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Determination of Insulin in a Single Islet of Langerhans by High-Performance Liquid Chromatography with Fluorescence Detection

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Rodents (rat and mouse) have two types of insulin (insulin I and II; each contains a universal chain A and a different composition of each type BI chain or type BII chain). The physiological role for each isomer is not yet clarified because of the lack of an appropriate separative determination method for these isomers. Thus, in this paper, a sensitive and selective HPLC-fluorescence determination method for the isomers was developed, which includes derivatization with a fluorogenic reagent for thiols, 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate, in the presence of a reducing agent, TCEP, a nonionic surfactant, *n*-dodecyl *β*-D-maltopyranoside, and EDTA. The resultant chain A, BI, and BII derivatives were separated on a reversed-phase column (TSK gel ODS-120T, 250 × 4.6 mm i.d.) with a mobile phase containing 5 mM phosphate buffer (pH 7.0) and were detected at 505 nm with excitation at 380 nm. The detection limits for chain A, BI, and BII derivatives were 2.2, 3.4, and 3.7 fmol on column, respectively. The method was applicable to the determination of rodent insulin in a single islet of Langerhans, and the results indicated its feasibility for the investigation of the pathophysiological roles of the isomers in diabetes in the rodent.

Insulin is synthesized in the *β*-cells of the islets of Langerhans via a precursor, proinsulin, and is secreted into the bloodstream, where it regulates glucose metabolism[1] and influences cell growth or gene expression of many enzymes and proteins.[2] In human, only a single type of insulin (5808 Da) exists, which consists of chains A (21 amino acids) and B (30 amino acids) linked by disulfide bonds, whereas, in rodents such as the rat and mouse, there are two different nonallelic insulins (type I, 5804 Da, and type II, 5796 Da); Both contain chains A (21 amino acids) and B (30 amino acids), and they are different by only two amino acids in chain B (Pro9, Lys29 and Ser9, Met29 for I and II, respectively).[3] However, the difference in the physiological role between insulin types I and II in rodents remains unclear. Therefore, the separative determination of the insulin types I and II is required to study the relative importance of both isomers with a disease state such as diabetes in these experimental animals.

As to the determination of insulin, three main procedures are available. There are bioassay,[4] immunoassay,[5,6] and chromatographic methods.[7,8] Insulin bioassay is now applied to standardization of therapeutic insulin, where insulin is administered to animals and blood glucose level is monitored. However, because of its low sensitivity, it cannot be applied to the determination of insulin in biological samples. Since the advent of radioimmunoassay (RIA), developed by Yalow and Berson,[5] the immunoassay of insulin has been widely used and the immunoenzymometric assay (IEMA) is now popular for the determination of insulin in biological specimens.[6] However, cross-reaction and nonspecific binding with the coexistent biomolecules against anti-insulin antibody are the major interferences with the precise determination of insulin in biosamples.[9] Further, as far as we know, there have been no antibodies to differentiate the rodent insulin type I from type II.

In contrast to immunoassays, the chromatographic method is more reliable for selectivity because of the separation step included. High-performance liquid chromatography (HPLC) with UV detection[7] and, more recently, mass spectrometry (MS) with isotope dilution assay (IDA)[8] have been used for the determination of insulin. HPLC with UV detection, however, has poor sensitivity to detect insulin in biological samples; a picomole-level injection on column should be achievable. The IDA method allows precise determination of human insulin at physiological concentrations in blood but requires labeled insulin with stable isotopes as an internal standard and an expensive instrument, so that the application of the method for routine use is limited only to specialized laboratories.

Considering this background, we concluded that HPLC with the fluorescence detection method is best suited for the routine determination of both the rodent insulin types I and II. Fluorescence detection is facile as well as sensitive and selective, and moreover, the fluorescence detector is not expensive compared

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(1) Cherrington, A. D. *Diabetes* **1999**, *48*, 1198−1214.
(2) O'Brien, R. M.; Granner D. K. *Biochem. J.* **1991**, *278*, 609−619.
(3) Lomedico, P.; Rosenthal, N.; Efstratiadis, A.; Gilbert, W.; Kolodner, R.; Tizard, R. *Cell* **1979**, *18*, 545−558.
(4) Kowarski, C. R.; Bado, B.; Shah, S.; Kowarski, D.; Kowarski, A. A. *J. Pharm. Sci.* **1983**, *72*, 692−693.
(5) Yalow, R. S.; Berson, S. A. *Nature* **1959**, *184*, 1648−1649.
(6) Chevenne, D.; Trivin, F.; Porquet, D. *Diabetes Metab.* **1999**, *25*, 459−476.
(7) Ohkubo, T. *Biomed. Chromatogr.* **1994**, *8*, 301−305.
(8) Kippen, A. D.; Cerini, F.; Vadas, L.; Stocklin, R.; Vu, L.; Offord, R. E.; Rose, K. *J. Biol. Chem.* **1997**, *272*, 12513−12522.
(9) Clark, P. M. *Ann. Clin. Biochem.* **1999**, *36*, 541−564.

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to the mass spectrometer. Among the methods, the precolumn fluorescence derivatization of nonfluorescent biomolecules allows us to detect analytes at the femtomole range in HPLC.[10] Rodent insulins have several functional groups such as two or three amino, five carboxyl, and three oxidized thiol groups. Therefore, we could derivatize the groups with fluorogenic reagents followed by separation and fluorometric detection in HPLC. Since the reduced thiol group is highly reactive to nucleophiles as compared with an amino group, we selected the derivatization of thiol groups with a fluorogenic reagent following the reduction of oxidized thiol groups to reduced thiols in the insulin molecule.

