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Isolation and characterization of recombinant eel growth hormone expressed in *Escherichia coli*

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ABSTRACT

To obtain information about the microheterogeneity of recombinant protein, recombinant eel growth hormone II (EGH) analogues expressed in *Escherichia coli* were isolated and characterized. The modification was classified into three types: monodeamidation of Asn, oxidation of Met and N-terminal formylation. Monodeamidated EGH was isolated by ion-exchange chromatography. The major deamidation site (Asn147) was determined by peptide mapping using the substrate specificity of trypsin. Oxidized EGH and N-terminal-formylated EGH were isolated by reversed-phase high-performance liquid chromatography. Oxidized EGH was identified by amino acid composition analysis and N-terminal-formylated peptide by mass spectrometry.

INTRODUCTION

Growth hormones (GH) complementary deoxyribonucleic acid (cDNA) have been isolated from several vertebrate species[1–6] and some of these GH were produced by recombinant techniques in *Escherichia coli*[1–3,7]. Human GH has been applied for dwarfism, bovine GH is being developed as an animal medicine for growth promotion and to increase milk production and fish GH is expected to be applied in fish culture[8,9].

Eel GH (EGH) was isolated from eel pituitaries by Kishida *et al.*[10] and the primary structure of EGH was determined by Kishida *et al.*[9] and Yamaguchi *et al.*[11]. Molecular cloning of EGH cDNA and its expression in *Escherichia coli* were performed by Saito *et al.*[12]. The refolding and purification process of *Escherichia coli* derived EGH was established and its growth-promoting activity *in vivo* was confirmed by Sugimoto *et al.*[13].

Recombinant protein medicines for humans and other vertebrates should be highly purified and uniform in structure for safety and efficacy. However, several methods have revealed that small amounts of analogues exist in purified recombinant protein preparations[14,15]. It is very important to detect and determine such analogues in order to prevent their appearance and to separate them.

We have found minor analogues in purified recombinant EGH by several

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analytical methods. In this study, native EGH and five analogues were isolated and their structures were established.

EXPERIMENTAL

Materials

Trypsin-TPCK was purchased from Worthington (Freehold, NJ, U.S.A.), α-chymotrypsin from Sigma (St. Louis, MO, U.S.A.) and *Achromobacter* protease I from Wako (Osaka, Japan). DEAE Toyopearl 650 M for ion-exchange chromatography and a TSK gel ODS 120T reversed-phase high-performance liquid chromatographic (RP-HPLC) column (30 cm × 4.6 mm I.D.) were obtained from Tosoh (Tokyo, Japan), YM-10 ultrafiltration membrane from Amicon (Danvers, MA, U.S.A.), TEFCO-gel for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) from TEFCO (Tokyo, Japan), Determiner NH_3 ammonia assay kit from Kyowa Medex (Tokyo, Japan) and a protein assay kit from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Preparation of recombinant EGH Lot E-21-0

Recombinant EGH expressed in *Escherichia coli* as inclusion bodies was refolded and purified more than 95% pure, judging from gel filtration HPLC and SDS-PAGE[13]. The percentage purity was based on the peak areas of the chromatogram and of the scanning profile of the SDS-PAGE gel. The refolding and purification process was as follows: *Escherichia coli* cells containing EGH inclusion bodies were harvested from 6 l of broth and broken by passage through a Manton Gaulin homogenizer. The resulting homogenate was centrifuged at 8000 *g* for 40 min at 4°C and a 20-g pellet was solubilized by stirring for 5 days at 4°C in 2 l of 5 *M* guanidine hydrochloride–50 m*M* Tris–0.005% Tween 80 at pH 8.0. The solution was centrifuged, as above, to remove any insoluble materials and then dialysed against 60 l of 10 m*M* Tris–HCl buffer (pH 8.0) three times at 4°C. The dialysate was clarified by centrifugation. The supernatant, containing 890 mg of protein, was loaded onto a DEAE Toyopearl 650M column (25 cm × 7 cm I.D.) equilibrated with 10 m*M* Tris–HCl buffer (pH 8.0) and eluted with a linear gradient from 0 to 250 m*M* NaCl in 10 m*M* Tris–HCl buffer (pH 8.0) in a 3-l volume. The flow-rate was 1 l/h. Pooled fractions, containing 360 mg of protein, were concentrated to 0.5 mg/ml on a YM-10 filter. The final sample was poured into 5-ml glas containers and freeze-dried. Each container was sealed with a plastic cap and stored at 4°C with desiccant. The contents of each container of EGH Lot E-21-0 were redissolved in 5 ml of ice-cold water just before use.

