Structure and Activity Dependence of Recombinant Human Insulin-like Growth Factor II on Disulfide Bond Pairing*

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The complete peptide map of purified folded recombinant human insulin-like growth factor II (rhIGF-II) was determined to verify its sequence and disulfide bonding scheme. Each peptide generated by digestion with pepsin was purified and characterized by amino acid analysis, amino acid sequence analysis, and fast atom bombardment/mass spectrometry. Some peptides were also sequenced using tandem mass spectrometry. The rhIGF-II peptide map was compared to that of rat insulin-like growth factor II and to that of a disulfide-bonded isomer of rhIGF-II. The data obtained in these studies are consistent with the conclusion that the rhIGF-II obtained from Escherichia coli has the correct amino acid composition, sequence, and the native disulfide-bonded structure. The binding affinities of these forms of recombinant IGF-II for IGF carrier proteins were measured in an IGF binding protein assay. The disulfide isomer of rhIGF-II was 160-fold less potent than native rhIGF-II in the competitive protein binding assay. These studies illustrate the need to characterize recombinant polypeptides containing disulfide bonds to allow the native structure to be verified before characterizing the biological properties of such molecules in hopes of elucidating their physiologic functions.

Insulin-like growth factors I and II from human serum are polypeptide growth factors with insulin-like activity and sequence homology to insulin. IGF-I is postulated to be the mediator of growth hormone action on skeletal tissue, while IGF-II may play a role in fetal growth (1). The physiologic role of these polypeptides is not clear due to the limited supply from natural sources. These growth factors have been synthesized by recombinant DNA methods recently, which will enable their biologic properties to be investigated and correlated with their tertiary structure (2-5).

The amino acid sequence of human IGF-II was determined in 1978 (6) and confirmed when the cDNA sequence, which codes for the precursor form of IGF-II, was elucidated in 1984 (7, 8). IGF-II is a single polypeptide chain with three disulfide bonds and has a molecular weight of 7471. The disulfide bonding scheme, assigned on the basis of homology to the insulin sequence, has never been verified experimentally (9). In preparing authentic human IGF-II from recombinant sources, it is important to confirm that the disulfide bonding scheme is the same as for IGF-II isolated from natural sources, since a mismatching of disulfide bonds could have a major influence on any biological activity, as has been observed with disulfide-bonded isomers of insulin (10).

Since human IGF-II isolated from plasma is very scarce, it was not available for comparison to recombinant human IGF-II (rhIGF-II). We therefore relied on comparative studies with rat IGF-II, isolated from BRL-3A cells, and carried out extensive chemical and biological characterization of rhIGF-II to confirm its identity as native IGF-II. Amino acid analysis agreed with the expected composition. NH₂-terminal sequence analysis through the first 27 amino acids confirmed its primary structure as predicted from the DNA sequence of the synthetic IGF-II gene. Its activity in the competitive protein binding assay is comparable to rat IGF-II, and its potency in stimulating the uptake of aminoisobutyric acid in fibroblasts has also been established (2). Human IGF-II isolated from bone had identical mitogenic activity as rhIGF-II in a rat osteoblast cell line (11). The regional distribution of Type II receptors in rat brain was studied using rhIGF-II (12, 13). Recombinant human IGF-II has also been used to isolate and clone the Type II receptor, identify it as the mannose 6-phosphate receptor and study biological activities of IGF-II mediated through the Type II receptor (14-16).

The complete peptide map of purified folded rhIGF-II was determined to verify its sequence and disulfide bonding scheme and is reported here. A comparison of the peptide map of rat IGF-II to rhIGF-II confirmed the native disulfide bonding scheme in the recombinant form. In addition, we have isolated a disulfide-bonded isomer of rhIGF-II and compared its peptide map, disulfide bonding scheme, and binding protein affinity to native rhIGF-II. Each peptide generated during the digestion of native rhIGF-II with pepsin was purified and characterized by amino acid analysis, amino acid sequence analysis, and fast atom bombardment mass spectrometry (FAB/MS). Some peptides were also sequenced using tandem mass spectrometry (MS/MS). The binding affinities of these forms of rhIGF-II were measured by their ability to displace IGF-II from IGF binding proteins. The data obtained in these studies are consistent with the conclusion that the rhIGF-II obtained from Escherichia coli has the correct amino acid composition, sequence, and the proper disulfide bonds. These studies illustrate the need to characterize recombinant polypeptides containing disulfide bonds to permit verification of the native structure before character-