A preliminary trial by use of a combination of reduction with tributylphosphine (TBP) and fluorogenic derivatization of thiol groups with 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F) [11] or 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F)[12] has already been reported for the determination of oxidized form of low molecular weight compounds such as homocysteine, cystine-containing peptides, and oxidized glutathione[13] and identification of the peptide fraction containing oxidized thiol groups in big molecules such as egg albumin in HPLC.[14] However, it has never been applied to the determination of compounds with a molecular weight of more than a few thousand.

In this paper, to determine rodent insulin types I (5804 Da) and II (5796 Da), we used human insulin as a model for the derivatization studies with SBD-F following the reduction of oxidized thiol groups, since human insulin is easily available in a pure form and a large scale and has the same three oxidized thiol groups in the molecule as rodent insulin. SBD-F was selected because of its property to afford a less hydrophobic derivative. Then, the HPLC separation condition of rodent insulin types I and II derivatives was examined. Further, to demonstrate its feasibility, the proposed method was applied to the separative determination of the isomers in a single islet of rodent Langerhans. The larger content of the type I than the type II in the Goto-Kakizaki (GK) rat, a model for type II diabetes, suggested the different response between the two isomers to hyperglycemia.

EXPERIMENTAL SECTION

Cleavage of Disulfide Bridges with TCEP. Twenty-five microliters of 120 μM human insulin was mixed with the same volume of 1.4 or 14 mM tris(2-carboxyethyl)phosphine (TCEP) dissolved in 0.2 M borate buffer (pH 9.0) in the temperature range of 20−60 °C. The time course (2−60 min) for these reactions were investigated by periodic 10-μL injection onto the HPLC column.

Effect of Surfactants on the Derivatization and Identification of the Derivatives. Fifty microliters of 1 μM human insulin was mixed with the same volume of 3.5 mM TCEP, 8.5 mM SBD-F, 10 mM EDTA, and finally a surfactant adjusted to above the critical micelle concentration (cmc) was added. Brij 35, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS), *n*-dodecyl β-D-maltopyranoside (DM), and sodium lauryl sulfate (SDS) were tested as surfactants. Each reaction mixture was allowed to stand at 40 °C for 3 h, and a 10-μL aliquot of the reaction mixture was injected onto the HPLC column. Unless otherwise mentioned, each reagent was dissolved in 0.5 M borate buffer (pH 9.0).

To examine the spectral shift of the maximum fluorescence wavelength of the labeled chains A and B, both derivatives were synthesized and purified as follows: Human insulin (100 μM) dissolved in 0.5 M borate buffer (pH 9.0) was derivatized with 1.7 mM SBD-F at 40 °C for 3 h in the presence of 0.7 mM TCEP, 2 mM EDTA, and 1 mM DM, and consequently, chain A and B derivatives were obtained. The reaction mixture containing both derivatives was injected onto HPLC column (TSKgel ODS-120T, Tosoh). Then, the eluent containing each derivative was collected and was evaporated to remove excessive acetonitrile. Each residue was redissolved in 0.5 M borate buffer (pH 9.0), and the fluorescence spectra of each derivative before or after addition of a surfactant were measured on a fluorescence spectrophotometer.

The peak fractions corresponding to the chain A and B derivatives (each about 10 nmol) were collected and subjected to mass spectrometry using electrospray ionization (ESI). Each fraction was infused from a glass syringe at 5 μL/min with a syringe pump and was scanned from m/z 500 to 3000 in 1.0-s steps with a dwell of 2.5 ms.

Effect of pH, Temperature, Reaction Time, and Additives on the Derivatization. First, to optimize the pH of reaction mixture, 0.2 μM human insulin was derivatized in the pH range of 8.0−10.0 at 40 °C in the presence of 0.7 mM TCEP, 1.7 mM SBD-F, 2 mM EDTA, and 1 mM DM. Next, the reaction temperature was examined in the range of 30−50 °C at pH 9.0 in the presence of 0.7 mM TCEP, 1.7 mM SBD-F, 2 mM EDTA, and 1 mM DM. Finally, the final concentrations of each reagent was examined in the following ranges: 0−7 (TCEP), 0−8.5 (SBD-F), 0−20 (EDTA), and 0−10 mM (DM). The time course (0−8 h) for these reactions was investigated by periodic 10-μL injections onto the HPLC column.

Determination of Insulin Types I and II in a Single Islet in the Rodent. Pancreases from Wistar and GK rats (male and 10 weeks old each, $n = 3$, Charles River) and ICR mice (male and 10 weeks, $n = 3$, Clea) were digested with collagenase,[15] and islets and nonislet tissues from Wistar rats (as a negative control) were picked up with a stereomicroscope. Each single islet of Langerhans or nonislet tissue was dissolved in 50 μL of 0.1 M NaOH. Then, each sample was mixed with the same volume of 3.5 mM TCEP, 8.5 mM SBD-F, 10 mM EDTA, and 5 mM DM, respectively. This mixture was allowed to stand for 3 h at 40 °C. After this derivatization, a 20-μL aliquot of the reaction mixture was injected onto the HPLC column. Calibration curves were constructed by plotting the peak area versus the amount of chain A, BI (derived from insulin type I), and BII (derived from insulin type II) derivatives in the following ranges: 10−500, 7−350, and 6−150 fmol on column, respectively.