Rechromatography on DEAE Toyopearl

A 50-mg amount of EGH Lot E-21-0 was redissolved, dialysed against 10 l of 10 m*M* Tris–HCl buffer (pH 8.0) at 4°C and loaded onto a DEAE Toyopearl 650M column (14 cm × 3 cm I.D.) equilibrated with 10 m*M* Tris–HCl buffer (pH 8.0). Following washing with 100 ml of equilibration buffer, EGH was eluted with an NaCl gradient (from 0 to 250 m*M* in equilibration buffer) in 600 ml at a flow-rate of 50 ml/h. The volume of each fraction was 4 ml.

PAGE

PAGE at pH 9.5 and staining with Coomassie brilliant blue were carried out essentially as described by Davis[16], using TEFCO gel. The stacking gels contained 4% (w/v) acrylamide and the separation gels 14% (w/v) acrylamide. EGH fragment fractions from RP-HPLC were evaporated and redissolved in the sample buffer.

RP-HPLC

RP-HPLC was carried out on a TSK gel ODS-120T column (30 cm × 4.6 mm I.D.) with a JASCO Tri Rotar SR2 HPLC system. Linear gradient elutions from 40 to 70% acetonitrile in 0.1% trifluoroacetic acid (TFA) for EGH analogue separation and from 0 to 70% acetonitrile in 0.1% TFA for peptide mapping were performed in 60 min. The column oven temperature was kept at 35°C. The flow-rate was 0.7 ml/min. UV detection was carried out at 220 nm.

Amino acid composition analysis

Hydrolysis of protein and peptide was performed in 6 *M* HCl at 110°C for 22 h. Amino acid analysis was carried out by the Waters Pico Tag method of Bidlingmeyer *et al.*[17]. Amino acid standard solution was obtained from Wako.

N-Terminal sequence analysis

N-Terminal sequence analysis was performed with a gas-phase sequencer (Applied Biosystems Model 470A). EGH analogues and EGH fragment fractions from RP-HPLC were concentrated to about 0.1 ml and applied to the sequencer.

Enzymatic digestion

Digestion of EGH and its fragments was carried out with trypsin-TPCK, *Achromobacter* protease I (API) and α-chymotrypsin at pH 8.0 and 37°C for 4 h. The ratio of substrate to enzyme was 100:1 (w/w). The digestion was stopped by acidification to pH 2–3 with 1 *M* HCl.

Deamidation

Deamidation of EGH and its fragment was performed in 25 m*M* Tris–HCl buffer (pH 10) containing 250 m*M* NaCl at 37°C for 2 days. Detection of ammonia was carried out with the Determiner NH_3[18,19]. EGH fragment fractions from RP-HPLC were evaporated and redissolved in 10 m*M* Tris–HCl buffer (pH 8.0).

Mass spectrometry

Secondary ion mass spectra were obtained with a Hitachi M-80 B spectrometer. EGH fragment fractions from RP-HPLC were freeze-dried and redissolved in acetic acid and mixed with glycerol.

Protein concentration determination

Assay of protein was carried out with the Bio-Rad[20] protein assay kit. Bovine serum albumin was used as a standard.

RESULTS AND DISCUSSION

Isolation of EGH analogues

EGH preparation Lot E-21-0 was 95% pure (see Experimental for calculation) judging from gel filtration HPLC and SDS-PAGE[13]. Analogues of EGH, however, were detected in EGH Lot E-21-0 by PAGE (Fig. 1, lane a) and RP-HPLC (Fig. 2c). Here we call the EGH analogues corresponding to bands A and B in PAGE (Fig. 1, lane a) EGH-A and EGH-B, respectively, and EGH analogues corresponding to peaks α, β and γ in RP-HPLC (Fig. 2c) EGH-α, EGH-β and EGH-γ, respectively. The contents of both EGH-A and EGH-B were 50% and those of EGH-α, EGH-β and EGH-γ were 5%, 85% and 10%, respectively. Natural EGH derived from eel pituitaries[10] had the same mobility as EGH-A in PAGE and the same retention time as EGH-β in RP-HPLC (data not shown).

