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재조합 소성장호르몬의 구조적 특성

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The Structural Characterization of Recombinant Bovine Somatotropin Expressed in *Escherichia coli*

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ABSTRACT

In this paper we have described the structural characterization of recombinant bovine somatotropin produced in *Escherichia coli*. Recombinant bovine somatotropin consists of 191 amino acid residues with a calculated molecular weight of 21,802 Da. For fragmentation of recombinant bovine somatotropin, we have used trypsin, *Staphylococcus aureus* V8 protease, CNBr, and mild acid hydrolysis method. Digestion and cleavage with these proteases and chemicals yielded peptides of various size for amino acid sequence determination. The N-terminal sequence analysis was carried out up to thirty residues. Because the design of the recombinant bovine somatotropin gene for expression was such that the coding sequence begins with an initiation codon, AUG, before Ala, the first amino acid of bovine somatotropin, we could expect the initial amino acid as N-formyl Met. But the first amino acid of this protein, expressed in *E. coli* cells as inclusion bodies, was Ala. And the amino acid composition of RP-HPLC purified recombinant bovine somatotropin was determined and no essencial difference was observed. The amino acid sequence of the recombinant bovine somatotropin was identical to that predicted from its recombinant gene. There was no processing or replacement of amino acid residues in recombinant bovine somatotropin expressed in *E. coli*. The hydropathy plot of recombinant bovine somatotropin revealed a hydrophobic region at the NH_2-terminus and hydrophilic region at the COOH-terminus. The *E. coli* expression system is thought to be valuable for the expression of recombinant bovine somatotropin because protein was processed to remove the N-terminal Met residue by methionyl-aminopeptidase autonomously.

INTRODUCTION

Even though many useful proteins including growth hormones have been produced by recombinant DNA technology, few structural comparisons of the recombinant protein with the natural counterpart have been studied (1-3). Unusual recombinant proteins having different amino acid sequences from those expected from the respective cDNA sequences were sometimes encountered in the expression of the recombinant genes (4, 5). In the cases when proteins are pro-

duced in prokaryotic cells such as *E. coli*, it may be difficult to expect the proper post-translational modifications, *i.e.*, processing including extra Met removal, folding, and glycosylation. The resulting product may lead to biological malfunction, especially antigenicity, when used therapeutically (6-8).

Genes for several growth hormones, such as human, bovine, porcine etc., have been cloned (9-14) and some of them have been expressed in *E. coli* (9, 11, 12, 15-17). Among them, only recombinant human (18-21), recombinant eel (22, 23), and recombinant chicken growth hormone (24) have been reported for structural comparison, but the structural characterization of recombinant bovine somatotropin has not been reported.

Bovine somatotropin (bovine growth hormone) is a single polypeptide composed of 191 amino acids. It was initially synthesized as a pre-form containing an amino-terminal extention of 26 amino acid residues in bovine pituitary (25). Bovine somatotropin is being developed as an animal medicine for growth promotion and to increase milk production in dairy cow (26, 27). Administration of bovine somatotropin to dairy cow significantly increased the milk production and reduced the amount of feed required for given output of milk (26). The availability of sufficient supply of bovine somatotropin might be useful in animal husbandry for more economical food production. Since mammalian growth hormones are species specific for their biological activity, alternative sources to bovine somatotropin can not be used for this purpose.

In this paper, we describe the amino acid composition and primary structure of recombinant bovine somatotropin and the value of Met removal from recombinant protein autonomously in the *E. coli* expression system. This is the first report on the complete amino acid sequence of recombinant bovine somatotropin expressed in *E. coli*.

MATERIALS AND METHODS

Materials

Recombinant bovine somatotropin expressed in *E. coli* (36) was obtained from the LUCKY R&D Center, Biotechnology. The purity was judged by same method used in previous study (28).