Sixty islets of Langerhans from Wistar rat (male, 10 weeks, Charles River) were adopted for the identification of the chain A, BI, and BII derivatives by mass spectrometry, and the mass spectra were obtained using the same condition as described in the identification of human insulin derivatives (described above).

(10) Boppana, V. K.; Miller-Stein, C.; Politowski, J. F.; Rhodes, G. R. *J. Chromatogr.* **1991**, *548*, 319−327.
(11) Toyo'oka, T.; Imai, K. *Anal. Chem.* **1984**, *56*, 2461−2464.
(12) Imai, K.; Toyo'oka, T.; Watanabe, Y. *Anal. Biochem.* **1983**, *128*, 471−473.
(13) Uchiyama, S.; Santa, T.; Okiyama, N.; Fukushima, T.; Imai, K. *Biomed. Chromatogr.* **2001**, *15*, 295−318.
(14) Toyo'oka, T.; Imai, K. *Anal. Chem.* **1985**, *57*, 1931−1937.
(15) van der Vliet, J. A.; Meloche, R. M.; Field, M. J.; Chen, D. J.; Kaufman, D. B.; Sutherland, D. E. *Transplantation* **1988**, *45*, 493−495.

Chromatographic Condition. HPLC separation was performed with a TSKgel ODS-120T column (pore size 12 nm, 250 × 4.6 mm i.d.) (Tosoh, Tokyo, Japan). Mobile phases for the analysis of unlabeled chains A and B, which have reduced thiol groups, were (A) 20 mM ammonium formate (pH 6.5) and (B) 20 mM ammonium formate (pH 6.5) in 50% acetonitrile. The gradient cycle for the UV detection (280 nm) of chains A and B was as follows: Initial condition was 60% B, which was increased to 100% B within 18 min. The flow rate was 1.0 mL/min. Mobile phases for the analysis of labeled chains A and B derived from human insulin were (A) 50 mM ammonium acetate (pH 7.0) and (B) 50 mM ammonium acetate (pH 7.0) in 50% acetonitrile. The gradient cycle for the fluorescence detection of the labeled chains A and B was as follows: Initial condition was 40% B, which was increased to 100% B within 30 min. The flow rate was 0.5 mL/min. The following mobile phases were also used for the analysis of labeled chains A, BI, and BII derived from rodent insulins: The eluents were (A) 5 mM phosphate buffer (pH 7.0) and (B) 5 mM phosphate buffer (pH 7.0) in 50% acetonitrile. The gradient cycle and the flow rate were the same as those used for the analysis of the labeled chains A and B derived from human insulin. Unless otherwise specified, the column temperature was kept at room temperature, and the fluorescence detector was set at 380 and 505 nm for excitation and emission, respectively.

Apparatus. The HPLC system was constructed with a HP 1090 series II system (Hewlett-Packard GmbH) and a FP-2025 plus fluorescence detector (Jasco). Fluorescence spectra were measured on a F-4500 fluorescence spectrophotometer (Hitachi). Mass spectra were obtained on a API-300 mass spectrometer (Applied Biosystems/MDS Pesciex) using ESI.

Chemicals. Recombinant human insulin (P/N, I 0259) was purchased from Sigma (St. Louis, MO). SBD-F, DM, and CHAPS were purchased from Dojindo Laboratories (Kumamoto, Japan). Brij 35 and SDS were purchased from Wako Pure Chemical Industries (Oosaka, Japan). TCEP was purchased from Tokyo Kasei (Tokyo, Japan). All other chemicals were analytical or guaranteed reagent grade. Rat insulin standards (P/N, 8013) was purchased from Linco Research (St. Charles, MO) and purified to remove contaminants such as albumin by HPLC. The same HPLC condition as for the analysis of unlabeled chains A and B was used (described in Chromatographic Condition). Purified insulin types I and II eluted from the column were collected, and the solvent was evaporated to dryness. The residue was stored at −20 °C until the determination. The recovery of the purified insulin was more than 76%, and the concentrations of the insulins were corrected.

RESULTS AND DISCUSSION

Cleavage of Disulfide Bridges with TCEP. Since there are no reduced thiol groups in the insulin molecule[16] (5808 Da, 51 amino acids, Figure 1), the reduction of the disulfide bridges (three oxidized thiol groups) to produce reduced thiols was required with a reducing agent such as TBP, which was described in our previous paper,[17] where some low molecular weight compounds containing oxidized thiol groups such as cystine and oxidized glutathione were the target molecules. They were reduced with TBP, and the thiols produced were derivatized with SBD-F followed by HPLC separation and fluorescent detection. In our preliminary experiment, however, TBP was not able to reduce the disulfide bridges of insulin. Therefore, a stronger reducing agent, TCEP,[18,19] was examined, which is advantageous in the present aqueous derivatization condition (later described) because of its good solubility in water (300 mg vs less than 1 mg/mL water for TCEP and TBP, respectively). According to some trials on the reduction with TCEP, 0.7 mM TCEP under alkaline conditions was enough to produce six thiols in the insulin molecules (data not shown). TCEP also obstructed the oxidation of the thiols produced, and hereafter, the experiment was performed in the presence of 0.7 mM TCEP in the reaction medium.