To separate EGH-A and EGH-B, EGH Lot E-21-0 was dialysed against 10 m*M* Tris–HCl buffer (pH 8.0) and rechromatographed on DEAE Toyopearl (Fig. 3). A slower flow-rate and a gentle gradient gave a good resolution. EGHs were eluted in two peaks, the first and second corresponding to bands A and B in PAGE (Fig. 1, lanes b and c), respectively. As it was found that both EGH-A and EGH-B were divided into three peaks (α, β and γ) (Fig. 2a and b), a total of five analogues (EGH-A-α, EGH-A-γ, EGH-B-α, EGH-B-β and EGH-B-γ) were detected in addition to native EGH (EGH-A-β). The contents of the analogues were 2.5% for EGH-A-α and EGH-B-α, 42.5% for EGH-A-β and EGH-B-β and 5% for EGH-A-γ and EGH-B-γ.

It is suggested that the modification causing heterogeneity in hydrophobicity among α, β and γ was independent of the modification causing heterogeneity in charge between A and B, because both the retention times and compositions of EGH-A-α, EGH-A-β and EGH-A-γ in RP-HPLC were identical with those of EGH-B-α, EGH-B-β and EGH-B-γ, respectively (Fig. 2). This indicates that each analogue can be characterized from the difference between EGH-A (mixture of EGH-A-α, EGH-A-β

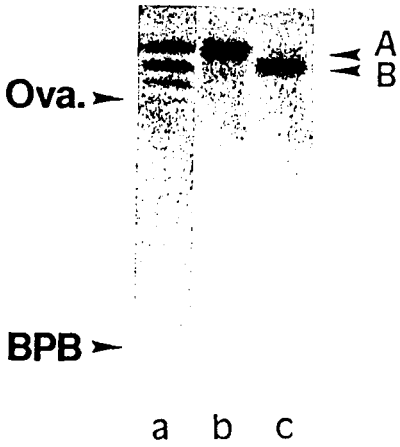

Fig. 1. PAGE of recombinant EGH. Recombinant EGH preparation Lot E-21-0 (lane a) was rechromatographed on DEAE Toyopearl (Fig. 3) and fractionated into two pools, EGH-A (lane b) and EGH-B (lane c). Ova, and BPB indicate the mobilities of ovalbumin and bromophenol blue, respectively.

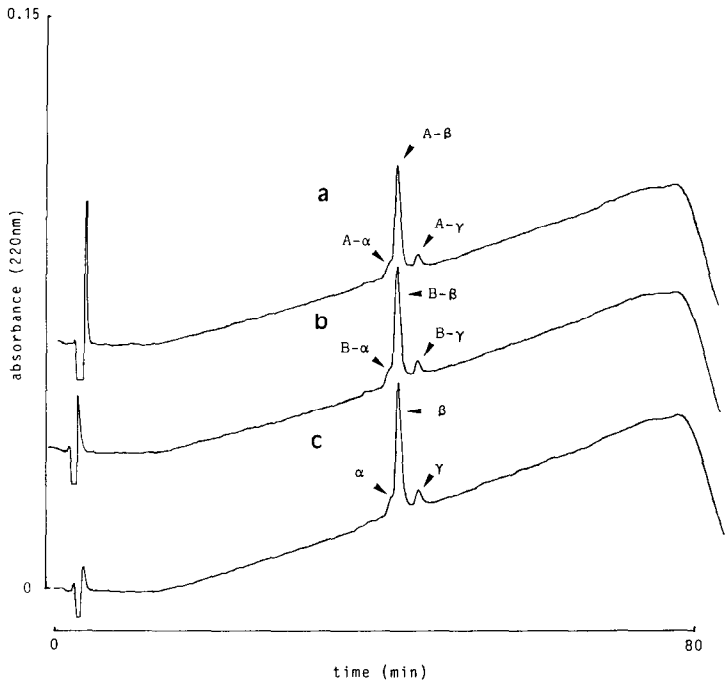

Fig. 2. RP-HPLC of recombinant EGH. EGH-A (a) and EGH-B (b) in addition to recombinant EGH preparation Lot E-21-0 (c) were divided into three peaks, α, β and γ. The peaks corresponding to α, β and γ in EGH-A are referred to as A-α, A-β and A-γ, respectively. Peaks in EGH-B were named in the same way. EGH analogues corresponding to peaks A-α, A-β, A-γ, B-α, B-β and B-γ are referred to as EGH-A-α, EGH-A-β, EGH-A-γ, EGH-B-α, EGH-B-β and EGH-B-γ, respectively. No measurable peak was seen in a blank chromatogram.