Trypsin (E.C.3.4.21.4), sequencing grade, and *Staphylococcus aureus* V8 protease (E.C.3.4.21.19), sequencing grade, were purchased from Boehringer Mannheim Biochemica (Mannheim, Germany). HPLC-grade trifluoroacetic acid (TFA) (Pierce, Rockford, IL, U.S.A.), HPLC-grade acetonitrile (Baxter Diagnostics Inc., MI, U.S.A.), analytical grade reagents and distilled, deionized Water (Millipore, Bedford, MA, U.S.A.) were used. And the following materials were obtained from the indicated commercial sources: ammonium acetate, β-mercaptoethanol, cyanogen bromide, EDTA, formic acid, guanidine HCl, sodium hydroxide, Trizma base, and 4-vinylpyridine (Sigma Chemical Co., St. Louis, U.S.A.), calcium chloride (Fluka Chemie AG, Switzerland), HCl (Pierce, U.S.A.), Tris-HCl (Boehringer Mannheim Biochemica, Germany).

All other chemicals were of the highest commercially available grade.

Nomenclature of Peptides

The first fractionation of tryptic (Fig. 2) and *S. aureus* V8 protease digested recombinant bovine somatotropin (Fig. 4) are designated by BT and BG. Mark T, G, CN, and H designate peptides produced by digestion, cleavage, or hydrolysis with trypsin (Fig. 3), *S. aureus* V8 protease (Fig. 5), CNBr (Fig. 6), and mild acid (Fig. 7), respectively.

Enzymatic Digestion

Trypsin Digestion

The tryptic digested peptides of recombinant bovine somatotropin were obtained by employing the following conditions (28). 10 mg sample of recombinant bovine somatotropin was dissolved in 40 ml of cold 1 mM NaOH. Immediately upon dissolution, 5 ml of 1 M Tris-HCl, 10 mM calcium chloride buffer (pH 8.3) was added to make 0.1 M Tris-HCl, 1 mM calcium chloride condition.

This was followed by the addition of 5 ml of 0.1 mg/ml RP-HPLC trypsin prepared in cold 1 mM HCl. The ratio of trypsin to recombinant bovine somatotropin was 1:20 (w/w). The mixture was incubated at 24 ℃ for 24 hrs and quenched by freezing at -20℃.

The purification of tryptic peptides was performed by two-dimensional HPLC descrived else where (28). The tryptic digested rbST was loaded directly on a Protein-Pak™ SP-8HR Strong Cation Exchange Column for the first dimensional peptide mapping. The column was equilibrated with 20 mM sodium phosphate, pH 2.5 (buffer A). This peptides were eluted at a flow rate of 0.8 ml/min with a three-step gradient programming from buffer A to the buffer B (20 mM sodium phosphate, 1 M NaCl, pH 7.0); Initial to 5 min, 0~35 % buffer B; 5 to 30 min, 35~100 % buffer B; 30 to 35 min, 100 % buffer B.

This aliquots were applied to the RP-HPLC column and eluted for 28 min at a flow rate of 1.0 ml/min with a step gradient; Initial to 10 min, 0~30 % buffer B; 10 to 28 min, 30~46 % buffer B. The buffer A in the second dimensional peptide mapping was 0.1 % TFA in water and the buffer B was 0.1 % TFA in acetonitrile.

S. aureus V8 Protease Digestion

8 mg of recombinant bovine somatotropin was digested with *S. aureus* V8 protease in 100 mM ammonium acetate (pH 4.0) for 48 hrs at 37℃ at enzyme to substrate ratio of 1:50 (w/w) (29).

The *S. aureus* V8 protease digested recombinant bovine somatotropin was loaded directly on a Shodex Protein WS-803F Column™ (300 Å, Shodex Co., Japan) for the first dimensional peptide mapping. The column was equilibrated with 0.1 % TFA in water. This peptides were fractionated at a flow rate of 0.7 ml/min.

This aliquots were applied to the RP-HPLC column and eluted for 30 min at a flow rate of 1.0 ml/min with a step gradient; Initial to 10 min, 0-60 % buffer B; 10 to 22 min, 60~65 % buffer B; 22 to 30 min, 65~100 % buffer B. The buffer A in the second dimensional peptide mapping was 0.1 % TFA in water and the buffer B was 0.1 % TFA in acetonitrile.

Chemical Cleavage

Reduction and S-Pyridylethylation

Recombinant bovine somatotropin was reduced and s-pyridylethylated by the method of Fullmer (30). The s-pyridylethylated protein was obtained as a lyophilizate after desalinization by RP-HPLC. This s-pyridylethylated recombinant bovine somatotropin was subjected to CNBr cleavage.