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MATERIALS AND METHODS

Recombinant human IGF-II was isolated from E. coli as previously described (2) with the following modifications. An additional cation-exchange chromatography step was introduced after the folding reaction to separate the isomer of rhIGF-II from the native form of rhIGF-II, IGF-II and the disulfide-bonded isomer were eluted from a Mono S column (HR 16/10, Pharmacia LKB Biotechnology Inc.) at room temperature with a linear gradient over 150 min from 200 to 300 mM NaCl in 20 mM malonic acid, pH 2.5, 7 M urea. The disulfide-bonded isomer eluted with 230 mM NaCl and native rhIGF-II with 260 mM NaCl. IGF-II was further purified by reversed-phase high performance liquid chromatography (RP-HPLC) and gel filtration as previously described (2). The S-carboxymethylated derivative of purified native rhIGF-II was prepared (17) and desalted over a Sephadex G-25 column. Rat IGF-II was purified from conditioned media as described (18). Pepsin (EC 3.4.23.1), 3165 units/mg, was used as received from Sigma. All other chemicals were reagent grade and used without further purification.

Amino Acid Sequence Studies—A semi-quantitative NH₂-terminal sequence analysis was conducted on an S-carboxymethylated derivative of rhIGF-II shown by amino acid analysis to be fully derivatized. The sequence analysis was conducted using automated Edman degradation in a Beckman 890D spinning cup sequenator equipped with a microaccessor controller. The resulting phenylthiohydantoin (PTH) were separated by RP-HPLC and detected at 269 nm. A thiol adduct of PTH-serine was used to identify and quantitate serine. The sequenator was programmed to allow for extra reaction times at proline residues, thus minimizing the incomplete reactions often associated with this residue.

Peptides from the peptic digest were either sequenced using the system described above or using an Applied Biosystems gas-phase sequenator, according to the manufacturer’s directions.

Peptide Mapping—Pepsin was used to digest rhIGF-II at room temperature in 0.01 N HCl at pH 7.5, for 4 h, with an enzyme to substrate ratio of 1:5. The reaction mixture was injected onto the Brownlee column and eluted using the gradient system described above.

Refolding Studies—The disulfide-bonded isomer and native form of rhIGF-II were reduced by dissolving 1 mg of each in 10 mM Tris, 7 M urea, pH 8.0, and adding a 120-molar excess of diethiothreitol. The reaction was quenched after 20 min by desalting on a Sephadex G-25 column equilibrated on 0.01 N HCl. The reduced IGF-II was refolded at 67 μg/ml in 20 mM glycine, pH 10.0, at 4 °C in the presence of a 36-molar excess of cysteine. The folding reaction was quenched after 20 h by the addition of 1 M acetic acid to pH 4.7. The samples were analyzed by RP-HPLC using a Du Pont Zorbax C8 column in 0.2 mM (NH₄)₂SO₄, 0.1 M H₂SO₄ with a linear gradient from 20 to 26% acetonitrile in 30 min with a flow rate of 1.0 ml/min at 40 °C.

Competitive Protein Binding Assay—The competitive protein binding assay for IGF-II has been described previously (20) as well as the modifications used (2).

RESULTS AND DISCUSSION

The biosynthesis of polypeptide hormones in host organisms such as E. coli by recombinant DNA technology offers a convenient method for obtaining large quantities of otherwise scarce proteins. Frequently the physiologic role of the cloned protein is unknown, as is the case with IGF-II, and having a large supply of authentic protein can aid in determining its function. Establishing the authenticity of a recombinant protein such as IGF-II, which contains three disulfide bonds and hence has the potential of forming disulfide-bonded isomers, is therefore a prerequisite for studies aimed at elucidating function. In addition, the disulfide bonds for IGF-II have previously been assigned on the basis of the homology of the cysteine residues to the insulin sequence but have never been determined experimentally.