and EGH-A-γ) and EGH-B (mixture of EGH-B-α, EGH-B-β and EGH-B-γ) and that between EGH-α (mixture of EGH-A-α and EGH-B-α), EGH-β (mixture of EGH-A-β and EGH-B-β) and EGH-γ (mixture of EGH-A-γ and EGH-B-γ). To determine the difference between EGH-A and EGH-B, we used EGH-A and EGH-B, which were

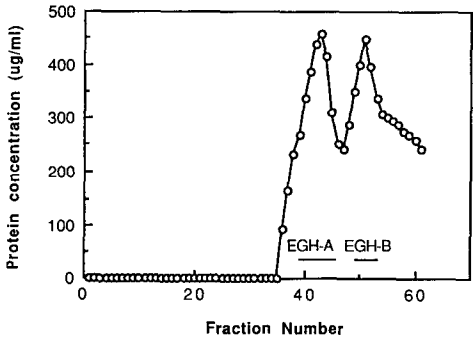

Fig. 3. Ion-exchange chromatography of recombinant EGH preparation Lot E-21-0 on DEAE Toyopearl. For conditions, see Experimental. Bars indicate the pooled fractions as EGH-A and EGH-B.

fractionated by DEAE chromatography. To determine the difference between EGH-α, EGH-β and EGH-γ, we used EGH-α, EGH-β and EGH-γ, which were fractionated by RP-HPLC.

Each analogue was denatured under reducing conditions and refolded as above to clarify whether these analogues were caused by different types of disulphide bond formation or by conformational changes of the peptide chain. No changes were observed, indicating that these analogues were formed in some other way.

Characterization of EGH-A and EGH-B

Heterogeneity of human and bovine GH in PAGE was reported by Lewis *et al.*[21]. They and others[21–27] suggested that deamidation was a possible cause of the electrophoretic heterogeneity of preparations of GH. That conclusion was based on the facts that (1) ammonia is liberated when the GH are exposed to an alkaline medium; (2) no change in amino acid composition occurs, nor are new end terminal amino acids detected in altered samples; (3) heterogeneity is not seen when the electrophoresis is carried out at pH 4, indicating suppression of ionization of carboxyl groups; (4) the converted forms have the same molecular weight as the major band; and (5) proteolytic inhibitors failed to prevent the alteration.

EGH-A was treated at alkaline pH to examine the deamidation of EGH. Band B in PAGE was formed, releasing a stoichiometric amount of ammonia (Fig. 4). As this suggests that EGH-B is a deamidated form of EGH-A, peptide mapping was performed to determine the deamidation site.

Tryptic mapping of EGH Lot E-21-0 had been performed in a previous study[13]. Peaks were assigned to fragments from EGH and it was confirmed that disulphide bonds were formed at the same position as human GH.

The tryptic mapping patterns of both EGH-A and EGH-B by RP-HPLC were

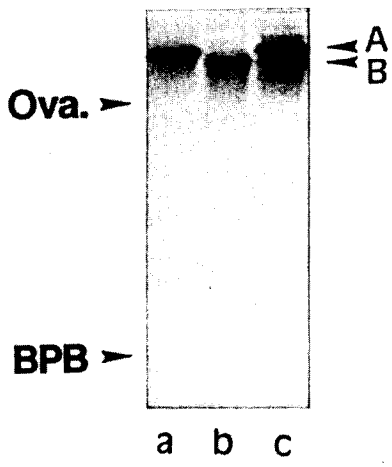

Fig. 4. PAGE of deamidated EGH-A. Lane a, EGH-A; lane b, EGH-B; lane c, EGH-A treated at alkaline pH. Ova. and BPB indicate the mobilities of ovalbumin and bromophenol blue, respectively. For treatment conditions, see Experimental.