CNBr Cleavage

Recombinant bovine somatotropin was cleaved with a 100-fold molar excess CNBr of expected number of Met residues in 70% formic acid for 24 hrs at room temperature (31). The cleaved sample was diluted about 10-fold with water and lyophylized with Speed-Vac Concentrator. This peptides were fractionated for 45 min at a flow rate of 1.0 ml/min with following way; Initial to 10 min, 0~30 % buffer B; 10 to 40 min, 30~100 % buffer B; 40 to 45 min, 100 % buffer B. The buffer A was 0.1 % TFA in water and buffer B was 0.1 % TFA in acetonitrile.

Mild Acid Hydrolysis

Recombinant bovine somatotropin was cleaved in the following condition (32). 2 mg of protein was dissolved in 500 μl of 0.03 N HCl in an ampule. After flushing the solution with nitrogen gas and sealed. It was kept for 3 hrs at 105 ℃ and dried in a Speed-Vac Concentrator (Savant, NY, U.S.A.). This hydrolysate was loaded directly on μBondapak™ C18 column and eluted for 76 min at a flow rate of 1.0 ml/min with a step gradient; Initial to 10 min, 0 % buffer B; 10~15 min, 0~30 buffer B; 15~70 min, 30~70 buffer B; 70~75 min 70~100 % buffer B; 75~76 min, 100 % buffer B. The buffer A was 0.1 % TFA in water and buffer B was 0.1 % TFA in acetonitrile.

Amino Acid Composition Analysis

Amino acid composition analysis was per

formed with a Pico Tag™ amino acid analysis system (Waters, MA, U.S.A.), (33). In the analysis using the Waters instrument, protein was hydrolysed with 5.7 M HCl containing 0.2 % phenol in solution at 110 ℃ for 24 hrs.

Determination of Amino Acid Sequence

Amino acid sequencing of peptides were carried out according to the modified method reported by Applied Biosystems Inc.. The sequences of a given peptides were determined by Edman degradation in an Applied Biosystems model 471A Protein/Peptide Sequencer (Applied Biosystems Inc., CA, U.S.A.). The detail procedures of this method have been described elsewhere (34).

RESULTS AND DISCUSSION

The expression of recombinant bovine somatotropin produced in *E. coli* has been reported by several groups. However, the reports on this protein have only focused on expression and purification, and there have been no experiments studying the primary structure of recombinant bovine somatotropin. Sometimes recombinant products are different from the expected amino acid sequence when they are expressed in bacterial or eukaryotic cells. These phenomena may be caused by different modification steps in the host cell. Therefore it is very important to perform a structural characterization of the recombinant protein.

In this paper we have described the primary structural study of bovine somatotropins produced in *E. coli*. For determining the amino acid sequence of the recombinant bovine somatotropin, we have used trypsin, *S. aureus* V8 protease, CNBr, and mild acid hydrolysis method for fra-