Recombinant folded human IGF-II was analyzed by NH₂-terminal analysis and digested with pepsin to verify its native structure and disulfide bonding scheme. Pepsin, which is active at pH 2, was used to digest rhIGF-II so as to prevent a possible disulfide interchange reaction. A typical peptide map of rhIGF-II is shown in Fig. 1. The peak assignments shown correspond to the peptic cleavage sites for rhIGF-I1 in Fig. 2 and described in Table I. The sequence analysis was conducted using automated Edman degradation in a Beckman 890D spinning cup sequenator equipped with a microaccessor controller. The resulting phenylthiohydantoin (PTH) were separated by RP-HPLC and detected at 269 nm. A thiol adduct of PTH-serine was used to identify and quantitate serine. The sequenator was programmed to allow for extra reaction times at proline residues, thus minimizing the incomplete reactions often associated with this residue.

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raphy and RP-HPLC (peptides 9,6 and 1B,3A for instance). All peptides isolated by anion-exchange chromatography at pH 7.5 had identical retention times to the peptides in the original peptide map which were kept at low pH. Therefore, under the conditions used for the purification, no disulfide bond rearrangement has taken place.

![Diagram](human-igf-ii.png)

**FIG. 2.** Peptic cleavage sites of rhIGF-II as determined by amino acid analysis, FAB/MS, MS/MS, and Edman sequencing of isolated peptides as described in Table I. Bold lines indicate the major cleavage site while lighter lines show minor cleavage sites. *Shaded residues* correspond to the homologous residues in the human insulin A and B chains.

The presence of the putative native disulfide bonds, Cys²⁻Cys¹⁷, Cys¹⁷⁻Cys⁶⁰, and Cys⁶⁰⁻Cys¹⁵¹, in rhIGF-II was demonstrated as follows. Isolation of peptide 4-10 clearly established the presence of the native disulfide pair Cys¹⁷⁻Cys⁶⁰. The possibility of mismatched disulfide bonds in peptide 2-7 cannot be excluded on the basis of amino acid composition and FAB/MS data. In order to verify the presence of native disulfide bonds in this peptide, rat IGF-II, which is homologous to human IGF-II in all peptides except 4 and 5, was digested with pepsin and the HPLC map compared to that of rhIGF-II, as shown in Fig. 3. Fragment 2-7 eluted in the same position on both maps, indicating that the folded rhIGF-II possesses the same disulfide pairs as IGF-II isolated from a natural source. This comparison does not allow assignment of the disulfide-bonded structure in peptide 2-7 but only the conclusion that rhIGF-II has the same disulfide-bonded structure as rat IGF-II isolated from a natural source.

A structural isomer of IGF-II formed during the disulfide interchange reaction used to generate native IGF-II and eluted earlier in the HPLC chromatogram (Fig. 4). The isomer was separated from native IGF-II by cation-exchange chromatography and further purified by RP-HPLC. Both forms of IGF-II had identical amino acid compositions and NH₂-terminal sequences. Each form of IGF-II was reduced with excess cysteine and allowed to refold in the presence of air to deter-

