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Characterization of N^{α}-acetyl methionyl human growth hormone formed during expression in *Saccharomyces cerevisiae* with liquid chromatography and mass spectrometry

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Abstract

We found a new variant of human growth hormone (hGH) from the recombinant hGH expression process in *Saccharomyces cerevisiae*. The variant was identified as N^{α}-acetyl methionyl hGH which may be formed by N^{α}-acetylation of met-hGH during the intracellular expression of hGH in *S. cerevisiae*. The variant was isolated from manufacturing process of LG Life Sciences' hGH product. The variant was subjected to trypsin digestion and RP-HPLC analysis, resulting in a delayed retention time and an increased mass (173 Da) of T1 tryptic peptide. The amino acid composition and amino acid sequence of the peptide showed the same result with T1 peptide of met-hGH except the N-terminal modification on methionine in the variant peptide. With collision induced dissociation (CID) experiments of the variant T1 tryptic peptide, we found the sequence and the a_1 fragment of N-terminal residue matched with those of acetyl-methionyl hGH. Within our production process, we produce the methionyl hGH first and then use the aminopeptidase to cut the N-terminal methionine. So the acetylation may inhibit the aminopeptidase to remove methionine and produces N^{α}-acetyl methionyl hGH. And the biological activity of the variant was comparable to one of the unmodified hGH when tested by rat weight gain bioassay.

Keywords: Human growth hormone; Modification; Variant; Mass spectrometry; Aminopeptidase

1. Introduction

Commercial preparations of hGH are available from several manufacturers. Variants of hGH can be divided into two categories depending on their origin: process-related variants and product-related variants. The product-related variants of hGH result from degradation of hGH product either during long-term storage or by stress conditions such as high temperature, extreme pH and light. They include desamido forms [1,2], oxidized forms [3,4], clipped form, des-Phe-Pro-hGH [5], dimers [6], and aggregates [7] and are shown to also occur throughout the manufacturing process. However, the process-related variants are specific for its production process. Trisulphide derivative [8], des-Phe variant [9,10], and clipped forms [11,12] are examples found during expression of hGH in *Escherichia coli*. Methionyl hGH [13,14] is also a type of process-dependent variants. Up to now most of commercial hGH products are produced from *E. coli* and accordingly its variants derived from recombinant *E. coli* have been extensively identified and elucidated well. However, a variant of hGH specific for expression in yeast has not been reported. LG Life Sciences produces hGH product through intracellular expression in *Saccharomyces cerevisiae*. Here, we report a new variant resulting from yeast expression.

Proteins synthesized in eukaryotic cells undergo two common types of post-translational modifications at their N-termini: initiator methionine cleavage and N^{α}-acetylation

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[15]. These reactions are catalyzed by two classes of enzymes, the methionine aminopeptidases (MetAP) [16] and N^{α}-acetyltransferases [17,18]. The initiator methionine can usually be removed by MetAP if the penultimate amino acid is small and uncharged [19]. However, it has been found that incomplete processing of methionine could occur either when the penultimate amino acid of proteins is too big or when the proteins are expressed as insoluble inclusion bodies in the production of recombinant proteins [20,21].

LG Life Sciences' recombinant human growth hormone (hGH) product is intracellularly expressed within yeast *S. cerevisiae*. The recombinant human growth hormone was synthesized as met-hGH forms because methionine aminopeptidase of the yeast might not work due to either big penultimate amino acid (phenylalanine) or insoluble expression. During purification process, N-terminal methionine is processed by a specific aminopeptidase. However, some part of met-hGH was found as N^{α}-acetylated form and seemed to be resistant to the aminopeptidase treatment. The variant was identified as N^{α}-acetyl methionyl hGH by extensive structural studies and its physicochemical and biological properties are provided in this publication.

2. Materials and methods

2.1. Materials

Recombinant hGH (Eutropin™ or Valtropin™) was obtained from LG Life Sciences Ltd. (Korea) and N^{α}-acetyl methionyl hGH was derived from commercial process of LG Life Sciences' hGH production in which hGH is synthesized as methionyl hGH form in *S. cerevisiae*. Desamido hGHs were generated by storing an hGH solution at 37 °C for 21 days. N^{α}-Acetyl methionyl hGH form was isolated from a waste pool during purification process of LG Life Sciences' hGH product. Anion exchange column of small particle size is applied to the isolation of N^{α}-acetyl methionyl hGH form under the same condition as in analytical DEAE-HPLC experiment below. The isolated sample was lyophilized and stored at −20 °C until use.

