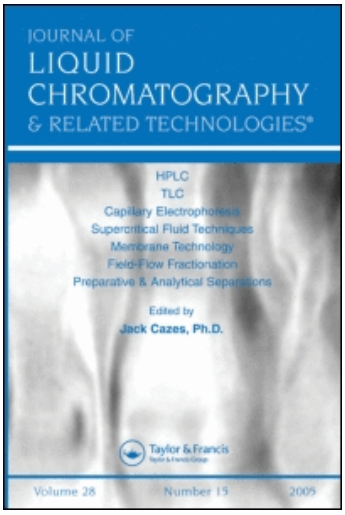


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# Tryptic Map Variation of Erythropoietin Resulting from Carboxypeptidase B-Like Activity

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# TRYPTIC MAP VARIATION OF ERYTHROPOIETIN RESULTING FROM CARBOXYPEPTIDASE B-LIKE ACTIVITY

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## ABSTRACT

The recombinant human erythropoietin (r-HuEPO) is monitored by tryptic mapping for its purity and identity. Occasionally, these maps have produced an additional peak not evident in the reference standard. This peak was isolated, sequenced and characterized by mass spectrum. It is derived from a known r-HuEPO tryptic peptide 144-150 with the C-terminal arginine removed. Its formation can be reduced or totally eliminated by the addition of inhibitors of carboxypeptidase B activity. These results indicate the presence of residual carboxypeptidase B-like activity in some r-HuEPO lots. Similar enzymatic activity has been shown in varying amounts in the concentrated diafiltered media before purification. The peptide 144-150 is one of the first to be affected by the carboxypeptidase B-like activity. This peptide seems to locate in the most trypsin-sensitive part of r-HuEPO.

## INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone that induces mammalian red blood cell differentiation (1-7). Recombinant human EPO

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(r-HuEPO, also called EPO in this text) is marketed worldwide for the treatment of the anemia associated with chronic renal failure. Also, in the U.S, r-HuEPO is marketed for the anemia associated with human immunodeficiency virus (HIV)-infected patients under zidovudine (azidothymidine, AZT)- therapy and for anemia related to chemotherapy for cancer. The EPO molecule has been extensively characterized (8-12) and consists of 165 amino acids with two disulfide bonds and about 40% of carbohydrate by weight. The EPO used in the formulations is rigorously tested for its identity and purity by several analytical techniques. One of these tests is tryptic mapping which is a well established technique for protein identity and purity determination. As a routine specification test for EPO reference standard and bulk lots, it requires the chromatogram produced for the sample tryptic map to correlate with that of the reference standard.



Occasionally, an extra peak has appeared in some EPO lots, not evident in the reference standard. Interestingly enough, this anomaly was not observed by any other analytical tests and did not affect the activity. Regardless, the presence of the extra peak was persistent enough in some lots to prompt the study of its identity and the source of the unexpected fragmentation. This report describes the successful determination of the unknown peak as an anomalous EPO peptide fragment. Various experiments are described in the investigation to determine the cause of this unusual fragmentation pattern.

## MATERIALS AND METHODS

### Materials

Trifluoroacetic acid (TFA) was protein sequencer grade (Applied Biosystems, Foster City , CA) and acetonitrile was high performance liquid

chromatographic (HPLC) grade (Baxter, Burdick & Jackson, Muskegon, MI). All the other chemicals were of reagent grade (Sigma Chemical Co., St. Louis, MO). The TPCK-treated trypsin solutions were prepared in distilled water fresh daily. Carboxypeptidase B was diluted with distilled water as required in the experiments. The solutions of ethylenediamine tetraacetic acid, disodium salt (EDTA) , L-arginine, L-histidine and 2,2'-dipyridyl were also prepared in distilled water in the concentrations specified in the Results section.

## Chromatography

The EPO peptide mapping method consisted of a reversed phase (RP) C4 column (250 x 4.6 mm, 5 micron, 300 A, Vydac 214TP54) and a gradient mobile phase of 0.06% TFA in water (A) and in acetonitrile (B). Gradient 1, which was used in the routine tryptic map method, had Step 1: 0% to 55% B in 125 min and Step 2: 55% to 75% B in 10 min. This gradient was modified to Gradient 2 , used in this study, which consisted of Step 1: 0% to 38% B in 80 min and Step 2: 38% to 100% B in 10 min. The column temperature was ambient and the detection was by ultra-violet (UV) at 215 and 280 nm.