### Table I

**Characterization of human IGF-II peptic peptides**

| Peptide         | Sequence | Amino acid composition | Mass  |
|-----------------|----------|------------------------|-------|
| NH₂ terminus    | 1        | T,S,E,P,A,L,Y,R        | 935²  |
| 1A              | 1-8      | S,E,P,A,Y,R            | 721²  |
| 1B              | 1-7      | T,S,E,P,A,Y,R          | 822²  |
| Double disulfide region | 2-7  | D,S,E,2G,4C,L,F,R      | 1435² |
|                 | 7        | T,D,S,2E,2G,4C,2L,F,R  | 1647² |
| 2A              | 7        | D,S,E,2G,4C,L,F,R      | 1548² |
| 2B              | 7        | D,S,E,2G,4C,L,F,R      | 1548² |
| Single disulfide region | 4-10 | D,T,S,E,P,2G,2A,2C, V,Y,2F,K,R | 1866² |
|                 | 4        | D,T,S,E,P,2G,2A,2C, V,Y,2F,K,R | 1866² |
| 4B-10           | 4-10     | D,T,S,E,P,2G,2A,2C, V,Y,2F,K,R | 2029² |
| 4B              | 10       | D,T,S,E,P,2G,2A,2C, V,Y,2F,K,R | 1065² |
| 4-10A           | 19-26    | D,T,S,E,P,2G,2A,2C, V,Y,2F,K,R | 2006² |
| 4               | 10       | D,T,S,E,P,2G,2A,2C, V,Y,2F,K,R | 1065² |
| C-peptide       | 5        | 4S,P,G,A,V,F,5R        | 1618² |
| 5A              | 28-33    | 2S,P,A,F,R             | 663²  |
| 6               | 42-44    | E,V,I                  | 359²  |
| 8               | 53-55    | A,2L                   | 315²  |
| 9               | 56-58    | T,E,L                  | 361²  |

* Amino acid sequence verified by MS/MS.
\^ Amino acid sequence determined by Edman degradation.
* Molecular weight verified by FAB/MS is based on isotopic masses.
Structure and Activity Dependence of rhIGF-II

A
Human IGF-II

B
Rat IGF-II

Fig. 3. A, comparison of human and rat IGF-II amino acid sequences and the double disulfide-bonded peptide generated from digesting IGF-II with pepsin. B, comparison of rat IGF-II and rhIGF-II peptide maps on the reversed-phase system described in the text. The hatched peaks represent the 2-7 peptides or double disulfide-bonded region of IGF-II.

Fig. 4. Reversed-phase HPLC analysis of refolded IGF-II from reduced native rhIGF-II and reduced isomeric rhIGF-II on a Zorbax C8 column as described in the text. The early eluting peak around 1440 s is the isomer of rhIGF-II (4%), followed by native IGF-II (40%) around 1600 s, and the late eluting peaks between 2300 and 2500 s are IGF-II disulfide-bonded polymers (56%).

Fig. 5. Reversed-phase HPLC peptide map of rhIGF-II isomer and native rhIGF-II. The only peaks with different elution positions are hatched and correspond to those peptides, 2-7, 2B-7, and 2A-7, containing the double disulfide-bonded region of IGF-II, as described in Table I.

Peptide mapping of the isomer was conducted to further elucidate the exact nature of the mismatched disulfide bonds in the isomer. A comparison of the HPLC peptide maps of native IGF-II and the IGF-II isomer in Fig. 5 shows that the main difference between the two forms was due to the 2-7 peptide (9-13)-(45-52), which is the double disulfide-bonded region of IGF-II. The other disulfide-bonded peptide 4-10
eluted in the same position as the 4-10 peptide from native IGF-II, which eliminated the possibility of the Cys41-Cys40 disulfide bond being mismatched. The 2-7 peptide from the isomer digest was isolated by HPLC and further digested with trypsin to cleave at Arg49. This second digestion with trypsin can distinguish between the structure shown below for isomer III, with a disulfide bond between two adjacent cysteine residues 46 and 47, and isomers I and II. A single peptide is generated after trypsin digestion in the case of isomer I and isomer II, compared to two peptides from isomer III.

**Isomer I (native)**

45 Glu-Cys-Phe-Arg-Ser-Cys-Asp
   | Cys-Gly-Gly-Leu
   9  13

**Isomer II**

45 Glu-Cys-Phe-Arg-Ser-Cys-Asp
   | Cys-Gly-Gly-Leu
   9  13

**Isomer III**

45 Glu-Cys-Phe-Arg-Ser-Cys-Asp
   | Cys-Gly-Gly-Leu
   9  13

**two peptides**

The elution position of the 2-7 peptide from isomeric IGF-II and native IGF-II changed after digestion with trypsin, but two smaller peptides were not generated (Fig. 6). NH2-terminal sequence analysis of the trypsin-digested 2-7 peptide confirmed that the double disulfide-bonded peptide is still intact and has been cleaved at Arg49. These results showed that neither isomeric IGF-II nor native IGF-II contained the disulfide-bonded structure of isomer III shown above. However, these results cannot distinguish between the presumed native structure, isomer I, and isomer II, since the 2-7 peptide from either would yield a single peptide after trypsin digestion. Yet, our results would suggest that the mismatched form of IGF-II most likely has the structure of isomer II.