2.2. Equipment

The HPLC experiments were carried out using Agilent 1100 HPLC system (Waldbronn, Germany) consisting of a binary pump G1312A, a diode array detector G1315B, a thermostated autosampler G1329A and a column thermostat G1316A. For peptide mass mappings and molecular weight measurements, mass spectra acquired with a Finnigan MAT LCQ mass spectrometer (San Jose, CA, USA). A Micromass Q-TOF2 mass spectrometer (Manchester, UK) and a JMX-HX110/110A tandem mass spectrometer (Jeol, Tokyo, Japan) were used to perform the low and high-energy collision induced dissociation (CID) experiments.

2.3. Methods

2.3.1. Separation of N^{α}-acetyl met-hGH

N^{α}-Acetyl met-hGH was isolated from process sample in commercial production. The process sample containing N^{α}-acetyl met-hGH variant was processed by small scale Macro-Prep 25Q (Bio-Rad, Hercules) column chromatography. The chromatography was performed by linear gradient of NaCl (0–50 mM) under the buffer containing 10 mM histidine (pH 5.7) and 0.5 M urea. The latter peak behind hGH corresponding to N^{α}-acetyl met-hGH was pooled, lyophilized and stored at −20 °C until use.

2.3.2. Physicochemical analysis of N^{α}-acetyl met-hGH

In terms of physicochemical characterization, N^{α}-acetyl methionyl hGH showed no difference with hGH by SEC-HPLC, and SDS-PAGE. It was found that the variant could be distinguishable to hGH by IEF, RP-HPLC, and DEAE-HPLC. The isoelectric focusing was performed using a pre-cast IEF gel ranging p*I* 3–7 (Novex) and stained with a colloidal Coomassie blue according to the manufacturer's instruction.

RP-HPLC analysis of hGH variants was performed using Vydac C4 (4.6 mm × 250 mm, 214ATP54) at flow 0.5 mL/min and column temperature 45 °C. Mobile phase was 50 mM Tris (pH 7.5)/*N*-propanol (71/29). DEAE-HPLC analysis was conducted with TSK gel DEAE-5PW column (10 μm, 7.5 mm × 750 mm, TOSOH Corp, Japan). The buffer consisting of 10 mM histidine (Sigma) and 0.5 M urea (Sigma) at pH 5.7 was used as mobile phase. Elution was carried out by a linear gradient from 0 to 100% of 50 mM NaCl with 1 mL/min flow rate and 280 nm UV detection wavelength.

2.3.3. Molecular weight measurement

Positive-ion mass spectra of whole proteins were obtained using a Finnigan MAT LCQ mass spectrometer equipped with the electrospray ionization source operated at flow rate 0.1 mL/min, needle voltage 4.3 kV and capillary temperature 250 °C. Methanol/water/acetic acid (50:50:1, v/v/v) solution was used to inject 5 μL of hGH or N^{α}-acetyl met-hGH samples (∼1 mg/mL).

2.3.4. Peptide mapping of N^{α}-acetyl met-hGH

For tryptic peptide mapping, N^{α}-acetyl methionyl hGH and hGH were dissolved or diluted in 50 mM Tris (pH 7.5) separately. Trypsin digestions were performed by adding a trypsin of sequencing grade at 3:100 (w/w) of the enzyme to hGH ratio for 4 h at 37 °C. The reaction was stopped by freezing. RP-HPLC analysis of tryptic peptide was performed using Aquapore RP300 C8 (4.6 mm × 250 mm, 7 μm, 300Å, Brownlee) at flow 1 mL/min and column temperature 30 °C. The mobile phase was the acetonitrile gradient in the presence of 0.1% (v/v) aqueous trifluoroacetic acid. The gradient used to separate the hGH tryptic digests was linear from 0 to 18% acetonitrile in 20 min, to 22.5% in 20 min, to 45%

in 25 min and 45% isocratic in 5 min. Tryptic peptides were detected by UV detector with 214 nm or mass spectrometer over 50–2000 in mass-to-charge ratio. Positive-ion mass spectra of tryptic digested peptides were obtained using a Finnigan MAT LCQ mass spectrometer equipped with electrospray ionization source operated at flow rate 0.1 mL/min, needle voltage 4.3 kV and capillary temperature 250 °C.