## Sample Preparation

In the trypsin digests, 200 µL of EPO sample in 20 mM citrate buffer was mixed with 10 µL of trypsin, both at concentration of 1.0 mg/mL, to reach the substrate to enzyme ratio of 20:1. For preparative studies, the original sample concentration (usually 1.5-2.0 mg/mL) was used and the added trypsin adjusted accordingly to maintain the 20:1 ratio. The length of incubation time at 37°C was usually 18 hours. All the samples were frozen immediately after the digestion and stored in the freezer (-20°C) until ready for analysis. Sample aliquots used for the chromatography

varied from 20 μL to 200 μL depending on the original sample concentration.

## Unknown Peak Isolation

Using a 200-μL column load, the extra peak was collected under the same chromatographic conditions as in the analytical studies. The collected peak was concentrated on SpeedVac to a final volume of about 50 μL, and the concentrate was used for further analysis.



## Unknown Peak Identification

The sequencing was done by an ABI 477A Pulsed Liquid Sequencer using the conditions recommended by the manufacturer. The Fast Atom Bombardment (FAB) mass spectral data were acquired using a Finnigan-MAT (San Jose, CA) TSQ70 triple stage mass spectrometer equipped with an Ion Tech saddle field source (ion gun) and high voltage conversion dynode (15kV) detection. The samples were prepared in a thioglycerol matrix and bombarded with 8 keV xenon atoms. The instrument was scanned initially from 500 to 1500 u (mass units) in a total time of 2 sec. Follow-up scans were acquired over the mass range of 1500 to 3000 u (scan time 3 sec).

# RESULTS

## Isolation and Identification of Peak 14

The typical tryptic maps of EPO reference standard (A) and the variant EPO lot (B), recorded at 215 nm, highlight the presence of the extra peak

14 (Figure 1, B). The peptide 18, that eluted at around 100 minutes, was not critical to the study results and was omitted to shorten the total runtimes. The sequences of all the tryptic peptide peaks 1-18 have been determined as shown in Figure 1. The complete 165-amino acid sequence of EPO structure has been previously published (10). The tryptic map chromatograms, recorded at 280 nm, on the other hand, showed only five major peaks, 8, 13, 16, 17 and 18 for (A) and also the new peak 14 for (B). These were the tryptic peptides that contained either tyrosine and/or tryptophan amino acids. The ultraviolet (UV) spectra of each peak were recorded by a photo-diode array detector during the chromatographic run. The scans for peaks 8, 13 and 14 indicated the presence of tyrosine and were identical for 13 and 14. Peak 16 showed the typical UV-pattern of a tryptophan containing peptide and 17 and 18 showed the mixture of tyrosine and tryptophan. In addition to the identical UV scans, the two peaks 13 and 14 were interrelated by the peak 13 decreasing as the peak 14 was forming. The peak 13 is a known EPO tryptic fragment, Val-Tyr-Ser-Asn-Phe-Leu-Arg (VYSNFLR), at a position of 144-150. This indicated that the unknown fragment 14 was some kind of derivative of the peptide 13, but less polar, due to its later elution in reversed phase HPLC.

The peak 14 in the variant EPO chromatogram was isolated by reversed phase HPLC and analyzed by sequencer and FAB-mass spectrometry. The amino-terminal sequence data revealed the peptide 14 to be a hexapeptide, Val-Tyr-Ser-Asn-Phe-Leu ( VYSNFL), which was the same sequence as for the heptapeptide 13, except the terminal arginine removed. A closely eluting heptapeptide peak 15, Ser-Leu-Thr-Leu-Leu-Arg (SLTTLLR), with arginine in sequence position 7, was interfering in the identification until very pure 14 was obtained. The FAB mass spectrum of the purified 14 yielded an intense signal with m/z 742, consistent with the sequence of VYSNFL. This mass ion differed only by

