

# Mishandling of the Therapeutic Peptide Glucagon Generates Cytotoxic Amyloidogenic Fibrils

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**Purpose.** Some therapeutic peptides exhibit amyloidogenic properties that cause insolubility and cytotoxicity against neuronal cells *in vitro*. Here, we characterize the conformational change in monomeric therapeutic peptide to its fibrillar aggregate in order to prevent amyloidogenic formation during clinical application.

**Methods.** Therapeutic peptides including glucagon, porcine secretin, and salmon calcitonin were dissolved in acidic solution at concentrations ranging from 1 mg/ml to 80 mg/ml and then aged at 37°C. Amyloidogenic properties were assessed by circular dichroism (CD), electron microscopy (EM), staining with  $\beta$ -sheet-specific dyes, and size-exclusion chromatography (SEC). Cytotoxic characteristics were determined concomitantly.

**Results.** By aging at 2.5 mg/ml or higher for 24 h, monomeric glucagon was converted to fibrillar aggregates consisting of a  $\beta$ -sheet-rich structure with multimeric states of glucagon. Although no aggregation was observed by aging at the clinical concentration of 1 mg/ml for 1 day, 30-day aging resulted in the generation of fibrillar aggregates. The addition of anti-glucagon serum significantly inhibited fibrillar conversion of monomeric glucagon. Glucagon fibrils induced significant cell death and activated an apoptotic enzyme, caspase-3, in PC12 cells and NIH-3T3 cells. Caspase inhibitors attenuated this toxicity in a dose-dependent manner, indicating the involvement of apoptotic signaling pathways in the fibrillar formation of glucagon. On the contrary to glucagon, salmon calcitonin exhibited aggregation at a much higher concentration of 40 mg/ml and secretin showed no aggregation at the concentration as high as 75 mg/ml.

**Conclusions.** These results indicated that glucagon was self-associated by its  $\beta$ -sheet-rich intermolecular structure during the aging process under concentrated conditions to induce fibrillar aggregates. Glucagon has the same amyloidogenic propensities as pathologically related peptides such as  $\beta$ -amyloid (A $\beta$ )<sub>1-42</sub> and prion protein fragment (PrP)<sub>106-126</sub> including conformational change to a  $\beta$ -sheet-rich structure and cytotoxic effects by activating caspases. These findings suggest that inappropriate preparation and application of therapeutic glucagon may cause undesirable insoluble products and side effects such as amyloidosis in clinical application.

**KEY WORDS:** aggregation; fibril toxicity; glucagon; salmon calcitonin.

## INTRODUCTION

Glucagon is a polypeptide hormone that consists of 29 amino acid residues and plays a central role in the maintenance of normal circulating glucose levels (1). The administration of glucagon induces an increase in hepatic glycogenolysis and gluconeogenesis, and attenuates the ability of insulin to inhibit these processes. Glucagon is widely used for peroral endoscopy, clinical diacrisis, and treatment of hypoglycemia. Water-insoluble glucagon is usually solubilized at acidic pH. However, there is at least one serious problem regarding the physicochemical property of glucagon in solution, resulting in glucagon forming gel-like fibrillar aggregates in dilute acid (2). Glucagon is largely unfolded with few stable intramolecular bonds under clinical usage, while the conformation in glucagon fibrils is mainly  $\beta$ -sheet-rich. Other therapeutic peptides such as insulin (3), GLP-1 analog (4), and growth hormone (5) also display conformational changes into  $\beta$ -sheet-rich fibrils. These insoluble products are attributed to the formation of partially unfolded intermediates with an exposed hydrophobic region that drives the aggregation toward the pharmaceutically undesirable form (6).