2.3.5. Amino acid composition analysis

N^{α}-Acetyl methionyl T1 peptide (tryptic digested peptide including N-terminal) as shown in Fig. 5 was isolated during the peptide mapping by injecting 200 μL tryptic digest of 2.3 mg/mL N^{α}-acetyl met-hGH sample. The isolated sample was dried using Speed Vac (Savant) and then dissolved in 200 μL of 50% acetonitrile and its amino acid composition was analyzed using 5, 10, and 15 μL of the dissolved sample. The peptide samples were hydrolyzed by 6N HCl at 115 °C for 22 h in triplicate. And then each hydrolysate was analyzed using AccQ Tag™ Method (Waters, MA, USA).

2.3.6. MS/MS analysis

To identify the structure of hGH variant, collision induced dissociation experiments were performed for the modified N-terminal peptide, N^{α}-acetyl methionyl T1. Low energy CID experiments were performed on a Micromass Q-TOF2 mass spectrometer equipped with a nanoflow-ESI source. The modified peptide was dissolved in 50% acetonitrile in 1% aqueous acetic acid and sprayed at flow rate 0.5 μL/min with NP 70-2208 Harvard syringe pump (Holliston, MA, USA) and cone-voltage was 3.5 kV. MS/MS spectrum was obtained using 10–15 psi argon gas and 30 eV collision energy. In high-energy CID experiments, protonated molecular ions were generated by the fast atom bombardment on sample, mixed with 3-nitrobenzyl alcohol (Sigma) matrix and pasted on the stainless steel probe, with 22 kV accelerated Xe ions. MS/MS experiments with high energy CID were performed on the four-sector ($E_1B_1E_2B_2$) instrument. Precursor ions were selected in MS_1 (E_1B_1) and excited by collision with helium gas in collision cell, which is between MS_1 and MS_2 (E_2B_2) with maintaining the precursor ion intensity as 70% of unexcited one. ^{12}C monoisotopic precursor peptide ions were excited and their fragments were detected in MS_2.

2.3.7. Biological activity of N^{α}-acetyl met-hGH

N^{α}-Acetyl met-hGH sample and hGH sample were diluted quantitatively with 0.1 M ammonium bicarbonate pH 8.0 buffer solution to obtain two different concentrations of each test solution, 20 and 80 μg/mL. Ten female rats of Sprague–Dawley hypophysectomized at 4 weeks old of age from an animal supplier, Harlan Sprague Dawley Inc. (Indianapolis, IN, USA) were tested with each dose to each sample. hGH and N^{α}-acetyl met-hGH were injected each day for 10 days subcutaneously. The body weight gain of each animal was recorded after 10 day treatment and the potency was computed using the completely randomized design analysis of parallel-line assay.

3. Results

3.1. Physicochemical properties of N^{α}-acetyl met-hGH

N^{α}-Acetyl met-hGH was analyzed by DEAE-HPLC and compared to normal hGH and desamido form. As shown in Fig. 1, the N^{α}-acetyl met-hGH form eluted latter than normal hGH. N^{α}-Acetyl met-hGH was clearly separated from the normal hGH and located between the normal hGH and the desamido form.

RP-HPLC is typically applied to separate hGH variants such as desamido forms and sulfoxidized form of hGH [2,4,22]. As shown in Fig. 2, RP-HPLC was also selective to separate N^{α}-acetyl met-hGH. Desamido form, normal hGH and N^{α}-acetyl met-hGH were separately eluted at different retention times of about 28, 33 and 38 min, respectively.

Isoelectric focusing (IEF) is a mode of electrophoresis in which proteins are separated by their migration in an electric field over a pH gradient. Fig. 3 shows the IEF pattern of hGH and related variants. Bands of hGH, mono-desamido form and di-desamido form appeared at p*I* 5.2, 5.0 and 4.9, respectively. The band of N^{α}-acetyl met-hGH appeared at p*I* 4.95 between mono-desamido form and di-desamido form.