The biological activity of isomeric IGF-II was measured in the competitive protein binding assay and compared to recombinant native IGF-II. Fig. 7 shows the displacement of iodinated IGF-II by native rhIGF-II and isomeric IGF-II from a partially purified preparation of bovine plasma binding proteins. The isomer of IGF-II was approximately 160-fold less potent than native rhIGF-II in its ability to compete with IGF-II for binding proteins. Scatchard analysis of these data gave an association constant of $4 \times 10^9$ M$^{-1}$ for native IGF-II and $1.4 \times 10^7$ M$^{-1}$ for isomeric IGF-II. Thus, a slight structural modification of IGF-II had a profound effect on the ability to form a complex with binding proteins.

Fig. 6. Reversed-phase HPLC analysis of rhIGF-II peptide 2-7 isolated from a pepsin digest of native rhIGF-II and isomeric rhIGF-II, and then digested with trypsin, as described in the text.

Fig. 7. Competitive protein binding assay of native rhIGF-II and isomeric rhIGF-II using native $^{125}$I-rhIGF-II and a crude preparation of bovine plasma binding proteins.
literature ($1.3 \times 10^{-7}$ to $1.44 \times 10^{-7}$M), assuming an equimolar reaction between IGF-II and each mole of binding protein (27, 28). These crude calculations show that if the disulfide bonds of IGF-II were improperly paired, between 42 and 45% of the IGF-II would be free IGF-II (~290 ng/ml) compared to 0.4 and 0.6% for native IGF-II (~3 ng/ml). Although these estimates are based on several assumptions, they do provide reasonable values of free native IGF-II and illustrate how a change in affinity of this order can influence the amount of free IGF-II. The consequences of free IGFs were illustrated by a comparison of the observed biological effects of IGFs administered to rats either by slow infusion or a bolus injection (26). Acute insulin-like effects were seen with the bolus injection when the binding capacity of the binding proteins was temporarily overridden, whereas growth-promoting effects without hypoglycemia were observed with the slow infusion. In fact, the regulation of the insulin-like activity of IGFs by their binding proteins is critical to the control of blood glucose levels, since IGFs are present in serum at about 1000 times the concentration of insulin. The potential effects of IGF-II disulfide bond isomerization on glucose homeostasis could therefore be devastating. The disulfide-bonded structures of other polypeptide hormones, such as epidermal growth factor, interleukin-2, and insulin, have been studied (10, 29, 30). The ability to isomerize has been noted in the case of interleukin-2 and insulin, and the biological consequences of isomerization have been described for insulin. The biological activity of two chemically synthesized disulfide isomers of insulin has been characterized. They are full agonists with about 20% of the potency of insulin in stimulating glucose oxidation and inhibiting lipolysis in fat cells in a rat hypoglycemia test and a mouse convulsion assay.

These peptide mapping studies with rhIGF-II, rat IGF-II, and a disulfide-bonded isomer of rhIGF-II are consistent with the conclusion that the rhIGF-II produced in our laboratory has the native conformation and the full set of proper disulfide bonds. The fact that a disulfide-bonded isomer forms during the refolding reaction used to generate native IGF-II from the S-sulfonate, and from fully reduced IGF-II, illustrates the need to completely characterize the chemical structure of recombinant proteins containing disulfide bonds. This is particularly true of proteins and polypeptides whose supply is limited and whose function is unknown, since isomeric forms of polypeptides can have altered biological activity and/or potency. The preliminary comparison between recombinant native human IGF-II and isomeric IGF-II biological activities show there are large differences which could lead to artificial characterization of its physiological function.