The generation of insoluble peptide/protein fibrils is well-confirmed in amyloidosis, complex disorders characterized by the polymerization and aggregation of normally innocuous and soluble proteins or peptides followed by extracellular insoluble fibrils with resistance to peptidases (7,8). There are at least 16 proteins forming amyloid fibrils in clinically diverse conditions, which include  $\beta$ -amyloid (A $\beta$ ) in Alzheimer's disease (9), amylin in type II (non-insulin-dependent) diabetes mellitus (10), prion protein (PrP) in Creutzfeldt-Jakob disease and spongiform encephalopathy (11), and polyglutamine in Huntington's disease (12). These pathologically related amyloidogenic protein/peptide fibrils share a distinct conformational feature in the richness of the  $\beta$ -sheet structure (13). In addition, there are similar characteristics of polarity, hydrophobicity, and the size of side-chain among certain segments containing 10–15 amino acid residues of amyloid-forming peptides such as insulin, A $\beta$ , and amylin, and these factors were indicative of a consensus sequence as a recognition motif of Congo Red, a specific dye for amyloidogenic protein/peptide fibrils with  $\beta$ -sheet dependency (14,15). Because there are similar neurotoxic effects of fibrils from pathologically related peptides and non-pathologically related peptides including glucagon (16), it is plausible that there is also a common toxic mechanism related to their sec-

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**ABBREVIATIONS:** A $\beta$ , amyloid  $\beta$  peptide; PrP, prion protein; WST-8, 4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolol]-1, 3-benzene disulfonate sodium salt; Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-1-al; Z-VAD-FMK, N-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone; TEM, transmission electron microscopy; CD, circular dichroism; ThT, thioflavin T; SEC, size-exclusion chromatography; DMEM, Dulbecco's modified Eagle's medium; LDH, lactate dehydrogenase.

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ondary and macromolecular structures for different amyloid-forming proteins/peptides.

In this investigation, we have characterized the physico-chemical and physiological properties of glucagon fibrils using biophysical techniques including circular dichroism (CD), transmission electron microscopy (TEM),  $\beta$ -sheet-imaging probes, and size-exclusion chromatography (SEC). As well as glucagon, we also investigated the characteristics of other therapeutic peptides, which include porcine secretin that exhibits 52% amino acid sequence homology to glucagon (17) and salmon calcitonin, a therapeutic peptide used for the treatment of osteoporosis, Paget's disease, and hypercalcemia (18). We have demonstrated that glucagon requires the lowest concentration for fibril formation among those three therapeutic peptides and the peptide fibrils of glucagon and salmon calcitonin possess the same conformational properties and cytotoxic apoptotic signaling pathways by activating caspases as fibrils derived from pathologically-related peptides including  $A\beta_{1-42}$  and PrP<sub>106-126</sub>. Here, we provide further insights into the associative behavior of glucagon, showing that its noncovalent aggregation was dependent on the condition for storage.

## MATERIALS AND METHODS

### Chemicals

Human glucagon and porcine secretin were synthesized by the solid-phase strategy employing optimal side-chain protection as reported previously (19). Salmon calcitonin,  $A\beta_{1-42}$  and PrP<sub>106-126</sub> were purchased from American Peptide Company (Sunnyvale, CA, USA). Congo Red and thioflavin T (ThT) were purchased from Wako (Osaka, Japan), and WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] was obtained from Dojindo (Kumamoto, Japan). Ac-DEVD-CHO and Z-VAD-FMK, caspase inhibitors, were purchased from Promega (Madison, WI, USA). Calibration standards for size-exclusion column chromatography (SEC) were obtained from Sigma (St. Louis, MO, USA). Human glucagon antibody YP040 was obtained from Yanaihara Institute, Inc. (Fujinomiya, Japan).

### Aging Treatment of Peptides

Glucagon, salmon calcitonin, and porcine secretin were dissolved in 0.01 M HCl at the concentration indicated in the text.  $A\beta_{1-42}$  and PrP<sub>106-126</sub> were prepared in sterile water as 5 and 10 mg/ml stocks, respectively (20,21). Peptides were incubated at 37°C for the periods indicated in the text and then diluted to the required concentration.