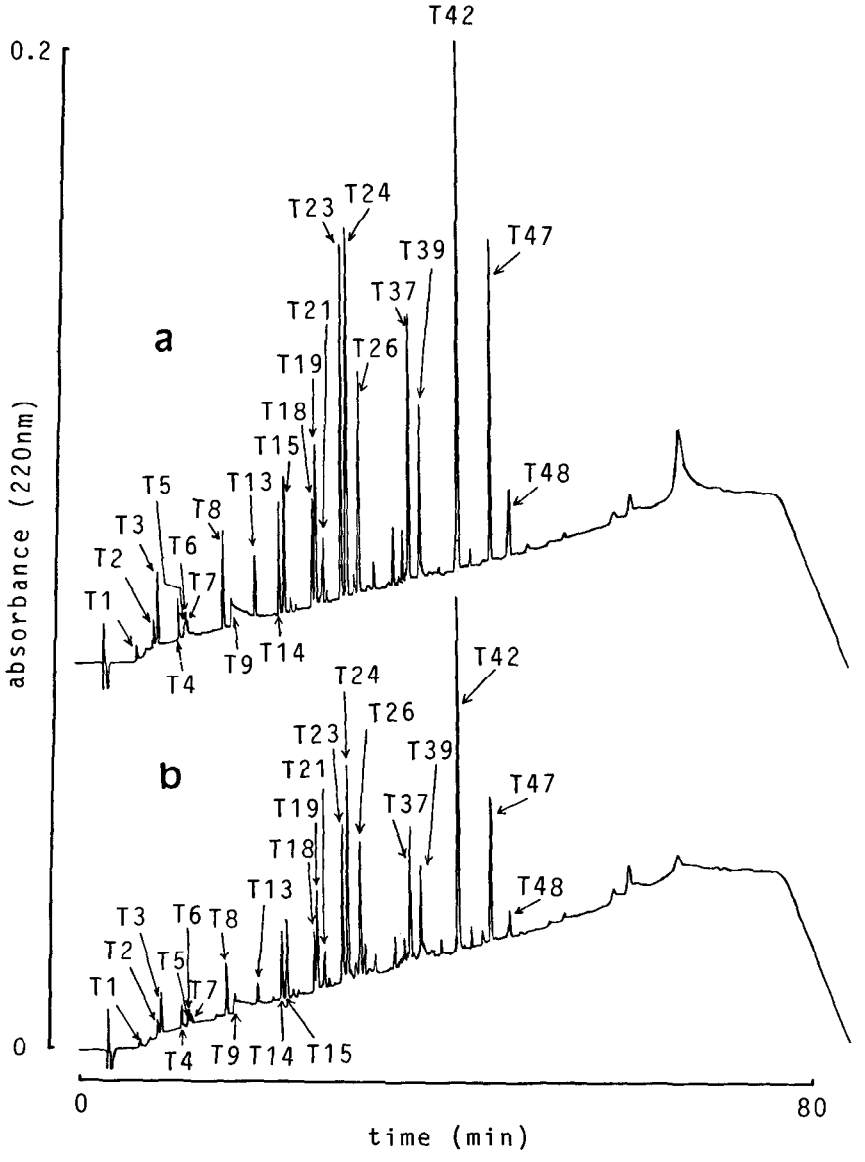

Fig. 5. Tryptic mapping by RP-HPLC of (a) EGH-A and (b) EGH-B. Volumes of 20 μl of digests were injected. Correspondence between peaks and fragments was confirmed previously[13]. No measurable peaks was seen in a blank chromatogram. For conditions of enzymatic digestion and mapping by RP-HPLC, see Experimental.

almost the same (Fig. 5). Each fragment peak had the same retention time within ± 12 s. One peak (T13) of the tryptic peptide of EGH-B, however, were markedly smaller than that of EGH-A. It is assumed that N- and/or C-terminal peptide bonds of the peptide derived from EGH-B were not hydrolysed completely. The peptide from

