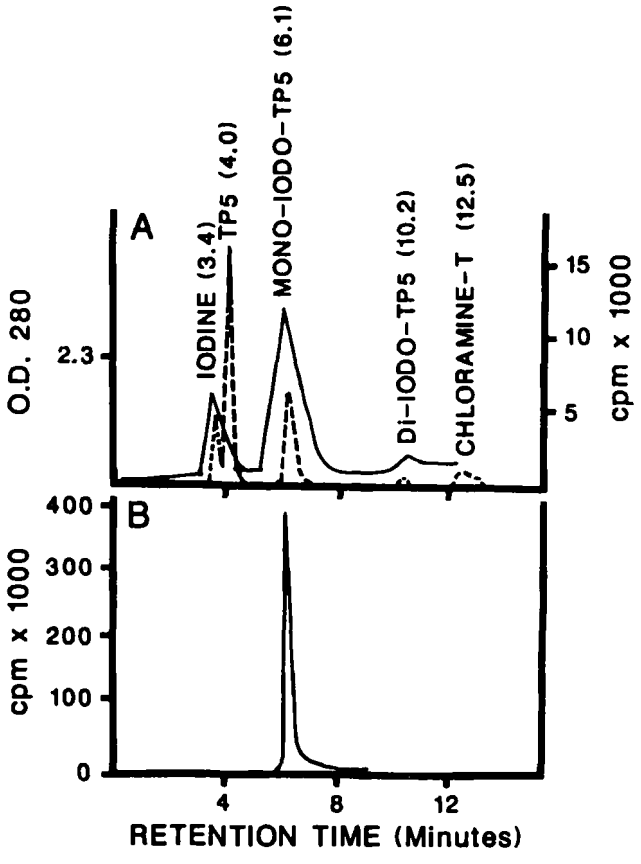

Figure 2. (A) HPLC purification profile of products obtained after chloramine-T iodination of thymopentin. The dotted line represents the UV absorption pattern. The radioactivity in each fraction is shown by the solid line. Retention time in minutes is shown in parenthesis. (B) Radioactivity pattern after rechromatography of the I-TP5 peak under the same conditions. Flow conditions differed from those shown in Fig. 1 so the fraction numbers do not correspond between these two experiments.

TP5 and the two iodinated forms were tested for biological activity using induction of cGMP in CEM cells as a bioassay (11). Both I-TP5 and I_2-TP5 were biologically active, although their potencies were different, while unreacted TP5 was inactive (Fig. 3). This surprising finding that TP5 was inactive is probably attributable to dilution during labelling and purification, since TP5 is known to be unstable at concentrations less than 1 mg/ml (unpublished data). Interestingly, not only was iodinated TP5 active, but the

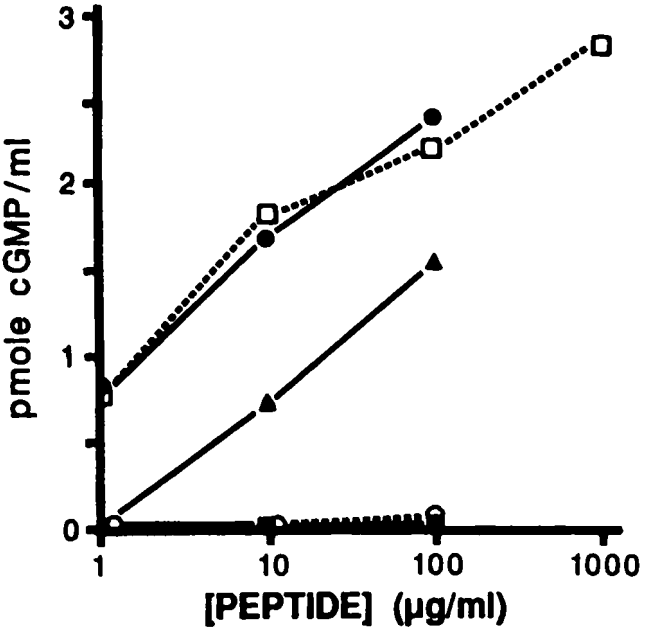

Figure 3. Intracellular cGMP levels in CEM cells after incubation with the purified reaction products after iodination of TP5. I_2 -TP5 (●) had the same activity as fresh TP5 standard (□); I-TP5 (▲) had 10 percent activity and the other products, unreacted TP5 (■) and free I_2 (o) had no biological activity.

substitution with iodine appears to have stabilized the molecule and preserved biological activity under conditions that denature intact TP5. It may be that the increased lipophilic nature of I_2-TP5 might be contributing towards the enhanced biological activity compared to I-TP5. The biological activity of iodinated TP5, coupled with high specific activity of the radioiodinated molecule, should prove useful in the study of binding of TP5 to specific receptors. Further work is necessary to 1) increase the bioligical activity of ^{125}I-TP5 and 2) increase the yield of $^{125}I_2$-TP5.

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