Preparation of Biologically Active and Site Specifically Radioiodinated Recombinant Human Insulin-like Growth Factor-I

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Recombinant human insulin-like growth factor-I (rhIGF-I) was iodinated using a lactoperoxidase-catalyzed labeling method. The labeled products were separated into more than five fractions by ion-paired reverse-phase high performance liquid chromatography (HPLC). A fraction (peak 1), which showed the highest yield and radioactivity, was found to be biologically active in the BALB/c 3T3 cell proliferating system. The site of the iodination was investigated by S-pyridylethylation followed by trypsinization and separation with HPLC using reverse phase columns. From the amino acid analysis of the peaks which were radioactive, the iodination site of peak 1 was revealed to be Tyr-24 and Tyr-60. This is the first report of the biological activity of radioactive peptide hormone with a defined labeled site.

Keywords iodination; ^{125}I; insulin-like growth factor-I; lactoperoxidase-catalyzed labeling; BALB/c 3T3 proliferation

Insulin-like growth factor I (IGF-I) is believed to mediate most of the growth-promoting actions of somatotropin.[1] This growth factor, which is structurally related to human proinsulin,[2] stimulates deoxyribonucleic acid (DNA) synthesis and cell replication in tissues of diverse origin and is one of the important mitogenic substances in serum.[3] Balance and metabolism studies of recombinant human (rh) IGF-I necessitate labeling of rhIGF-I. However, the iodination naturally resulted in a complex of variously labeled rhIGF-I. Iodination of the rhIGF-I may have some effect on its biological activity, and balance and metabolism studies are not meaningful if the labeled product is not biologically active. The best assumption may derive from the biologically active IGF-I with defined radio labeled position. Iodination by the lactoperoxidase-catalyzed labeling (LCL) method is done under milder conditions than the chrolamine T method.[4] In order to preserve the biological activity of IGF-I as much as possible, therefore, we labeled rhIGF-I by the LCL method, and obtained biologically active [^{125}I]rhIGF-I with a defined radio labeled position was obtained.

Experimental

Iodination of rhIGF-I rhIGF-I was iodinated according to the LCL method described by Stanton and Hearn.[5] rhIGF-I was kindly supplied by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan), and rhIGF-I (65.5 nmol, 500 μg/150 μl in 0.5 M sodium containing 5 μl of 26.2 μM of NaI and 5 μl of 20 mCi/ml of carrier-free ^{125}I (NEZ033A, Dupont/NEN)). Then, 83.5 μg/10 μl of lactoperoxidase (80 units/mg solid, Sigma) was added to the mixture. Reaction was started by adding 6 μl of 0.09% H_2O_2 and continued for 10 min at 25 °C. The reaction was terminated by adding 24 μl of 0.23% NaN_3 and 800 μl of 4% CH_3COOH. The mixture was then passed through a Sep-pak C_{18} cartridge (Waters). After the cartridge was washed with 10 ml of 4% CH_3COOH twice, the iodinated products were eluted with 5 ml of 100% CH_3OH. The eluate was completely concentrated *in vacuo* and chromatographed on a reverse-phase column. Since tyrosine, monoiodotyrosine and diiodotyrosine in acidic solution had very similar molar extinction coefficients at 270 nm, both iodinated and native IGF-I colud be analyzed quantitatively from the peak area of the high performance liquid chromatography (HPLC) chromatogram. Eluate was collected manually by observing absorbance at ultraviolet (UV) 270 nm. The radioactivity was counted with a γ-counter (Aloka, TDC-103). Major fractions were rechromatographed on the same column.

Reductive Alkylation and Tryptic Mapping The [^{125}I]rhIGF-I was reduced and S-pyridylethylated according to the method described by Miyata *et al.*[6] The [^{125}I]rhIGF-I (approximately 13 nmol) was dissolved in 840 μl of 0.5 M Tris–HCl, pH 8.5, containing 6 M guanidine hydrochloride and 10 mM ethylenediamine tetraacetic acid (EDTA). 2-Mercaptoethanol was added to give a 300-fold molar excess over the concentration of protein disulfide groups. Three mol of 4-vinylpyridine/mol of 2-mercaptoethanol was added to the reaction mixture and incubated for 90 min at room temperature. The reaction mixture was dialyzed against water using a Spectropore 7 dialysis tube (molecular weight cut-off 1000, Spectrum Medical Industries Inc.), and lyophilized.