3.2. Molecular weight measurement

The molecular weight of N^{α}-acetyl met-hGH was measured on a Finnigan MAT LCQ equipped with electrospray ionization. N^{α}-Acetyl met-hGH isolated by DEAE-HPLC was dialyzed with 1% acetic acid. Five microliters of sample was infused into LCQ ESI/MS with 0.1 mL/min. Mass spectra were deconvoluted into mass domain using the Biomass Deconvolution algorithm of Bioworks software from Thermo-Finnigan. Fig. 4 shows the mass spectra of hGH and N^{α}-acetyl met-hGH on the axis of mass rather than on the axis of mass-to-charge ratio by deconvoluting the mass spectra of multiply charged (+12 to +18) protonated protein molecular ions. We found that the molecular weight of N^{α}-acetyl met-hGH was 173 Da larger than that of hGH. After molecular weight measurements, we tried to determine N-terminal sequence to get the information around N-terminal of hGH variant, however, we only got noisy background. So we suspected the N-terminal site was blocked and some modification might happen at the terminal amine group [23].

3.3. Peptide mass mapping

To identify the modified site, the molecular weight of all the tryptic digest peptides from N^{α}-acetyl met-hGH and hGH were analyzed with LC/MS. The tryptic digests of hGH were separated by RP-HPLC and then identified by on line LCQ mass spectrometer. The tryptic map of N^{α}-acetyl met-hGH

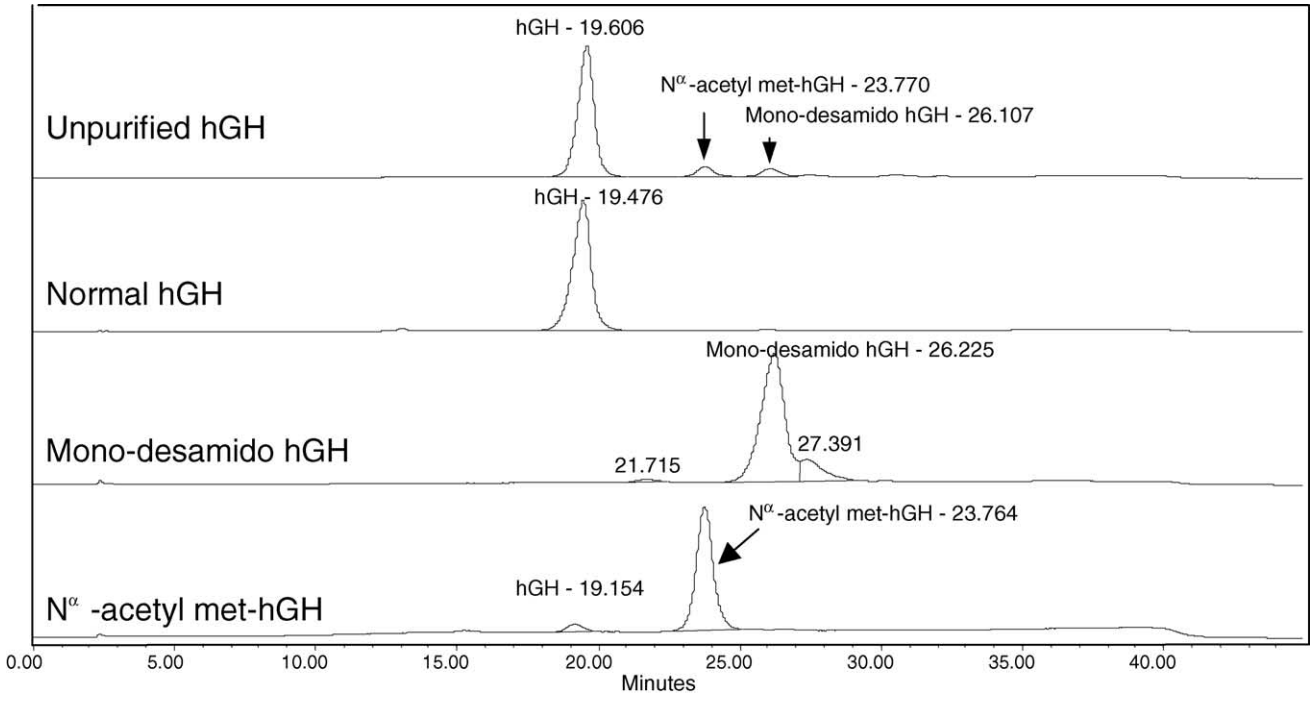

Fig. 1. Separation of N^{α}-acetyl methionyl hGH by DEAE-HPLC analysis.