Effect of Surfactants on the Derivatization. In the previous paper for the identification of disulfide bridges in big molecules such as egg albumin, SDS was used to accelerate the derivatization of thiol groups with ABD-F.[14] In the present experiment, several surfactants above the cmc were studied to enhance the derivatization of human insulin with SBD-F. Since insulin (Figure 1) consists of two chains (A and B) linked by disulfide bonds, two peaks should appear on the chromatogram after the reduction with TCEP and derivatization with SBD-F followed by HPLC separation and detection. Indeed, the two fluorescent peaks corresponding to the insulin chain A and B derivatives were obtained as shown in Figure 2. Peaks 1 and 2 were identified as the chains A and B labeled with SBD-F, respectively, because the mass numbers were observed as the expected multiply charged ions, $[M + 2H]^{2+}$ (1586.8 and 1911.7 Da, for the labeled chains A and B, respectively) and $[M + 3H]^{3+}$ (1057.9 and 1274.9 Da, for labeled chains A and B, respectively), by an ESI mass spectrometer. According to this HPLC, the determination of both chains was achieved. The results on the effect of surfactants are summarized in Figure 3. Any corresponding peaks were not observed without surfactants in the reaction mixture, indicating that surfactant was essential for the derivatization to proceed. Either DM or CHAPS greatly accelerated the derivatization of both chains A and B. However, the effect of SDS, which was used as denaturant for egg albumin,[14] was small for the derivatization of both chains, especially the chain B. To clarify the reason for the lesser effect of SDS, we examined the spectral shift of the maximum fluorescence wavelength of the labeled chains A and B by addition of the above-mentioned surfactants, since the spectral shifts are generally correlated with hydrophobicity around the fluorophore.[20,21] The blue shifts (∼10−20 nm) of the maximum fluorescence wavelength of the labeled chains in solution were observed with the addition of DM, CHAPS, and Brij 35, whereas only a small blue shift (less than 5 nm) was observed with SDS for both derivatives. Therefore, a small conformational change of the surroundings near the thiol groups could be the reason for the small effect of SDS on the derivatization. Miyairi et al. derivatized metallothionein (∼6000 Da) with SBD-F without any

(16) Smith, L. F. *Am. J. Med.* **1966**, *40*, 662−666
(17) Toyo'oka, T.; Uchiyama, S.; Saito, Y.; Imai, K. *Anal. Chim. Acta* **1988**, *205*, 29−41.
(18) Burns, J. A.; Butler, J. C.; Moran, J.; Whitesides, G. M. *J. Org. Chem.* **1991**, *56*, 2648−2650.
(19) Han, J. C.; Han, G. Y. *Anal. Biochem.* **1994**, *220*, 5−10.
(20) Kosower, E. M. *J. Am. Chem. Soc.* **1958**, *80*, 3253−3260.
(21) Kosower, E. M. *J. Am. Chem. Soc.* **1958**, *80*, 3261−3267.