The S-pyridylethylated [^{125}I]rhIGF-I was dissolved in 168 μl of 50 mM Tris–HCl, pH 9.0, containing 8 M urea. After dilution with 168 μl of 50 mM Tris–HCl (pH 9.0), 8.4 μl of tosylphenylalanyl chloromethyl ketone-treated trypsin (1 mg/ml in 0.1 N HCl, 12200 N_α-benzoyl-L-arginine ethyl ester (BAEE) units/mg solid) was added[7] to the solution and digested at 37 °C for 8 h. The incubation continued for an additional 4 h after supplementation of the same amount of trypsin. Digestion was stopped by freezing, and the sample was chromatographed on a reverse-phase C_{18} column after thawing. Highly radioactive fractions were rechromatographed using the same column pre-equilibrated in 5% CH_3CN–0.08% trifluoroacetic acid (TFA), and eluted with a 60 min linear gradient to 50% CH_3CN–0.08% TFA.

Amino Acid Analysis The isolated tryptic peptides were hydrolyzed using vapor phase 7 M HCl–10% TFA containing 0.1% phenol at 158 °C for 25 and 50 min.[8] The revelated amino acids were labeled with (dimethylamino) azobenzensulfonyl chloride (DABS-Cl) and were analyzed with a Hitachi HPLC system (L-4000).[9]

Bioassay of Labeled rhIGF-I The [^{125}I]rhIGF-I was added to Dulbecco's modified eagle medium (DMEM) deprived of 10% fetal calf serum (FCS) and the cell proliferation activity was measured. Briefly, 3×10^4 of BALB/c 3T3 cells in 100 μl of assay medium were added to each of 96 wells of a Falcon plate together with a series of diluted samples (100 μl). Native rhIGF-I was used as a positive control. After 46—48 h of incubation, the medium was aspirated out and 50 μl of 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide in assay medium (DMEM containing 0.2% bactopepton and 0.05% pluronic F-68) was added. The cells were incubated for another 240 min at 37 °C. At the end of the incubation, 50 μl of 5% glutaraldehyde was added to each well. After being fixed for 10 min at room temperature, the cells were washed 4 times with 300 μl of H_2O and dried overnight at room temperature. The produced dye was extracted with 100 μl of 0.04 N HCl–isopropanol. The optical density (OD) at 540 nm was measured after adding 100 μl of water to the extract with a plate reader (Intermed model NJ-2001).

Results and Discussion

rhIGF-I was iodinated with a trace amount of ^{125}I and a predominant concentration (>99%) of stable ^{127}I at a molar ratio of I to rhIGF-I of 2.8. This ratio was used by Strickland *et al.*[10] in their study of iodination sites of bovine thyroide stimulating hormone (bTSH). The labeled rhIGF-I was concentrated by a Sep-pak C_{18} cartridge and separated by reverse-phase HPLC with a linear CH_3CN gradient. The 5 major peaks which were different from native IGF-I were obtained by reverse-phase HPLC (Fig. 1A). Native IGF-I

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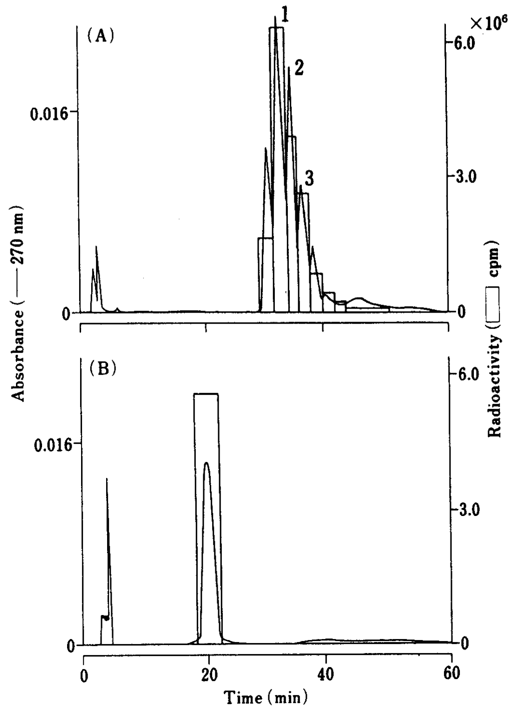

Fig. 1. Isolation of [^{125}I]rhIGF-I with HPLC

(A) Labeled products: equivalent to about 100 μg IGF-I. Column: Cosmosil $10C_{18}$ (4.6 × 250 mm). Solvent system: a) 27% CH_3CN in 0.08% TFA and 5 mM sodium pentanesulfonate; b) 38% CH_3CN in 0.08% TFA and 5 mM sodium pentanesulfonate. Linear gradient from a) to b) for 60 min. Flow rate: 1 ml/min. (B) Peak 1: equivalent to about 50 μg IGF-I. Column: Cosmosil $10C_{18}$ (4.6 × 250 mm). Solvent system: a) 27% CH_3CN in 0.08% TFA, b) 38% CH_3CN in 0.08% TFA. Linear gradient from a) to b) for 60 min. Flow rate: 1 ml/min.