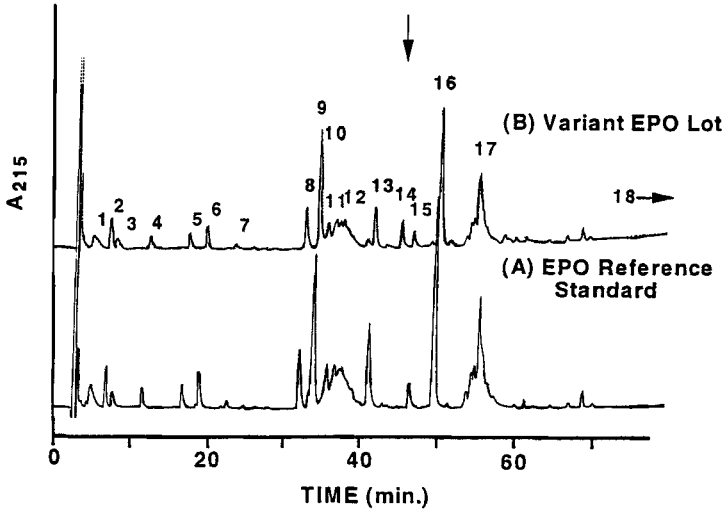


**Figure 1.** Typical tryptic-map chromatograms of a variant EPO lot (B) and EPO reference standard (A). Chromatographic conditions: Column, Vydac 214TP54, 5u, 300 A, 250 x 4.6 mm. Mobile Phase, A: 0.06 % TFA in water, B: 0.06 % TFA in aceto-nitrile. Gradient: Step 1, 0 to 38 % B in 80 min., Step 2, 38 to 100 % B in 10 min.
Detection was at 215 nm. Identified peak sequences: **1:** 1-4, **2:** not identified **3:** 111-116, **4:** 11-14, **5:** 98-103, **6:** 141-143, **7:** 140-143, **8:** 15-20, **9:** 132-139, **10:** 5-10 and 155-162, connected with disulfide bond CYS7-CYS161, **11:** 21-45*, **12:** 117-131*, **13:** 144-150, **14:** 144-149, **15:** 104-110, **16:** 46-52, **17:** 77-97*, **18:** 54-78.
* Indicates presence of carbohydrates.



one arginine (156 u) residue, from the molecular weight of 898 u for peak 13 (VYSNFLR). The loss of arginine from 13 was also consistent with the new peptide 14 being less polar in the reversed phase chromatogram. The synthetic peptide VYSNFL was also shown to coelute with the peak 14 in the tryptic map chromatogram (data not shown).

## Effect of Heat and Inhibitors

The presence of a metallo-protease in the variant EPO lots became a suspect when either a 3-minute preboiling (Figure 2) or addition of 8.5

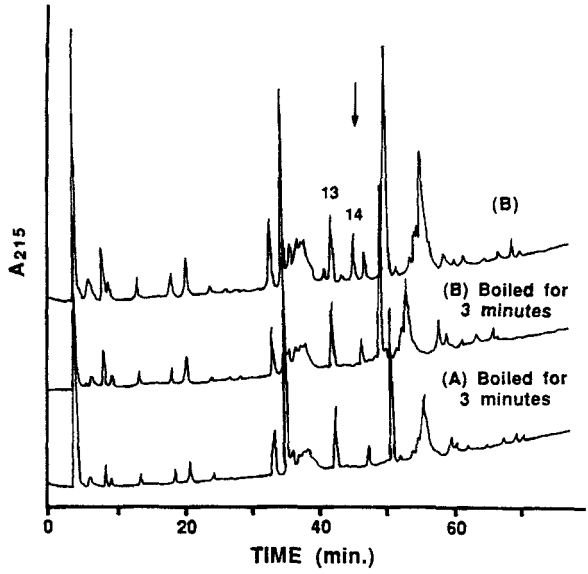


**Figure 2.** Effect of 3 minute boiling on the tryptic maps of a variant EPO lot (B) and EPO reference standard (A). Chromatographic conditions as in Fig. 1.



mM EDTA (Figure 3) prior to trypsin digestion, caused the extra peak to disappear. No effect was observed on the reference standard EPO under same conditions. Also, addition of 50 mM of specific Carboxypeptidase B inhibitors such as dipyridyl (Figure 4) or arginine hydrochloride (data not shown) to the samples before digestion caused the peak 14 to disappear. The inhibitor, L-lysine, was not equally effective in hindering the peak 14 formation, but 200 mM addition caused the peak to decrease (data not shown).

## Effect of Concentrated Diafiltered Media (CDM)

Addition of CDM (without EPO) in the samples before digestion had an opposite effect from the additives above. The undiluted CDM caused extensive changes in the EPO tryptic map. However, when the CDM

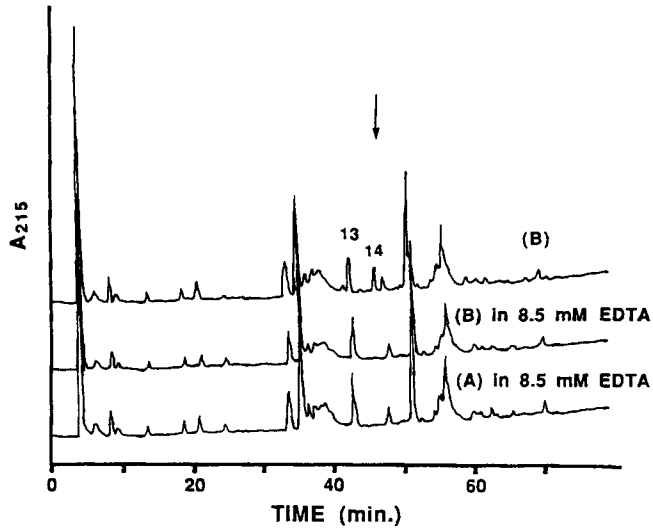


**Figure 3.** Effect of 8.5 mM of EDTA on the tryptic maps of a variant EPO lot (B) and EPO reference standard (A). Chromatographic conditions as in Fig. 1.