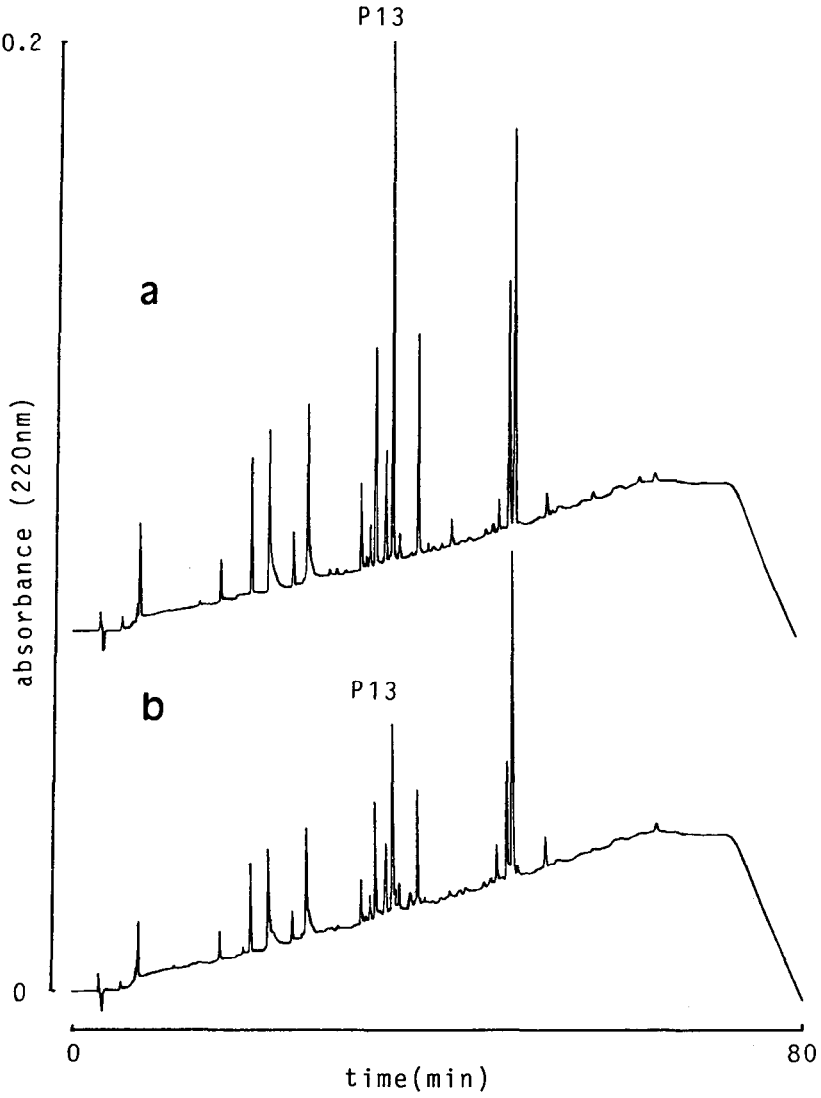

Fig. 6. API mapping by RP-HPLC of (a) EGH-A and (b) EGH-B. Volumes of 20 μl of digests were injected. No measurable peak was seen in a blank chromatogram. For conditions of enzymatic digestion and mapping by RP-HPLC, see Experimental.

EGH-A and EGH-B (Val122–Lys153), which includes tryptic peptide T13 (Asn147–Lys153), was isolated as peak P13 using lysyl endopeptidase API (Fig. 6). Amino acid analysis of all peaks from API mapping of EGH-B revealed no other peak corresponding to the fragment including peptide (Val122–Lys153).

The peaks P13 for EGH-A and EGH-B were further digested by trypsin (Fig. 7). The digest of peak P13 from EGH-B showed one more peak (PT5) in addition to peaks PT1–PT4 from EGH-A. Although amino acid analysis revealed that peak PT5

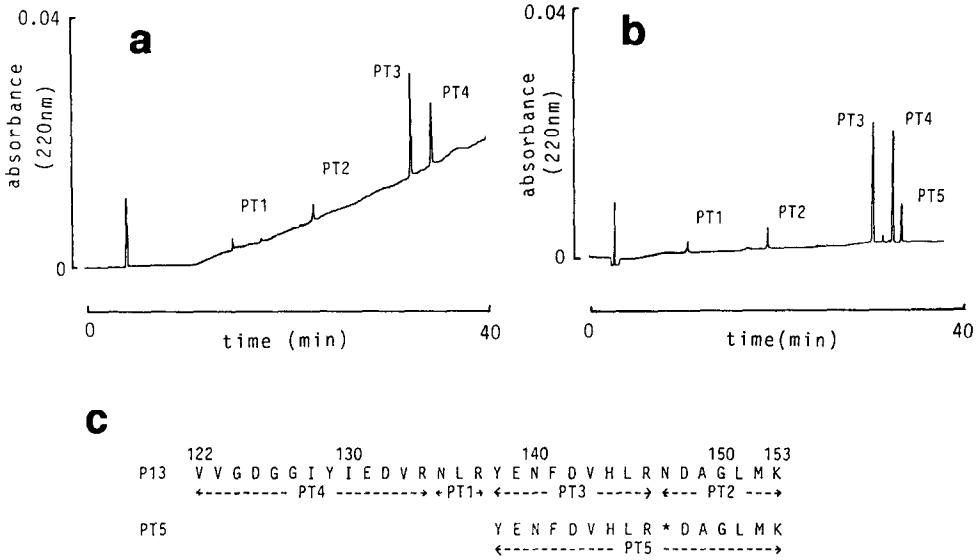

Fig. 7. Tryptic mapping of API peptide P13 (Val122–Lys153). (a) Fingerprint of the peptide from EGH-A; (b) from EGH-B. No measurable peak was seen in a blank chromatogram. For conditions, see Experimental. (c) Amino acid sequence of the peptide. The asterisk indicates an isoAsp residue.

correspond to peptide (Tyr138–Lys153), N-terminal sequence analysis of peak PT5 stopped at Asn147. As the Edman degradation reaction was continued but the remaining sequence was missed, some modification was detected in the backbone peptide bond of Asn147, other than in the side-chain of that residue. These data suggested that deamidation of Asn147 to an isoaspartyl residue occured.