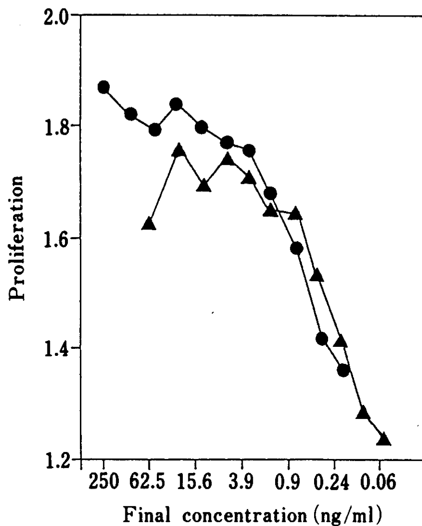

Fig. 2. BALB/c 3T3 Cell Proliferating Activity of Peak 1

▲, peak 1; ●, rhIGF-I.

(retention time: 27.4 min) was eluted between peak 2 and peak 3. All major collected as a single peak (Fig. 1B). A fraction (peak 1, retention time, 26.0 min) showed the highest yield and radioactivity. The specific activity of peak 1 was estimated at 167 nCi/μg rhIGF-I. The biological activity of peak 1 was confirmed by proliferation test of BALB/c 3T3 cells (Fig. 2).

To clarify the site of labelling, peak 1 was reduced, S-pyridylethylated and then digested with trypsin. The primary tryptic mapping of [^{125}I]rhIGF-I revealed the presence of three major highly radioactive peptides (T-15,

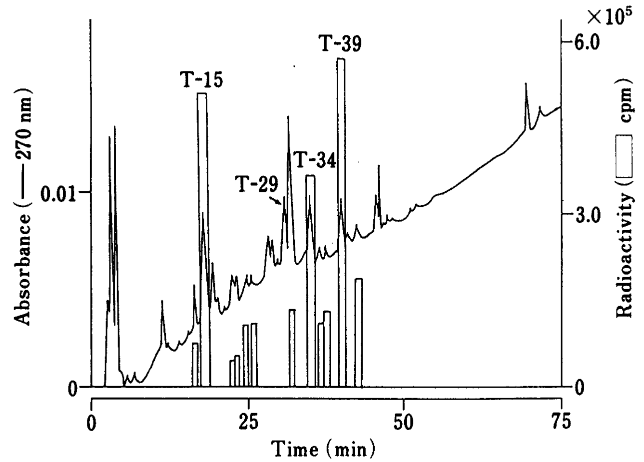

Fig. 3. Reverse Phase HPLC of Products with Trypsin Digestion

Column: Cosmosil $10C_{18}$ (4.6 × 250 mm). Solvent system: a) 5% CH_3CN in 64 mM NH_4HCO_3, b) 60% CH_3CN in 100 mM NH_4HCO_3. Linear gradient from a) to b) for 100 min. Flow rate: 1 ml/min.

34 and 39) on reverse-phase HPLC (Fig. 3). These peptides were rechromatographed and applied to amino acid analysis, and the amino acid compositions (pmol) peptides were determined as Ala (127) Glu (80), Lys (5), Leu (236), Met (148), Pro (123), Arg (41) and Tyr (67) for T-15, Phe (118), Gly (120), Lys (77), Pro (71), Thr (39) and Tyr (46) for T-34, and Ala (79), Glu (22), Lys (104), Leu (84), Met (35), Pro (80) and Tyr (15) for T-39. From the result of amino acid analysis, T-15, 34 and 39 were estimated to be IGF-I peptides 56—65 (Arg–Leu–Glu–Met–Tyr–Cys–Ala–Pro–Leu–Lys), 22—29 (Gly–Phe–Tyr–Phe–Asn–Lys–Pro–Thr) and 56—68 (Arg–Leu–Glu–Met–Tyr–Cys–Ala–Pro–Leu–Lys–Pro–Ala–Lys), respectively. Therefore, iodinated sites of peak 1 were assumed to be Tyr-24 and Tyr-60. Moreover, amino acid analysis of nonradioactive peak T-29 revealed that T-29 was IGF-I peptide 28—36 and that Tyr-32 was not iodinated. The result indicated it was possible to obtain a site of specifically iodinated rhIGF-I was possible. Biological activity and chromatographic behavior on HPLC of thus prepared [^{125}I]rhIGF-I was stable at least for two weeks.

Iodination of protein hormones has been reported for growth hormone,[11] insulin,[12] and luteinizing hormone.[13] However, these reports deal with the mixture of multiply labeled peptides. It was also reported that the four monoiodoinsulins do not have equal properties in assays based on biological activity.[14] Thus, it seems essential to specify the labeled tyrosine residue(s) together with their biological activities to assure that the metabolism research is accurate. Our data demonstrated that the iodination of rhIGF-I as used routinely for bTSH tracer preparation, labeled this growth factor in 2 of the available 3 tyrosine residues. Thus purified iodinated rhIGF-I would be very useful for balance and metabolism studies.

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