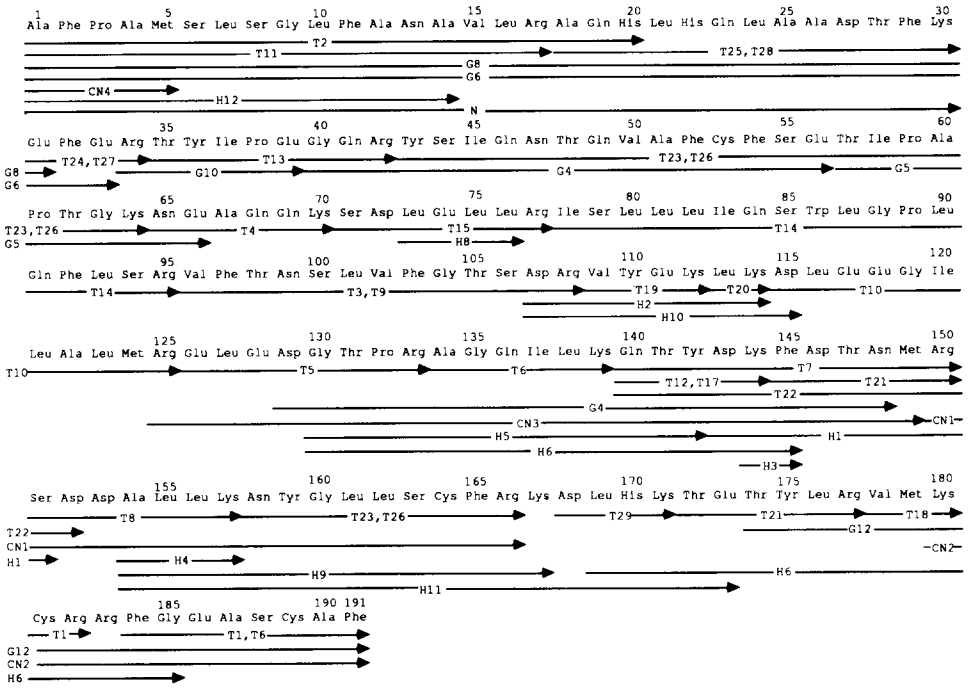

Fig 1. Amino acid sequence of recombinant bovine somatotropin. The figure shows peptides obtained by cleavage with Trypsin (T), *S. aureus* V8 protease (G), Mild acid hydrolysis (H), CNBr (CN), and direct N-terminal sequence analysis (N). Arrows indicate residues identified by N-terminal sequence analysis.

Table 1. N-terminal amino acid sequence analysis of the recombinant bovine somatotropin[a].

Cycle No.	PTH amino acid	Cycle No.	PTH amino acid
1	Ala(368.5)	16	Leu(176.9)
2	Phe(259.6)	17	Arg(169.1)
3	Pro(232.8)	18	Ala(156.7)
4	Ala(270.2)	19	Gln(80.23)
5	Met(191.8)	20	His(82.82)
6	Ser(33.82)	21	Leu(145.7)
7	Leu(204.7)	22	His(75.91)
8	Ser(28.25)	23	Gln(92.63)
9	Gly(196.8)	24	Leu(135.2)
10	Leu(185.1)	25	Ala(110.9)
11	Phe(173.2)	26	Ala(137.3)
12	Ala(193.2)	27	Asp(134.7)
13	Asn(189.7)	28	Thr(23.65)
14	Ala(191.4)	29	Phe(90.09)
15	Val(151.8)	30	Lys(80.64)

a Values in parentheses are the yields (pmol) of PTH-amino acid

gmentation. Digestion and cleavage with these proteases and chemicals yielded peptides of various size.

The recombinant bovine somatotropin to be sequenced was pure as judged by polyacrylamide gel electrophoresis with sodium dodecyl sulfate, RP-HPLC, and N-terminal sequence analysis. The results of automated N-terminal sequence analysis of the recombinant bovine somatotropin are shown in Table 1 and Fig.1. Analysis was carried out up to thirty residues. Because the design of the recombinant bovine somatotropin expression plasmid was such that the coding sequence begins with an initiation codon, AUG, before Ala (36), the first amino acid of bovine somatotropin, we could expect the initial amino acid to be N-formyl Met consisting of 192 amino acids. But protein, expressed in *E. coli* cells as inclusion bodies, was recombinant bovine somatotropin with Ala at the N-terminus, which as detected by direct N-terminal sequencing (Table 1), (28). The sequencing results indicated that recombinant bovine somatotropin was processed to remove the N-terminal Met residue by methionyl-

Table 2. Amino acid composition of recombinant bovine somatotropin.

Amino acid	Expected value[a] (residue)	Experimental value[b] (residue)
A(Ala)	15	15.6
C(Cys)	4	—
D(Asp)	16[c]	15.9
E(Glu)	24[d]	23.8
F(Phe)	13	13.0
G(Gly)	10	10.9
H(His)	3	2.75
I(Ile)	7	5.64
K(Lys)	11	10.6
L(Leu)	27	23.7
M(Met)	5[e]	3.46
P(Pro)	6	6.36
R(Arg)	13	12.4
S(Ser)	13	12.1
T(Thr)	12	11.8
V(Val)	6	5.70
W(Trp)	1	—
Y(Tyr)	6	5.16
Total	192	

[a] Calculated assuming Phe residues as 13.00.
[b] Calculated from the deduced sequence data of bovine somatotropin gene inrecombinant plasmid.
[c] This value is the summation of the number of Asp and Asn.
[d] This value is the summation of the number of Glu and Gln.
[e] This value includes an extra Met at its N-termius.