### Transmission Electron Microscopy

An aliquot (2  $\mu$ l) of the peptide gels or solution was placed on a carbon-coated Formvar 200 mesh nickel grid. The sample was allowed to stand for 15–30 s, and then any excess solution was removed by blotting. The samples were negatively stained with 2% (w/v) uranyl acetate and allowed to dry. The samples were then visualized under a Hitachi H-7000 transmission electron microscope operating at 75 kV. The magnification ranged from  $\times 12,000$  to  $\times 60,000$ .

### Circular Dichroism Analysis of Amyloidogenic Peptides

Aged preparations of peptides were dissolved in 20 mM Tris-HCl buffer (pH 7.4) or 50% methanol (MeOH)/20 mM Tris-HCl buffer, and circular dichroism (CD) spectra (average of ten scans) were collected from samples (2 ml) at 0.5 nm intervals between wavelengths of 200 and 400 nm using a Jasco model J-720 spectropolarimeter (Tokyo, Japan). Samples were incubated at room temperature and a baseline spectrum was subtracted from the collected data.

### Congo Red Binding Assay

Congo Red-reactive fibrils were measured as described previously (22). Aged preparations of peptides were adjusted to a concentration of 1 mg/ml, and 40  $\mu$ l of each dilution was added to 960  $\mu$ l of 25  $\mu$ M Congo Red in 20 mM potassium phosphate buffer (PBS, pH 7.4) containing 150 mM NaCl. After a 30-min incubation, absorbance was read at 540 and 477 nm, and the concentration of bound Congo Red ( $c_b$ ) was calculated from the equation,  $c_b = (A_{540}/25,295 - A_{477}/46,306)$ .

### Thioflavin T Binding Assay

Formation of peptide fibrils was fluorimetrically quantified by thioflavin T (ThT) binding (23). An aliquot of 20  $\mu$ l of each aged preparation was added to 1980  $\mu$ l of 5  $\mu$ M ThT in 20 mM PBS (pH 6.0) containing 150 mM NaCl. Fluorescence was immediately measured on an RF-5000 spectrofluorophotometer (Shimadzu, Tokyo, Japan) with excitation and emission maxima of 450 and 482 nm, respectively.

### Turbidity

Glucagon was aged at concentrations of 1.0, 2.5, or 5.0 mg/ml for 24 h in 0.01 M HCl. For turbidity analysis, samples aged at the concentrations of 2.5 and 5.0 mg/ml were diluted to a final concentration of 1 mg/ml with 0.01 M HCl. An aliquot of 200  $\mu$ l of each aged sample as well as non-aged glucagon (1.0 mg/ml) was transferred into a 96-well plate and turbidity (OD) was measured at 405 nm with a microplate reader (Bio-Tek, Winooski, VT, USA).

### Size-Exclusion Chromatography

Aged preparations of peptides were fractionated on a Zorbax GF-250 column (Agilent Technologies, Palo Alto, CA, USA) at 25°C using a Shimadzu LC-10A HPLC system. The column was equilibrated with the mobile phase (20 mM citrate buffer containing 130 mM NaCl, pH 3.0), and peptides were eluted under constant flow at 1.0 ml/min and monitored at 220 nm. The column was calibrated with blue dextran (2,000,000 Da) and a series of molecular weight protein standards including, sweet potato  $\beta$ -amylase (200,000 Da), human serum albumin (66,500 Da), chicken albumin (45,000 Da), human growth hormone (22,125 Da), porcine insulin (5,777 Da), and buserelin (1239 Da).

### Cell Cultures

Rat pheochromocytoma (PC12) cells were obtained from the RIKEN Cell Bank (Ibaraki, Japan). PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 5% (v/v) horse serum (HS, Gibco-BRL, Grand Island, NY, USA) and 5% (v/v) newborn calf serum (CS, Gibco-BRL) as described previously (24). NIH-3T3 cells were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supple-

mented with 10% CS. Cells were maintained in 5% CO<sub>2</sub>/95% humidified air at 37°C.