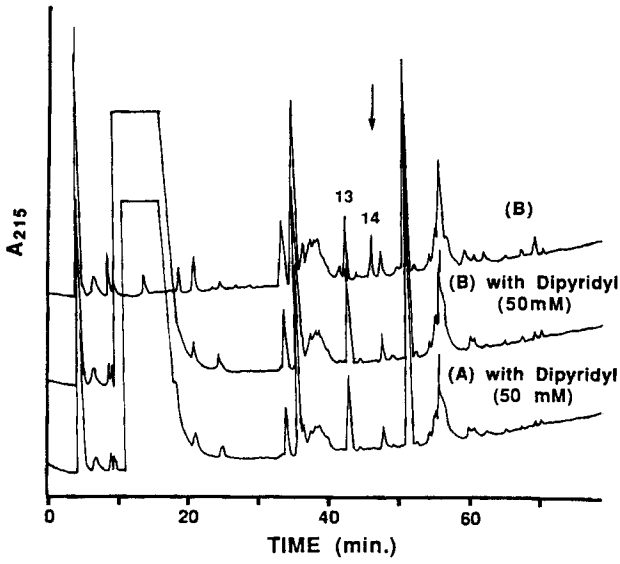


**Figure 4.** Effect of 50 mM of dipyridyl on the tryptic maps of a variant EPO lot (B) and EPO reference standard (A). Chromatographic conditions as in Fig. 1.

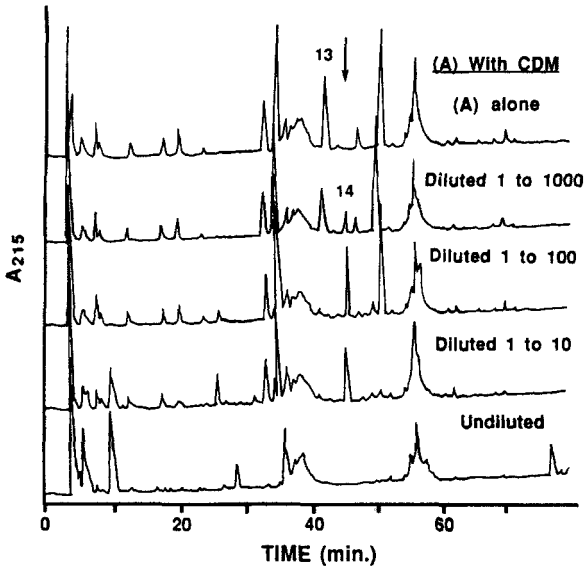


**Figure 5.** Effect of concentrated diafiltered media (CDM) on the tryptic map of EPO reference standard (A). Chromatographic conditions as in Fig. 1.



concentrate was diluted 1 to 10, 1 to 100 and 1 to 1000 with distilled water and then added to EPO reference standard, the extra peak 14 formation was observed. At the 1 to 1000 dilution level, the peak 14 size was close to those seen in the variant EPO lots (Figure 5, trace 2). The more concentrated dilution levels of CDM caused some additional degradations to occur (Figure 5, traces 3 and 4).

## Effect of Carboxypeptidase B (CPB)

Addition of CPB in the samples before digestion had the same effect as CDM. Concentrated CPB caused very extensive changes in the tryptic maps of EPO reference standard. However, when the enzyme level was reduced to around 1-20 mU/mL, the tryptic map became similar to that of

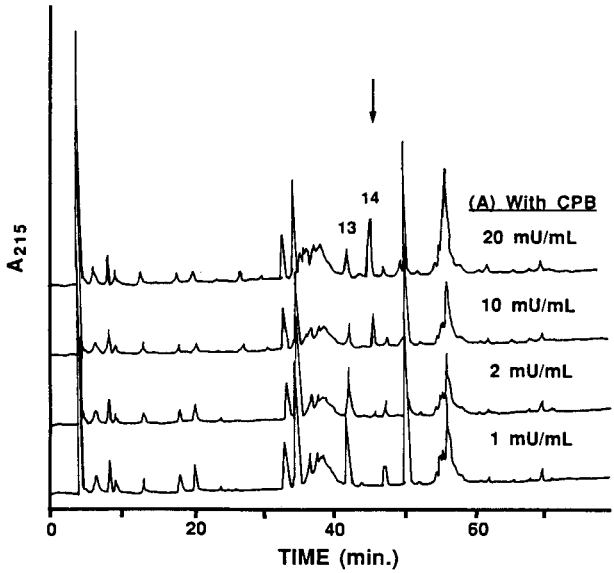


**Figure 6.** Effect of Carboxypeptidase B (CPB) on the tryptic map of EPO reference standard (A). Chromatographic conditions as in Fig. 1.