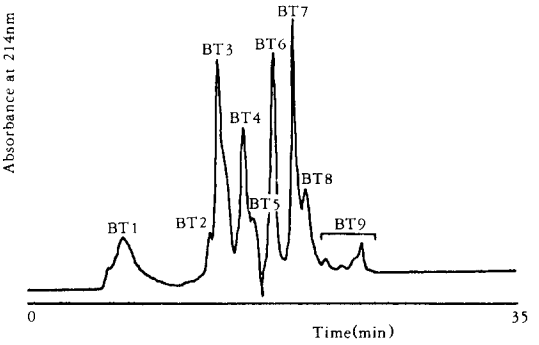

Fig 2. First fractionation of the peptides of the tryptic digest of recombinant bovine somatotropin on a Protein-PakTM SP-8HR Strong Cation-exchange column. Fractions in each peak were pooled and dried. These materials were resolved and applied to a reverse-phase column (See Fig 3).

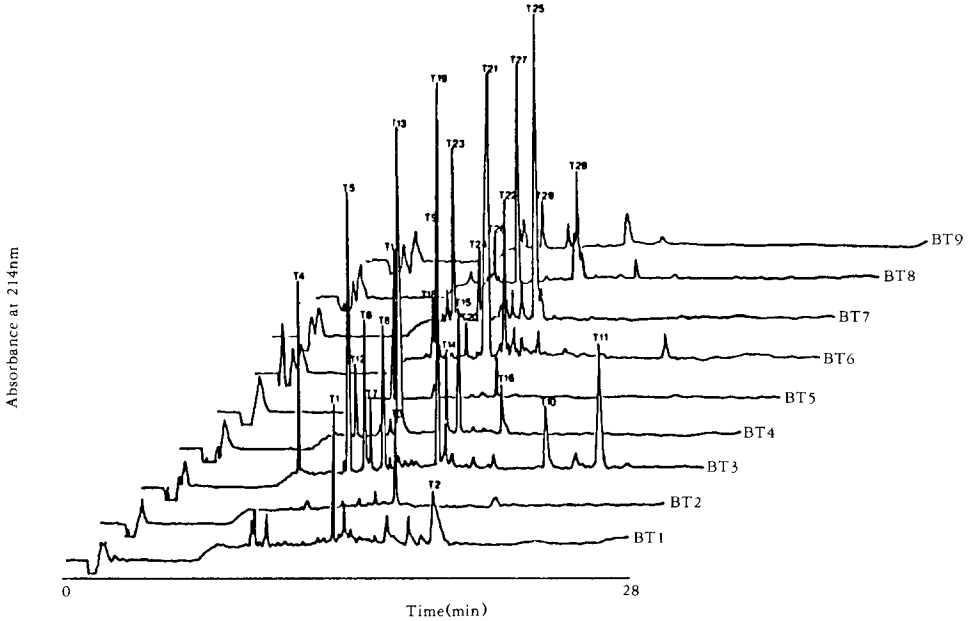

Fig. 3. Second fractionation of the chromatograms of peak BT1-9 of Fig. 2 on a reverse-phase Delta-Pak™ C18 column (3.9x150mm, 300 Å, Waters, MA, U.S.A.) as the second dimmension. The mark at the right of each chromatogram gives the fraction number from Fig. 2. The number in peaks give the positions of the peptide in recombinant bovine somatotropin sequence in Fig. 1.

aminopeptidase in *E. coli*. This processing has very important meaning because many recombinant growth hormones such as human, eel, and chicken have extra Met at their N-terminal end when they are expressed in the *E. coli* expression system. So these extra Met should be removed after purification with chemicals or aminopeptidases to be used pharmacotically.