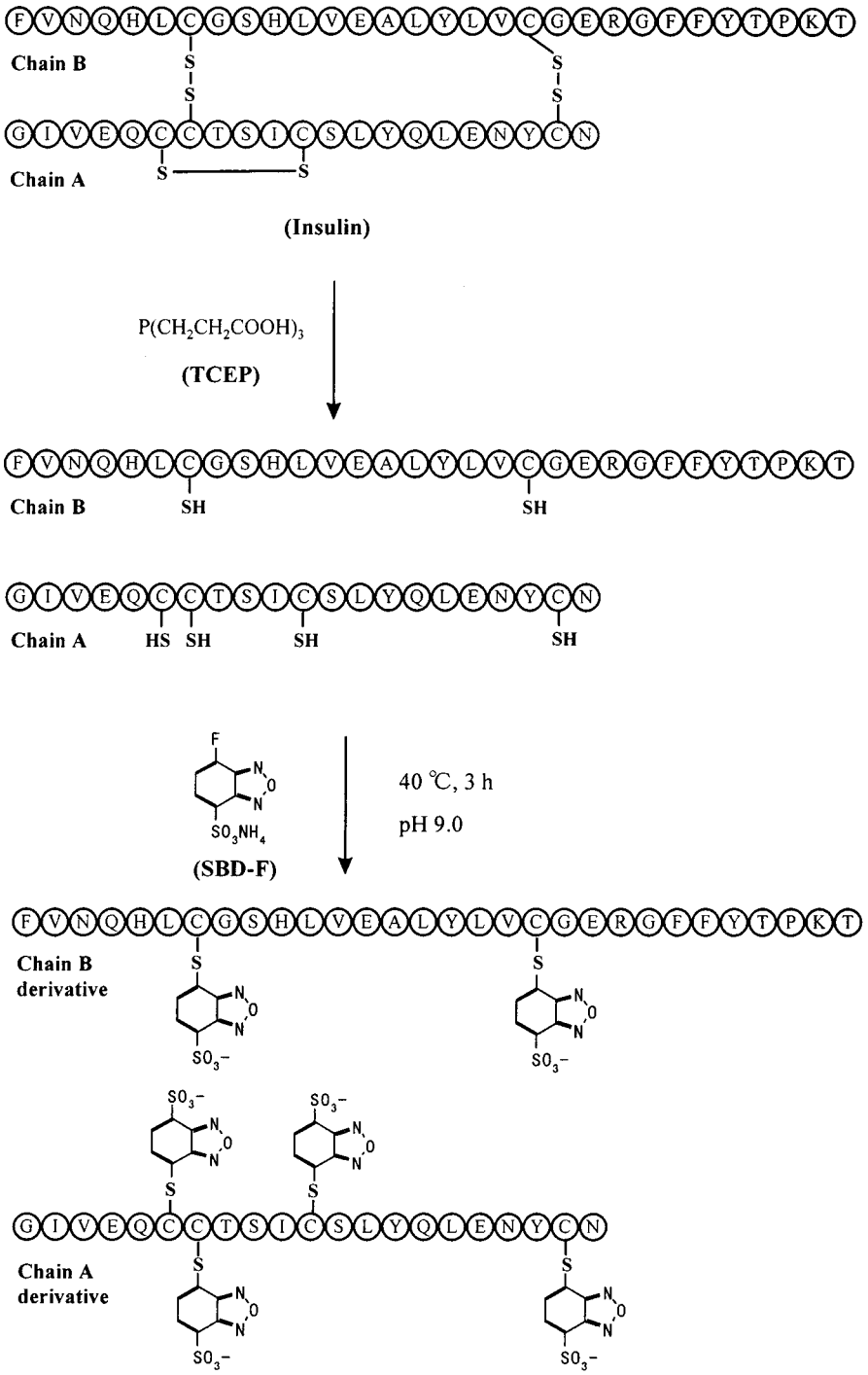

Figure 1. Derivatization scheme for insulin with SBD-F.

surfactant.[22] However, in our experiment, any derivatization of both the A and B chains of insulin was not observed without surfactant as depicted in Figure 3. The thiol moieties in both chains of human insulin could be more sterically hindered than those of metallothionein.

Effect of pH, Temperature, Reaction Time, and Additives on the Derivatization. Next, we examined the effect of pH, temperature, reaction time, and additives on the derivatization reaction. As in the case of low molecular weight compounds,[12] the maximum reaction yield was obtained at pH 9.0 (under 40 °C, 3 h, Figure 4). In the present experiment, a remarkable decrease of peak intensity was observed at pH 10.0, although the reaction was completed within 1 h. Cleavage of the peptide bonds may have occurred at pH 10.0 since some unknown fluorescent peaks were observed on the chromatograms after 1-h reaction. We also found that the rate for decrease of the chain A was faster than that of the chain B under alkaline conditions, so that the A chain may be more labile than the B chain under these conditions.

We also tested the effect of temperature on the formation of the derivatives (Figure 5). The maximal yield was observed under

(22) Miyairi, S.; Shibata, S.; Naganuma, A. *Anal. Biochem.* **1998**, *258*, 168−175.

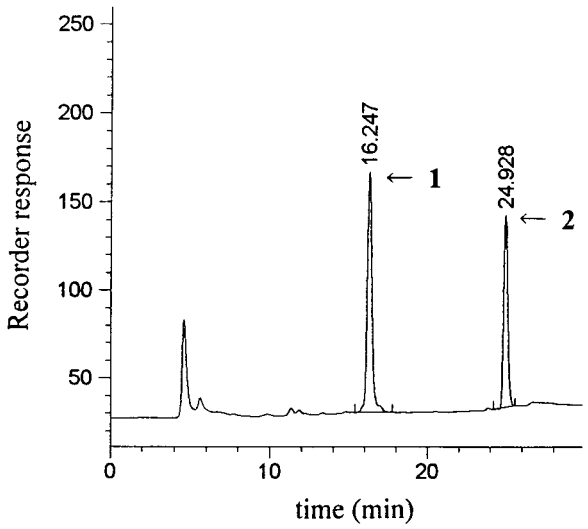

Figure 2. Representative HPLC chromatogram of human insulin derivatized with SBD-F. (1) Chain A derivative (2 pmol); (2) chain B derivative (2 pmol). Human insulin was derivatized with 1.7 mM SBD-F at pH 9.0 under 40 °C for 3 h in the presence of 0.7 mM TCEP, 2 mM EDTA, and 1 mM DM.

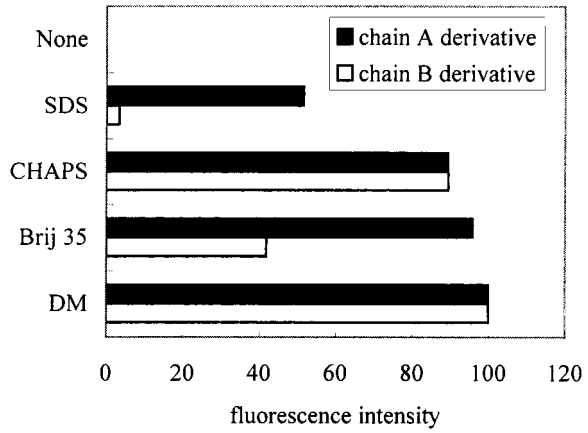

Figure 3. Effect of surfactants on the derivatization of human insulin with SBD-F. The concentrations of surfactants were set at as follows; SDS, 10 mM; CHAPS, 10 mM; Brij 35, 0.1%; DM, 1 mM. Human insulin was derivatized with 1.7 mM SBD-F at pH 9.0 under 40 °C for 3 h in the presence of 0.7 mM TCEP and 2 mM EDTA.

40 °C (pH 9.0, 3 h). The remarkable decrease of the peak intensity was observed at 50 °C with the appearance of unknown fluorescent peaks, whereas the derivatization of low molecular weight compounds was carried out under higher temperature (60 °C) at pH 9.5 without degradation.[12]

To avoid the oxidation of thiols with metal ions in alkaline conditions,[23,24] the effect of EDTA was examined. As shown in Figure 6, the peak of the chain A derivative was increased with EDTA up to 2 mM, while that of the chain B was almost constant regardless of the addition of EDTA. The concentration of SBD-F was also examined, and 1.7 mM was selected (data not shown).