the variant EPO lot (Figure 6). The minimum amount of CPB in EPO reference standard required to produce the peak 14, as seen in the variant EPO lots , was in the range of 2-10 mU/mL (Figure 6, traces 2 and 3). On the other hand, the addition 10 mU/mL of CPB into the variant EPO lot caused a total conversion of peak 13 to the new peak 14.

## DISCUSSION

Trypsin cleaves proteins at the carboxyl side of basic amino acids, arginine and lysine. On occasion, the trypsin digestion may be incomplete or variant, especially when adjacent proline, arginine or lysine residues are present, or when the bond is buried within the 3-

dimensional structure of a native protein. In general, variations in the peptide mapping can be caused by both structural changes in the protein and by differences in digestion and chromatography. The last two variables are usually minimized by running a reference standard side-by-side with the sample, as was done in our studies. Therefore, what was observed in this study was very unusual in that only one normal tryptic peptide lost the C-terminal arginine in the variant EPO lot digestion.

EPO carbohydrate heterogeneity did not seem to be the source of the anomaly. The extra peak 14 was sharp which was indicative of a non-glycosylated fragment. Also, based on the elution order on RP/HPLC, the peak 14 was less polar than the peptide peak 13, the potential parent peptide. Attached carbohydrate would have made the peptide more polar. In addition, the sequencing data gave a normal serine which was the most likely attachment site for the carbohydrate moiety on the peptide 14.

The total loss of the extra peak in the boiling and EDTA experiments, indicated that we were dealing with some type of metallo-protease. The boiling destroyed the enzyme activity, without altering the rest of the tryptic map, and EDTA formed a chelating complex with the divalent cation of the enzyme. Among the known metallo-proteases, there are a few specific ones that are known to cleave exclusively basic C-terminal amino acids, arginine and lysine. Carboxypeptidase B (CPB) is one such exopeptidase with a Zn-cofactor and a molecular weight at around 34,000, very close to that of EPO. It can be specifically inhibited by another complexing agent, dipyridyl or by L-arginine and L-lysine.

L-lysine is known to be a much less effective inhibitor (inhibitor constant, $Ki=13\times10^{-3}M$, pH 8) than L-arginine ($Ki=0.5\times10^{-3}M$, pH 8), which was also shown to be the case experimentally. Even 200 mM of L-lysine did not cause as much suppression of the extra peak formation as did 50 mM of L-arginine. One might suspect that the trypsin used in the study contained the metallo-protease as a trace contaminant. This, however, was not likely for two reasons: 1. CPB is known to be inactivated by

lyophilization, and the trypsin solution was made from lyophilized powder, and 2. The EPO reference standard, run side-by-side, did not produce the extra peak.

While the peak 14 formation was reduced or eliminated in the variant EPO tryptic map by various techniques, like adding 8.5 mM EDTA, it could also be produced in EPO reference standard mixed with 10 mU/mL of CPB or various levels of CDM. Since there was an indication of a high level of CPB-like activity in CDM, no C-terminal arginine could be assumed to be unaffected in native EPO. According to DNA sequence data, EPO should have an arginine in the C-terminal position of 166. However, no arginyl residue at 166 has been observed by C-terminal analysis by us and others (11). The 166-arginine clipping seems to be part of the post-translational modification of the EPO molecule.

It was also intriguing to note that mainly one specific peptide 13 (144-150) was the primary target of the CPB-like enzymatic activity. In addition, there never was a complete conversion of 13 into 14 in all the variant lots studied. It, therefore, seemed that this reaction consumed all the available enzyme in the sample. If another 10 mU/mL of CPB were added into a variant EPO lot, a total conversion of 13 to 14 was observed. Only when a large excess of CPB was added, did the other peptides become targets of attack, and very altered tryptic maps were observed. Why the tryptic peptide, 144-150, was the primary target for the residual CPB-like activity, is still under investigation. The reason may be that this peptide locates in the most trypsin-sensitive part of the molecule. The results, however, demonstrate the level of sensitivity that can be achieved by peptide mapping in detecting such minute enzymatic activity.



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