This observation was also supported by the suggestion of Hirel *et al.*(37) that extention of N-terminal methionine excision from *E. coli* proteins was governed by the side-chain length of the penultimate amino acid. The initiating Met followed by amino acid residues with a side-chain of small radius, such as Ala, could be removed in *E. coli*, but amino acid residues with side chain of large radius found in the cases of human, eel, and chicken could not be removed easily. Therefore, the *E. coli* expression system is thought to be valuable for the expression of recombinant bovine somatotropin.

The amino acid compositions of RP-HPLC purified recombinant bovine somatotropin and deduced from its recombinant gene sequence in plasmid are shown in Table 2, and no essencial difference was observed except for additional Met at the N-terminus.

The recombinant bovine somatotropin was initially digested with trypsin, and cleaved peptides were purified by two dimmensional HPLC. The first fractionation of trypsin digest was performed with strong cation-exchage chromatography (Fig. 2). From this chromatography we obtained nine fractionations (BT1-BT9), and each of these fractions were applied to RP-HPLC (Fig. 3). These peptides, designated T1-T29, were isolated and subjected to sequence determination (Fig. 1).

Digest of the recombinant bovine somatotropin with *S. aureus* V8 protease was separated in a similar manner in peptide purification of tryptic digested recombinant bovine somatotropin except

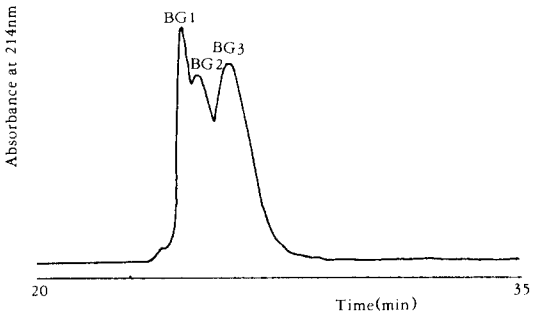

Fig 4. First fractionation of the peptides of *S. aureus* V8 protease digest of recombinant bovine somatotropin on a Shodex™ column. Fractions in each peak were pooled and dried. These materials were resolved and applied to a reverse-phase column (See Fig 5).

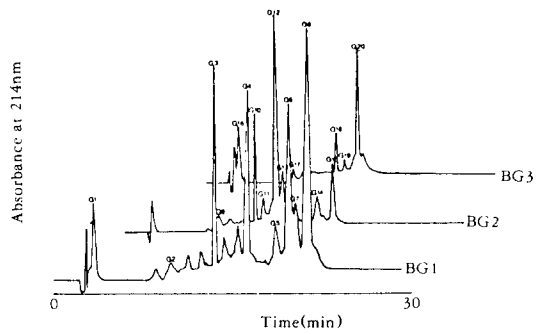

Fig 5. Second fractionation of the chromatograms of peak BG1-3 of Fig 4 on a reverse-phase Delta-PakTM C18 column as the second dimension. The marks at the right of each chromatograms give the fraction number from Fig 4. The number in peaks give the position of the peptide in recombinant bovine somatotropin sequence in Fig 1.

for the use of high-performance gel filtration chromatography instead of strong cation-exchange chromatography (Fig. 4). And each of these fractions, designated BG1-BG3, was applied to RP-HPLC (Fig. 5). These peptides, designated G1-G20, were analysed to determine the amino acid sequence (Fig. 1).

The peptide map of CNBr cleaved recombinant bovine somatotropin was shown in Fig. 6. We obtained 4 peptides in this step, and isolated peptides were subjected to sequence analysis. The amino acid sequences of these peptides, designed CN1-CN4, are shown in Fig. 1.

The recombinant bovine somatotropin was also cleaved with 0.03 N HCl solution at 105℃, and the digest was separated by RP-HPLC (Fig. 7). The amino acid sequences of these peaks, designated H1-H12, are shown in Fig. 1.

We previously reported that recombinant bovine somatotropin contained two disulfied bridge (28). In this paper two disulfied bridges were identified by sequencing the peptidesd containing Cys residue after tryptic digestion. The disulfide linkages of the recombinant bovine somatotropin were determined as Cys(53)-Cys(164) and Cys (181)-Cys(189).

The hydropathy plot of recombinant bovine somatotropin was shown in Fig. 9. The value of hydrophilicity was calculated with an average group length of seven amino acid residues. This analysis revealed a hydrophobic region at the NH_2-terminus and hydrophilic region at the COOH-terminus.