revealed the major difference in T1 peptide (the first tryptic digest peptide from N-terminal of hGH) as shown in Fig. 5, while the other tryptic peptides showed the same retention time with those of normal hGH. So N-terminal modified peptide, as previously expect, of N^{α}-acetyl met-hGH eluted on ~58 min and annotated as T1* peptide which was confirmed by tandem mass spectrometry (MS/MS) later section. T1* peptide of N^{α}-acetyl met-hGH [*m*/*z* 1103.5] showed the mass increment of T1 peptide by 173 Da compared to 930.5 Da of the normal one. The same mass differences as in total mass analysis proposed that whole modification took place at only N-terminal site as an additional modification with about one amino acid mass difference. Also the theoretically expected isotope distribution of T1 peptide was calculated. Due to the addition of a sulfur atom included in methionine, the isotope distribution of T1 peptide of variant hGH showed higher population at [M + 1], [M + 2], [M + 3] and [M + 4] than peptides without any sulfur atoms. For example, a similar peptide, KFPTIPLSR, shows lower population at heavier isotopic peaks. The population distribution of isotope peaks of AcMFPTIPLSR versus AcKFPTIPLSR is 100:64:27:9:2 versus 100:62:21:5:1. So, we may narrow down the candidates as cysteine and methionine, but both are not exactly explain the mass difference. So, we tried to characterize them

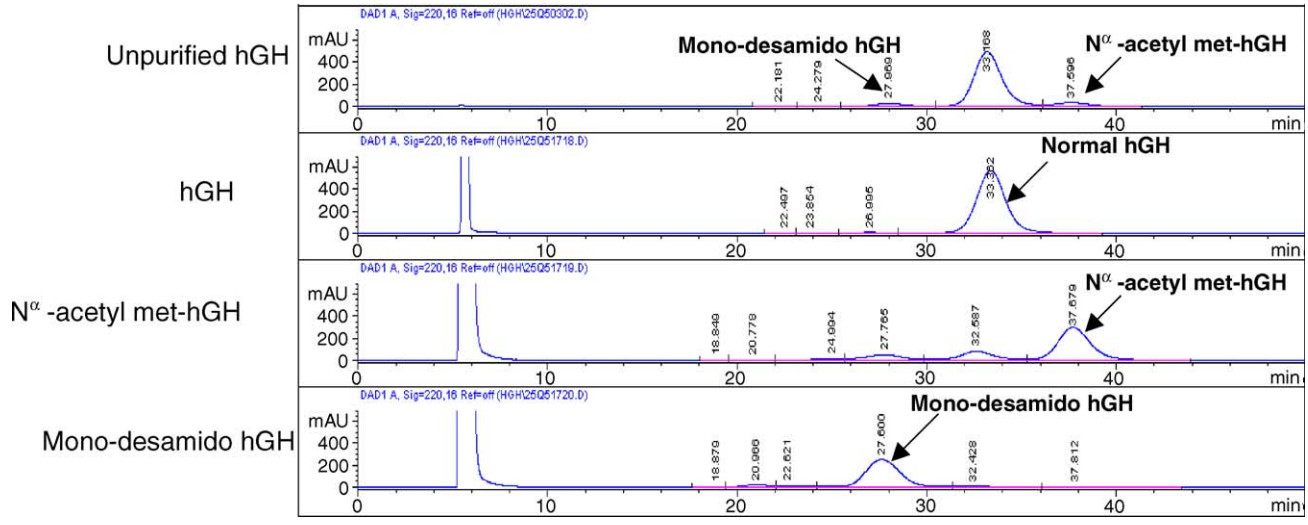

Fig. 2. Analysis of N^{α}-acetyl methionyl hGH by RP-HPLC analysis.