To clarify the deamidation of peptide (Val122–Lys153), the peptide from EGH-A was treated at alkaline pH and compared with the peptide from EGH-A and EGH-B by PAGE (Fig. 8). Two bands, X and Y, appeared for the peptide from EGH-B whereas a single band, X, was observed for EGH-A. Band X was converted to band Y, releasing a stoichiometric amount of ammonia.

These results suggested that EGH-B is the deamidated form of EGH-A and that the main component of EGH-B was deamidated EGH at Asn147. Based on the height of the peak PT1–PT5 in the mapping experiment, 25–30% of EGH-B was deamidated at Asn147. Given that EGH-B showed a single band on PAGE, EGH-B is apparently a mixture of monodeamidated EGH at some sites.

It has been reported that an acidic amino acid residue just before or after Arg and Lys interrupts cleavage of peptide bonds after those basic residues in trypsin digestions[28–30]. Hence it was concluded that conversion of Asn147 to IsoAsp interrupts enzymatic cleavage of the peptide bond between Lys146 and Asn147.

To determine a modification site, peptide mapping is useful. However, deamidated peptide was retained at the same time as non-deamidated peptide on RP-HPLC and all cleaved peptide can not be detected by PAGE and ion-exchange HPLC, because the charges of the peptides are variable. For these reasons it is difficult

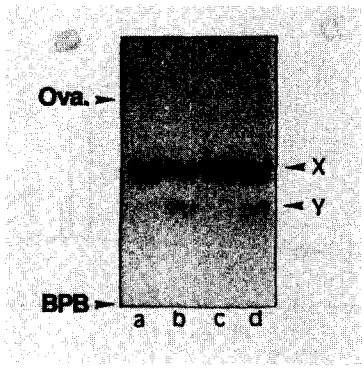

Fig. 8. PAGE of peak P13 from API mapping of EGH. Lane a: P13 from EGH-A; lane b: P13 from EGH-B; lane c: P13 from EGH-A, treated at alkaline pH at −20°C as a control; lane d: P13 from EGH-A, treated at alkaline pH at 37°C. Ova. and BPB indicate the mobilities of ovalbumin and bromophenol blue, respectively. For conditions of alkaline pH treatment, see Experimental.

to find the deamidation sites. Fortunately, Asn and Gln occur before or after Lys and Arg at selected positions in EGH. Here we successfully determined the one deamidation site using the substrate specificity of trypsin.

Characterization of EGH-α, EGH-β and EGH-γ

Amino acid composition was analysed for EGH-α, -β and -γ. One Met residue of EGH-α was oxidized to methionine sulphoxide whereas in EGH-β and -γ it was not.

Amino acid analysis of EGH Lot E-21-0 was unable to detect the conversion of Met to methionine sulphoxide because of the low content of the oxidized EGH. Separation of EGH-α was effective in establishing the oxidation of Met.

In N-terminal amino acid sequence analysis, the N-terminal sequence study of EGH-γ was blocked whereas EGH-α and -β started with an additional Met. N-Terminal API peptide of EGH-γ was further digested with chymotrypsin (data not shown). Mass spectra of N-terminal chymotryptic peptides of EGH-γ indicated that N-terminal additional Met was formylated (Fig. 9).

These results indicate that about 10% of EGH was formylated. It is also impossible to detect a 10% decrease in recovery by N-terminal sequence analysis. Separation of EGH-γ was effective in detecting the N-terminal blocked form of EGH. It has been reported that the formyl group is removed by deformylase[31]. The mechanism of inclusion body formation after expression is not clear, but it may be caused by a low level of deformylase or insufficient time for deformylation. EGH-β was not oxidized at Met nor formylated at the N-terminal position.

Although at most seven analogues could be formed by three types of independent modifications other than native EGH, oxidized and formylated EGH with and without deamidation were not detected. These analogues might be retained at almost the same position as EGH-β in RP-HPLC and/or the content might be less than 1%.