The amino acid sequence of the recombinant bovine somatotropin was identical to that predicted from its recombinant gene except for the removal of Met. It was consisted of 191 amino acids. Additionally, recombinant bovine somatotropin expressed in *E. coli* showed the biological activities too. It is thus concluded that the recombinant bovine somatotropin is indistinguishable from its natural counter part.

요 약

재조합 소성장호르몬을 트립신, S.aureus V8 단백질가수분해효소, CNBr, 그리고 산 가수분해법을 이용하여 단백질 일차구조 분석을 실시하였다. N-말단 분석은 30 잔기까지를 수행하였는데, 대장균 내에서 발현된 소성장호르몬은 E. coli 내에 존재하는 methionyl-aminopeptidase에 의해 해독개시인자로 넣어준 N-말단의 Met이 모두 제

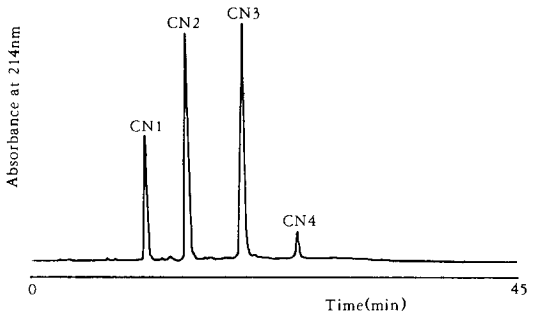

Fig 6. Separation of CNBr cleaved peptides of recombinant bovine somatotropin on the reverse-phase column (μBondapak™ C18, 3.9x300 mm, 100Å, Waters, MA, U.S.A.). The number in peaks give the position of the peptide in recombinant bovine somatotropin sequence in Fig 1.

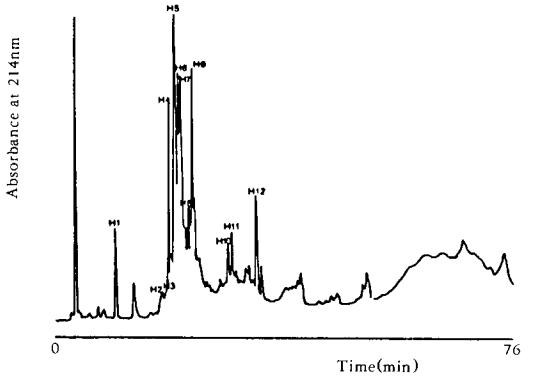

Fig 7. Separation of mild acid hydrolysed peptides of recombinant bovine somatotropin on the reverse-phase column (μBondapakTM C18, 3.9 x 300 mm, 100 Å). The number in peaks give the position of the peptide in recombinant bovine somatotropin sequence in Fig 1.

거된 형태로 나타났으며 아미노산 조성분석 결과 연역된 조성과 유사하게 나타났다. 효소와 화학물질로 절단한 소성장호르몬 조각들을 HPLC로 분리한 후 단백질 서열분석기를 이용하여 아미노산 서열을 분석하였다. 대장균에서 발현된 소성장호르몬은 191개의 아미노산으로 구성된 21,802 Da의 분자량을 갖

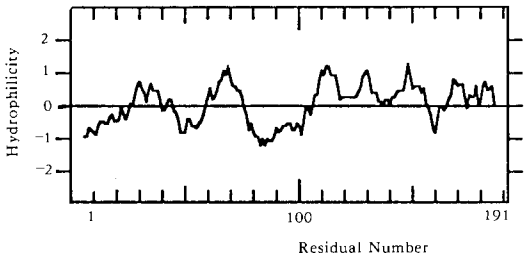

Fig 8. Hydrophobicity plot of recombinant bovine somatotropin. The hydropho-bicity was calculated by the method of Hopp and Woods (35) with an average group length of seven amino acid residues.

고 있는 단백질로 나타났다. 여기에서 을 갖고 있는 단백질로 나타났다. 여기에서 얻은 아미노산 서열을 바탕으로 hydropathy plot을 한 결과 N-말단에서는 소수성이 그리고 C-말단에서는 친수성 영역이 나타났다.

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