Considering the results, hereafter, the following derivatization conditions were selected: 0.5 M borate buffer (pH 9.0) with 1.7 mM SBD-F at 40 °C for 3 h in the presence of 0.7 mM TCEP, 2 mM EDTA, and 1 mM DM.

HPLC Separation of the Chain A and B Derivatives. First, the separation and determination of human insulin derivatives were examined. The molecular weights of the chain A and B derivatives are more than 3000 (3172 and 3824, respectively). Therefore, a wide pore-sized silica (∼>10 nm) is preferable to the narrow pore-sized one as stationary phase because of less steric hindrance for the analytes. The following two ODS columns were compared: TSKgel ODS-80TM (pore size 8 nm, 250 × 4.6 mm i.d., Tosoh) and TSKgel ODS-120T (pore size 12 nm, 250 × 4.6 mm i.d., Tosoh). As expected, the latter gave the sharper peak as compared with the former. With the pH of the mobile phase less than 3, the chain A derivative was not eluted from the column. It was derived from the hydrophobic property of the derivative because the chain A has Glu4, Glu17, and no basic amino acid, and the isoelectric point (p*I*) of the SBD derivative would be less than 4. According to the hydrophilicity of the sulfonic acid moiety of SBD, the derivatives were eluted with a rather small amount of acetonitrile in the mobile phase. Thus, with a gradient elution of 50 mM ammonium acetate (pH 7.0)/acetonitrile from 20% to 50% acetonitrile over 30 min, the labeled chains A and B were eluted and determined as shown in Figure 2.

Determination of Insulin Types I and II in a Single Islet in the Rodent. As described before, in the rodent, the islet of Langerhans produces two different nonallelic insulins (types I and II). They are different by two amino acid residues in the B chain (Pro9 and Lys29 in type I, and Ser9 and Met29 in type II, respectively). They also differ by four amino acid residues from human insulin in chains A (Asp4 in rodent, and Glu4 in human) and B (Lys3, Pro9, Lys29, and Ser30 in rodent type I, Lys3, Ser9, Met29, and Ser30 in type II, and Asn3, Ser9, Lys29, and Thr30 in human, respectively).[16] In a preliminary experiment, the chain BI derivative was not separated from the BII derivative under the mobile-phase condition adopted for the determination of human insulin. Then, we reexamined the mobile-phase composition for the separation of the labeled chain BI from the labeled chain BII derivative. Since the chain BI derivative has two lysine residues, which can interact strongly with the silanol moiety of the stationary phase at neutral pH with a low salt concentration, it may be eluted later as compared with the chain BII derivative. Then, we used the mobile phase containing 5 mM phosphate buffer (pH 7.0) instead of 50 mM ammonium acetate adopted for the separation of human insulin A and B derivatives. As expected, as shown in Figure 7, the chain BI derivative was eluted late and well separated from the BII derivative. The contribution of the silanol moiety to the separation of the isomers was also verified when a stationary phase of completely end-capped silica (Cadenza CD-C18 column, Imtakt) was adopted. No separation was observed with the same mobile phase (data not shown).

Three major peaks were observed on the chromatogram obtained from a rat single islet of Langerhans (Figure 7). These peaks (peaks 1, 2, and 3) were identified as the labeled chain A, BII, and BI derivatives from the mass numbers as the multiply charged ions, $[M + 2H]^{2+}$ (1582.8, 1916.2 and 1919.7 Da for A, BII, and BI, respectively) and $[M + 3H]^{3+}$ (1277.9 and 1279.9 Da for BII and BI, respectively), by ESI mass spectrometry. As shown in Figure 7, the present HPLC method was found to have enough

(23) Andrews, J. L.; Ghosh, P.; Ternai, B.; Whitehouse, M. W. *Arch. Biochem. Biophys.* **1982**, *214*, 386-396

(24) Kawahara, Y.; Hisaoka, M.; Yamazaki, Y.; Inage, A.; Morioka, T. *Chem. Pharm. Bull.* **1981**, *29*, 150−157.

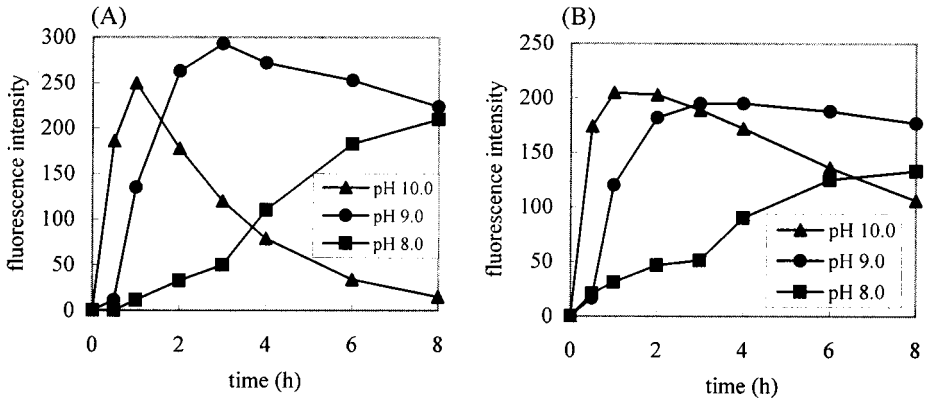

Figure 4. Effect of pH on the time course of the reaction yield of the derivatized human insulin. (A) Chain A derivative; (B) chain B derivative. Human insulin was derivatized with 1.7 mM SBD-F at 40 °C for 3 h in the presence of 0.7 mM TCEP, 2 mM EDTA, and 1 mM DM.