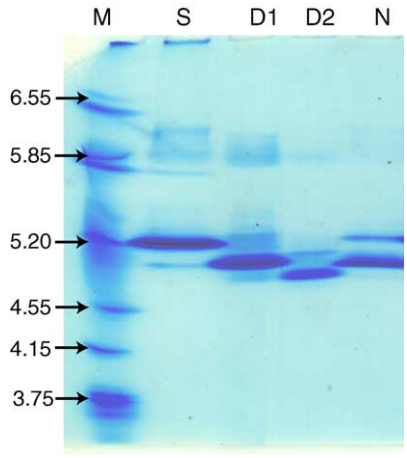

Fig. 3. Isoelectric focusing of N^{α}-acetyl methionyl hGH. Lane M: protein standards; Lane S: hGH; Lane Dl: mono-desamido form of hGH; Lane D2: di-desamido form of hGH; Lane N: N^{α}-acetyl methionyl hGH.

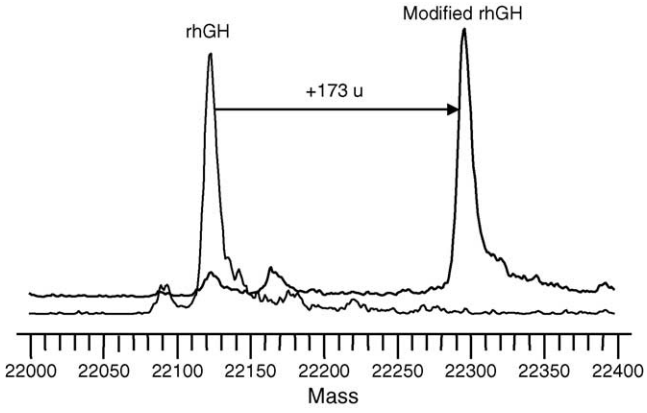

Fig. 4. Deconvoluted mass spectra of hGH and N^{α}-acetyl methionyl hGH were recorded on LCQ mass spectrometer with the electrospray ionization.

at molecular level with amino acid composition analysis and MS/MS experiments.

3.4. Amino acid composition analysis

By injecting tryptic digest above, T1* peptide of N^{α}-acetyl met-hGH was isolated during peptide mapping as shown in Fig. 5. The isolated sample was dried using Speed Vac and then dissolved in 200 μL of 50% acetonitrile and its amino acid composition was analyzed using 5, 10, and 15 μL of the dissolved sample. The amino acid composition profile of T1* peptide derived from N^{α}-acetyl met-hGH showed the major amino acid components, Ser, Arg, Thr, Pro, Met, Ile, Leu, and Phe. The result clearly shows that the T1* peptide of N^{α}-acetyl met-hGH had an additional methionine residue compared to T1 (FPTIPLSR) of normal hGH. Another clear evidence of the presence of methionine was the mass shift (+16 Da) in the mass