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Continued on next page.
Table II

| Amino acid | 1 | 2 | 3 | 4 | 5 |
|------------|---|---|---|---|---|
| Asp        | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Thr        | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Ser        | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Glu        | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Pro        | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Gly        | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Ala        | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Cys        | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Val        | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Ile        | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Leu        | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Arg        | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

Table III (Continued)

| Cycle | 1 | 2 | 3 | 4 | 5 |
|-------|---|---|---|---|---|
| V     | 199.4 | 228.8 | 199.4 | 228.8 | 199.4 |
| V     | 199.4 | 228.8 | 199.4 | 228.8 | 199.4 |
| V     | 199.4 | 228.8 | 199.4 | 228.8 | 199.4 |
| V     | 199.4 | 228.8 | 199.4 | 228.8 | 199.4 |
| V     | 199.4 | 228.8 | 199.4 | 228.8 | 199.4 |

* a indicates reanalysis because of ammonia interference.

Table III

Sequence Analysis of Peptide Generated Peptides from rHGF-II

| Peptide | Cycle | 1 | 2 | 3 | 4 | 5 |
|---------|-------|---|---|---|---|---|
| V       | 21.4 | 1.2 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) |
| V       | 21.4 | 1.2 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) |
| V       | 21.4 | 1.2 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) |
| V       | 21.4 | 1.2 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) |
| V       | 21.4 | 1.2 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) | 1.0 (1) |

* a indicates residue present but not quantified.
* b Numbers are in nmol and sequenced by Beckman 800C spinne.
Table IV

Amino Acid Analysis of S-Carboxymethyl-Cys-IGF-II (Lot T51-ZL7-9B)

| Amino Acid | Residues found |
|------------|----------------|
| Asp        | 3.0 (3)        |
| Thr        | 3.8 (4)        |
| Ser        | 4.3 (7)        |
| Glu        | 7.7 (7)        |
| Pro        | 3.2 (3)        |
| Gly        | 5.0 (5)        |
| Ala        | 5.0 (5)        |
| Asp-Cys    | 6.2 (6)        |
| Val        | 5.5 (4)        |
| Met        | 0.0 (0)        |
| Ile        | 0.6 (1)        |
| Leu        | 6.0 (5)        |
| Tyr        | 2.9 (3)        |
| Phe        | 3.9 (4)        |
| His        | 0.0 (0)        |
| Lys        | 1.0 (1)        |
| Arg        | 7.9 (9)        |

*Numbers in parentheses indicate theoretical value for the amino acid.*

Calculated values based on average mol of Asp, Glu, Gly, Ala, Leu, Lys, Arg. Samples were analyzed in triplicate as described under "Methods and Materials."

Table V

Sequence Analysis of S-Carboxymethyl-Cys-IGF-II (Lot T51-ZL7-9B)

| Cycle | PTH derivative | Residues found |
|-------|----------------|----------------|
| 1     | Ala            | 4.0 (3)        |
| 2     | Tyr            | 26.1           |
| 3     | Arg            | 11.7           |
| 4     | Pro            | 30.7           |
| 5     | Ser            | 28.9           |
| 6     | Glu            | 18.3           |
| 7     | Thr            | 8              |
| 8     | Leu            | 13.1           |
| 9     | S-Cm-Cys       | 11.0           |
| 10    | Gly            | 11.0           |
| 11    | Gly            | 11.7           |
| 12    | Gly            | 7.8            |
| 13    | Leu            | 7.1            |
| 14    | Val            | 6.9            |
| 15    | Arg            | 3.7            |
| 16    | Thr            | 39             |
| 17    | Leu            | 4.0            |
| 18    | Glu            | 3.1            |
| 19    | Phe            | 2.3            |
| 20    | Val            | 2.0            |
| 21    | S-Cm-Cys       | 2.0            |
| 22    | Gly            | 2.0            |
| 23    | Asp            | 1.1            |
| 24    | Arg            | 4              |
| 25    | Gly            | 1.8            |
| 26    | Phe            | 1.0            |
| Total yield | 65.2%           |
| Repetitive yield | 86.3% |

*Denotes PTH present but not quantified.*

S-Cm-Cys, S-Carboxymethylcysteine.