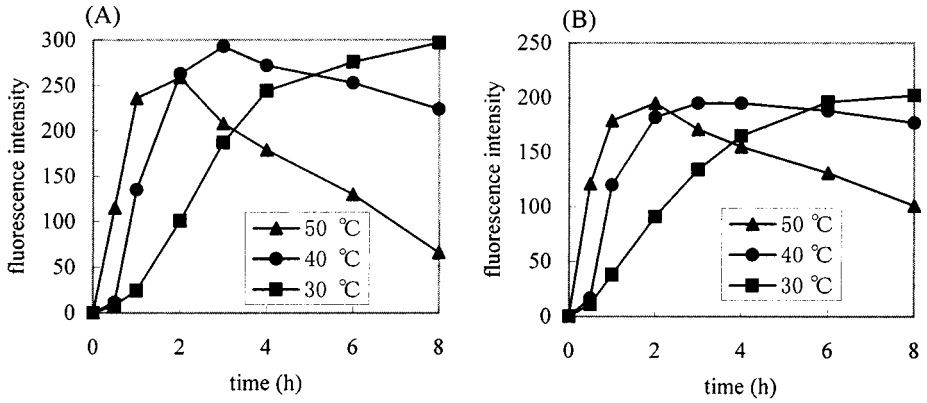

Figure 5. Effect of reaction temperature on the time course of the yield of the derivatized human insulin. (A) Chain A derivative; (B) chain B derivative. Human insulin was derivatized with 1.7 mM SBD-F at pH 9.0 for 3 h in the presence of 0.7 mM TCEP, 2 mM EDTA, and 1 mM DM.

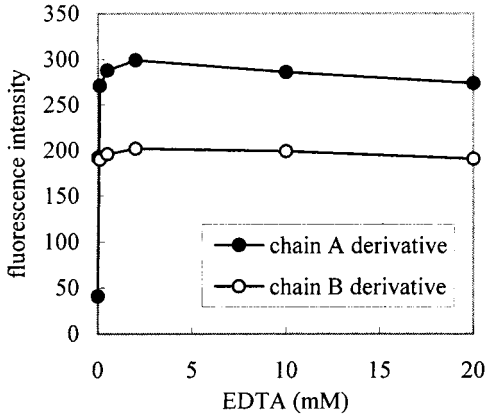

Figure 6. Effect of EDTA on the derivatiation of human insulin with SBD-F. Human insulin was derivatized with 1.7 mM SBD-F at pH 9.0 under 40 °C for 3 h in the presence of 0.7 mM TCEP and 1 mM DM.

sensitivity for the determination of rodent insulin in a single islet of Langerhans. The detection limits for chain A, BI, and BII derivatives calculated on the chromatograms obtained with 10-, 14-, and 6-fmol injections on column were 2.2, 3.4, and 3.7 fmol (signal/noise, 3), respectively. The calibration curves for the chain A, BI, and BII derivatives showed good linearity (r = 0.9996, 0.9996, and 0.9997, respectively) over the following ranges: 10−500, 7−350, and 6−150 fmol on column, respectively.

Then, the method was applied to the determination of rat and mouse insulin in a single islet. The total insulin content (type I plus type II) obtained from Wistar rats was calculated to be 137 ± 18 ng/islet (mean ± SD, n = 3). A similar value (118 ng/islet) was observed by HPLC with UV detection utilizing 20 islets.[7] Insulin content in mice (245 ± 59 ng/islet) was observed for the first time, although only the ratio of insulin types I to II obtained from mice was reported by Linde et al. with the use of more than 1400 islets.[25] The calculated ratio of types I to II obtained from rats and mice were 1.4 ± 0.2 and 0.57 ± 0.01, respectively, which were consistent with the previous data reported by Linde et al.[25] Kakita et al. reported that insulin type I is predominant in mice,[26] whereas the result in the present experiment and the data reported by Linde et al.[25] showed the predominance of type II. The reason for the discrepancy is not clear yet, but it should be noted that type II insulin is labile to oxidation because of the presence of Met29.[27] In the proposed method, the derivatization was performed under reduced conditions in the presence of TCEP and oxidation would not occur during the handling of the islet. In contrast, Kakita et al.[26] used gel electrophoresis for the separation of the isomers; oxidation of Met29 could occur if care was not taken.

Next, to demonstrate further the feasibility of the method, the determination was made for the GK rat, which is the model rat for type II diabetes. The total insulin content obtained, 123 ± 75

(25) Linde, S.; Nielsen, J. H.; Hansen, B.; Welinder, B. S. *J. Chromatogr.* **1989**, *13*, 462, 243−254.
(26) Kakita, K.; O'Connell, K.; Permutt, M. A. *Diabetes* **1982**, *31*, 841−845.
(27) Linde, S.; Nielsen, J. H.; Hansen, B.; Welinder, B. S. *J. Chromatogr.* **1990**, *530*, 29−37.