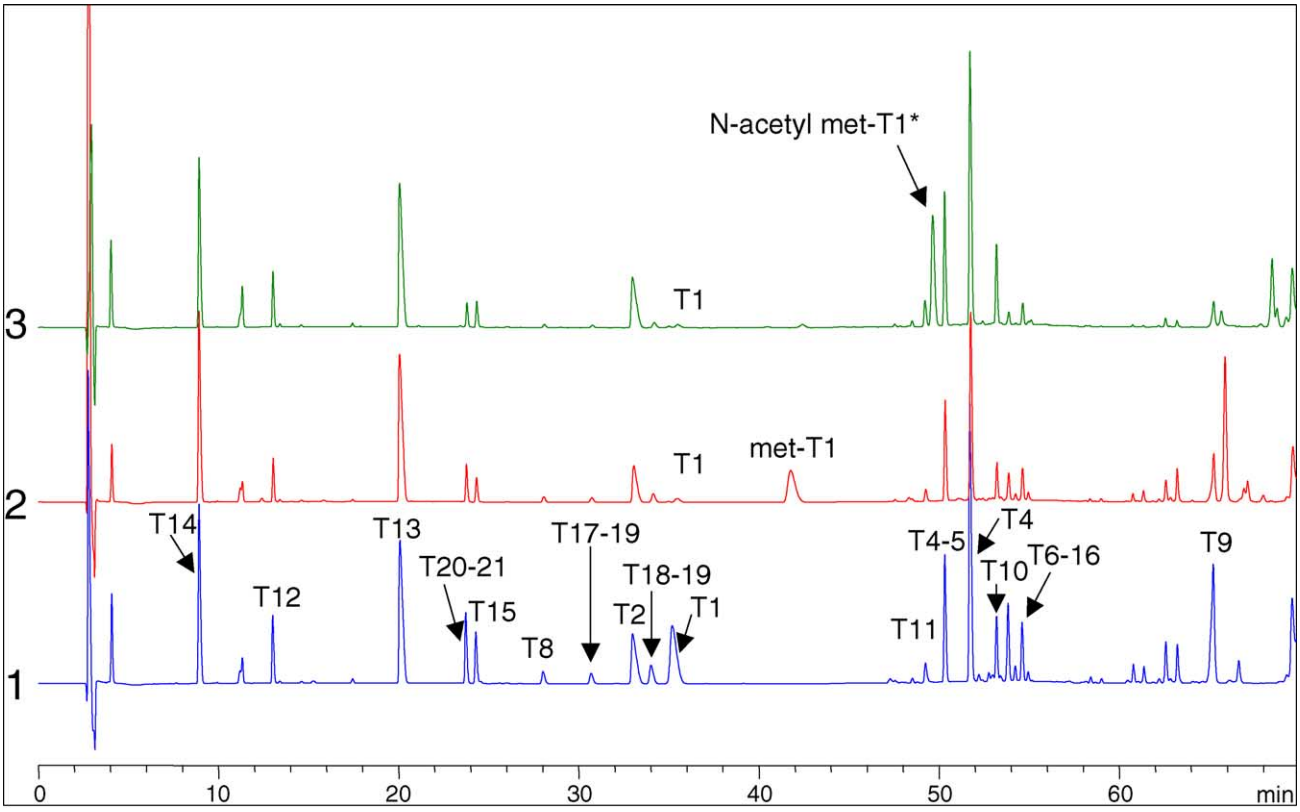

Fig. 5. RP/HPLC peptide map of tryptic digest peptides. The Ti peptide of N^{α}-acetyl methionyl hGH was eluted after the Tl peptide of normal hGH or met-hGH. Chromatograms 1, 2, and 3 represent peptide maps of normal hGH, met-hGH and N^{α}-acetyl met-hGH, respectively.

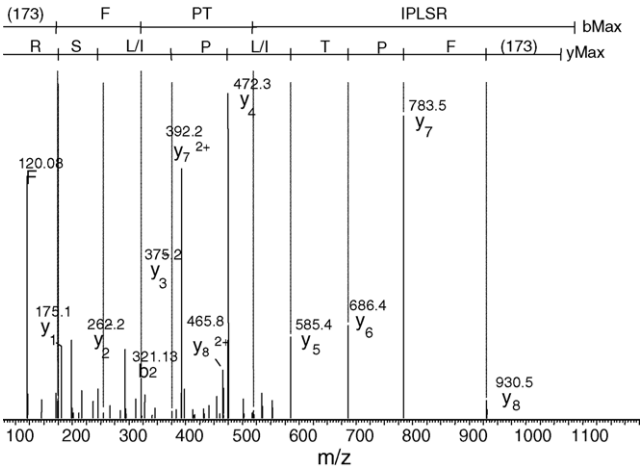

Fig. 6. Low energy collision induced dissociation experiments of tryptic digested T1* peptide of the variant hGH showed the partial sequence information from *y*-series (1, 4–8) which is consistent with the known sequence of Tl peptide in hGH.