In conclusion, native EGH and EGH analogues have been characterized by

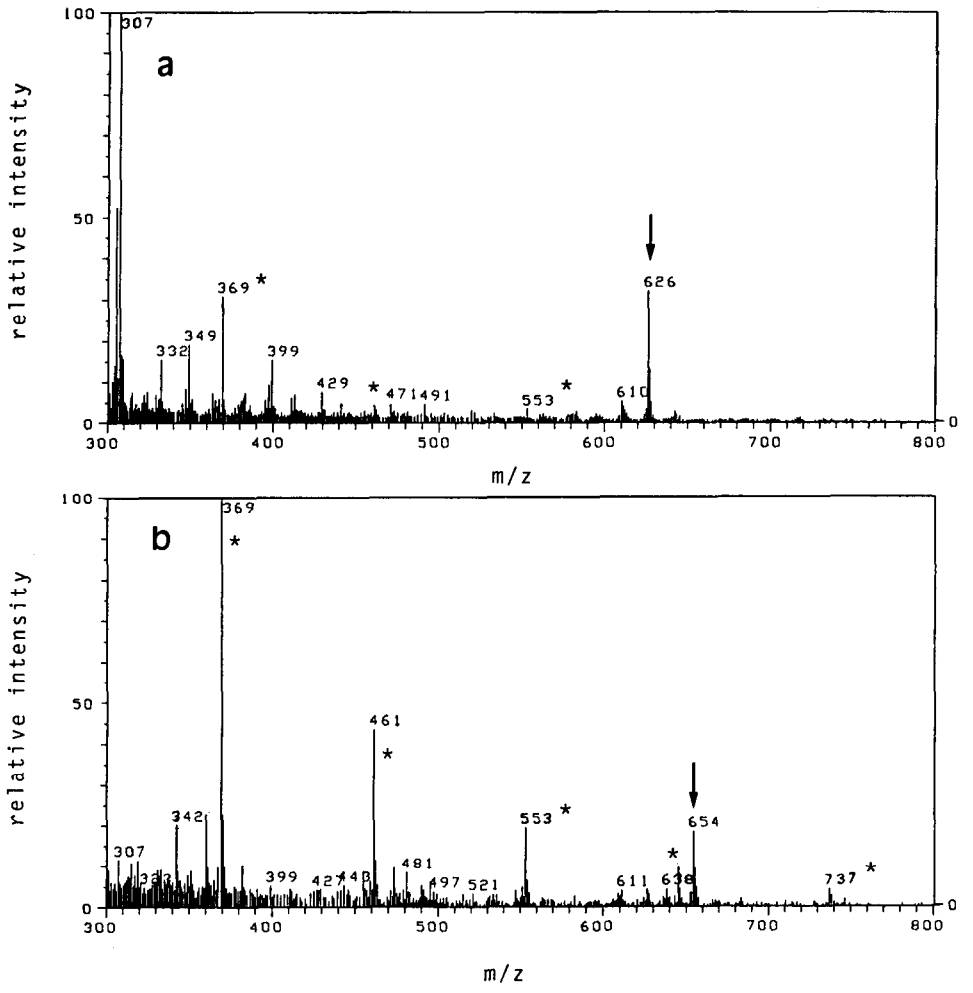

Fig. 9. Secondary ion mass spectra of N-terminal chymotryptic fragment of (a) EGH-β and (b) EGH-γ. Arrows indicate M^+. The peak at m/z 626 in (a) corresponds to the N-terminal fragment Met–Ile–Ser–Leu–Tyr and m/z 654 in b to the N-terminal fragment formylMet–Ile–Ser–Leu–Tyr. Signals marked with asterisks were derived from glycerol.

a combination of separations and proteolytic digestions. The modifications can be classified into three types: deamidation of Asn, oxidation of Met and formylation of N-terminal Met. EGH-A-β was the native structure and five other analogues were modified in one or two positions.

Recently, uniformity of recombinant protein medicine has become important and high purity is needed. To confirm the uniformity for recombinant proteins, SDS-PAGE and gel filtration–HPLC analysis have been routinely used. However, these techniques are inadequate for mixtures of analogues as in EGH preparation.

Here we have shown the importance of PAGE and RP-HPLC for the detection and isolation of analogues and the usefulness of peptide mapping, mass spectrometry, amino acid analysis and N-terminal sequence analysis for the determination of the modifications.

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