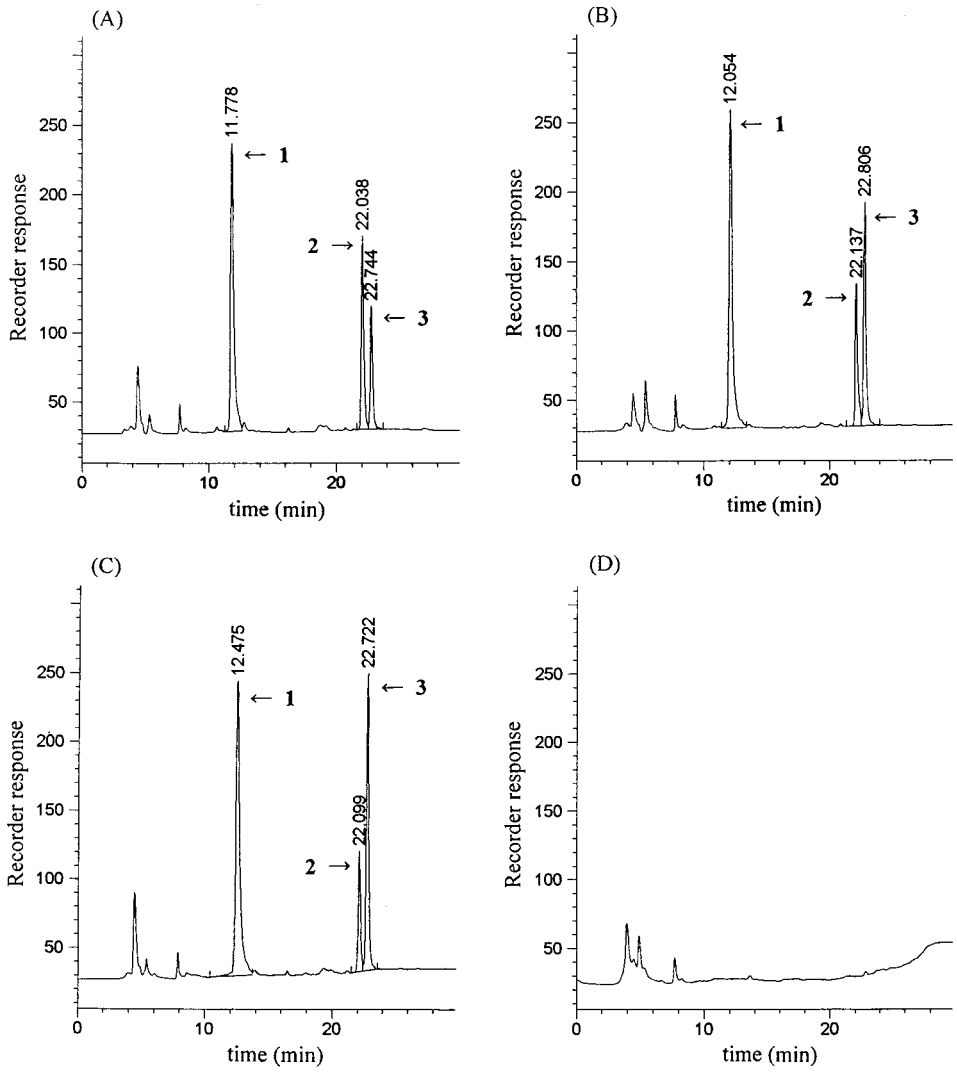

Figure 7. Chromatograms of the SBD-insulin derivatives obtained from a single islet of Langerhans and a nonislet tissue. Islet from (A) ICR mouse, (B) islet from Wistar rat, (C) islet from GK rat, and (D) nonislet tissue (10 μg) from Wistar rat (as a negative control): (1) chain A derivative; (2) chain BII derivative; (3) chain BI derivative. Islets and nonislet tissue were derivatized with 1.7 mM SBD-F at pH 9.0 and 40 °C for 3 h in the presence of 0.7 mM TCEP, 2 mM EDTA, and 1 mM DM.

ng/islet, was similar to that for the Wistar rat. However, the ratio of types I to II was 2.0 ± 0.2. The bigger value as compared to 1.4 ± 0.2 for Wistar rat could be related to a different response to hyperglycemia between the two isomers in type II diabetes, since Ling et al. reported that the increase of proinsulin I synthesis of islets cultured under a high glucose level was responsible for the elevation of the insulin type I as compared type II.[28] However, the large value could be derived from the accelerated release of the type II isomer from the islet. Thus, the concentration of both insulin types in blood should be determined under hyperglycemic conditions in the GK rat in order to clarify the bigger ratio in the islet of the GK rat.

The proposed method is more sensitive and selective as compared to the previous HPLC method using UV detection[7] in which more than 20 islets were required for the determination of insulin types I and II and, moreover, with many interfering peaks on the chromatogram. Further, the method should be superior to the immunoassay method, which is difficult for the separative determination of insulin type I from II unless each monoclonal antibody is prepared.

In conclusion, an appropriate method was developed for the sensitive and separative determination of both types of insulin in the rodent utilizing HPLC and fluorogenic derivatization with SBD-F. The proposed method was applicable to the determination of both isomers in a single rodent islet of Langerhans and thus might be useful for the investigation of the pathophysiology of diabetes in experimental animals.

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(28) Ling, Z.; Heimberg, H.; Foriers, A.; Schuit, F.; Pipeleers, D. *Endocrinology* **1998**, *139*, 491−495.