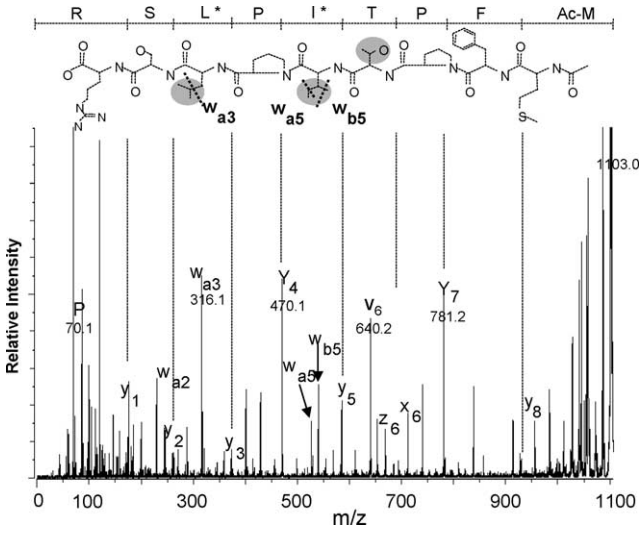

Fig. 7. With high energy collision induced dissociation experiments of tryptic digested T1* peptide of the variant hGH, we found an exact sequence which matched with Tl peptide in modified methionyl hGH.

spectrum of N^{α}-acetyl met-hGH by rapid oxidation at room temperature.

3.5. MS/MS analysis

Unsuccessful sequencing of the N-terminal site of N^{α}-acetyl met-hGH provided the evidence of modification location. We could assume that the T1 peptide moved to T1* due to modification on the N-terminal of hGH. So, we chose the mass spectrometric sequencing method to determine the structure of the N^{α}-acetyl met-hGH. From the low energy collision induced dissociation experiments [24,25] of tryptic digest peptide T1 of N^{α}-acetyl met-hGH, we found serial fragments of y_i ($i = 4$–8) as shown in Fig. 6. The analysis of the mass difference between sequential mass decrement of y_i fragment ions tell us the partial sequence of T1 peptides in N^{α}-acetyl met-hGH as F-T-P-L/I, which exactly matched to the one in hGH. From the mass difference, elemental composition, methionine presence and N-terminal blocking, we can suggest the acetylated methionine modification to the N-terminal of normal hGH.

For more specific sequence information, we also performed the high energy collision induced dissociation experiment with double focusing sector mass spectrometer. Due to various ions generated by high energy collisions, we were able to observed the typical fragment ions, W_{a5}, W_{b5} and W_{a3}, which can distinguish the amino acid leucine and isoleucine (Fig. 7) [26]. So the 5th and 7th amino acid residues from N-terminal may be isoleucine and leucine. In addition, various immonium ions of amino acid composing T1 peptide, proline, phenylalanine, serine, leucine/isoleucine, arginine and methionine, were observed in the low mass region of high energy collision dissociation mass spectrum. Especially, we found immonium ion of methionine and related peaks at 104 and 61 Da. From the high energy CID experiments, we can interpret the whole sequence, M-F-P-T-I-P-L-S-R, with additional information of the partial sequence from low energy CID experiments, immonium ions for residual composition and w_i, v_i, x_i, y_i series fragment ions. In biological respect, we found various papers to report the N-terminal acetylation by N^{α}-acetyltransferase especially dominant at the small amino acid residues like glycine, serine, methionine and aspartic acid.

3.6. Biological properties

By assuming a biological activity of hGH to be 3.0 IU/mg, the relative activity of N^{α}-acetyl met-hGH was determined to be 2.7 IU/mg. The biological activity of N^{α}-acetyl met-hGH seems to be equivalent to that of normal hGH form.

4. Conclusion and discussion

We found a new variant of hGH protein from yeast expression system with detail structural characterization by liquid chromatography and mass spectrometry and show the comparable biological activity of the variant. DEAE chromatography isolated the variant protein very specifically and mass spectrometric amino acid sequencing is a powerful tool to investigate the N-terminal blocked protein and peptide. Especially high-energy CID experiments provide the specific information of amino acid sequence and composition of the unknown peptides. So we could specify the sequence and structure of variant as N^{α}-acetyl met-hGH. The production process including the intermediate, methionyl hGH, also can explain the existence of the variant. Probably during the deletion process, acetyltransferase blocked the N-terminal with acetyl group and produced N^{α}-acetyl met-hGH, so aminopeptidase cannot delete the blocked methionyl terminus.

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