### LDH and WST-8 Assay

Cells were seeded at  $1 \times 10^4$  cells/well in 96-well plates coated with type I collagen (Becton Dickinson Labware, Bedford, MA, USA) at least 24 h before the experiment and cultured in serum-free DMEM supplemented with 2  $\mu$ M insulin. For preparation of peptide fibrils, therapeutic peptides, glucagon and salmon calcitonin, were incubated for 24 h at 5.0 mg/ml and at 60 mg/ml, respectively, and their fibril production was assessed by the Congo Red binding assay. Each aged or nontreated peptide was diluted and added to the cell culture at the indicated final concentrations. The degree of cell death was assessed by measurement of the activity of lactate dehydrogenase (LDH) released from the dead cells as reported previously (20). LDH activity in the culture medium was determined using a commercially available kit (Wako, Osaka, Japan) according to the manufacturer's protocol. In addition to LDH measurement in the medium, cell mortality was also assayed by WST-8 conversion (25). A volume of 10  $\mu$ l of WST-8 (5 mM WST-8, 0.2 mM 1-methoxy-5-methylphenazinium methylsulfate, and 150 mM NaCl) was added to each well and the reaction was continued for 4 h at 37°C. The absorbance of the sample at 450 nm was measured using a microplate reader (Bio-Tek) with a reference wavelength of 720 nm.

### Caspase-3-like Activity

Caspase-3-like activity in culture was measured using an Apo-ONE Homogeneous Caspase-3/7 Assay Kit (Promega) according to the manufacturer's instructions. Briefly, cells ( $5 \times 10^4$  cells/well) in type I collagen-coated 96-well plates (Becton Dickinson Labware) were rinsed twice with PBS. The cultures were incubated with or without the indicated stimulators in DMEM (50  $\mu$ l) at 37°C in an atmosphere of 95% air and 5% CO<sub>2</sub>. The cells were lysed in 50  $\mu$ l of Homogeneous Caspase-3/7 Buffer containing the caspase-3 substrate Z-DEVD-Rhodamine 110, and the cell lysates were incubated for 12 h at room temperature. After incubation, the fluorescence (excitation 480 nm and emission 535 nm) of the cell lysates (50  $\mu$ l) was measured with a GEMINIxs spectrofluorophotometer (Molecular Devices, Kobe, Japan).

### Statistical Analysis

Statistical evaluation was performed by the Student's *t* test or one-way analysis of variance (ANOVA) along with pairwise comparison by the Fisher's least significant difference procedure. *p* values less than 0.05 were considered to be significant in all analyses.

## RESULTS

### CD Spectral Analyses on Aged Peptides

CD is displayed when an optically active substance preferentially absorbs left or right-handed circularly polarized light and it provides useful information when conformational alterations occur in peptides/proteins (26). The CD spectrum of a  $\beta$ -sheet structure shows an intense positive band at 198

nm and a negative extremal band at 218 nm (27), and the CD spectrum of  $\alpha$ -helical structure shows an intense positive peak at 192 nm and two negative peaks at 209 nm and 222 nm (26). The well-known amyloidogenic peptides, PrP<sub>106–126</sub> and A $\beta$ <sub>1–42</sub>, gave typical CD spectra for the presence of a  $\beta$ -sheet structure after aging under both hydrophilic (20 mM Tris-HCl buffer, pH 7.4) and hydrophobic (50% MeOH/20 mM Tris-HCl buffer, pH 7.4) conditions (Figs. 1D and 1E). When glucagon was dissolved in the hydrophilic buffer at 10 mg/ml, non-aged glucagon gave the characteristic CD spectrum of a random coil conformation (Figs. 1A-I). However, when glucagon was aged for 24 h, a transition from random coil to  $\beta$ -sheet structure was observed for preparations dissolved at 5 mg/ml or higher. In the hydrophobic condition, the CD spectra of non-aged glucagon exhibited a typical  $\alpha$ -helical structure (Fig. 1A-II), the content of which was estimated to be 55% according to the calculation established by Greenfield&Fasman (26). When aged at 1 mg/ml for 24 h, the  $\alpha$ -helical content of glucagon was not changed, whereas aging at 2.5 mg/ml or higher resulted in a significant decrease in the  $\alpha$ -helical content to ca. 1%.

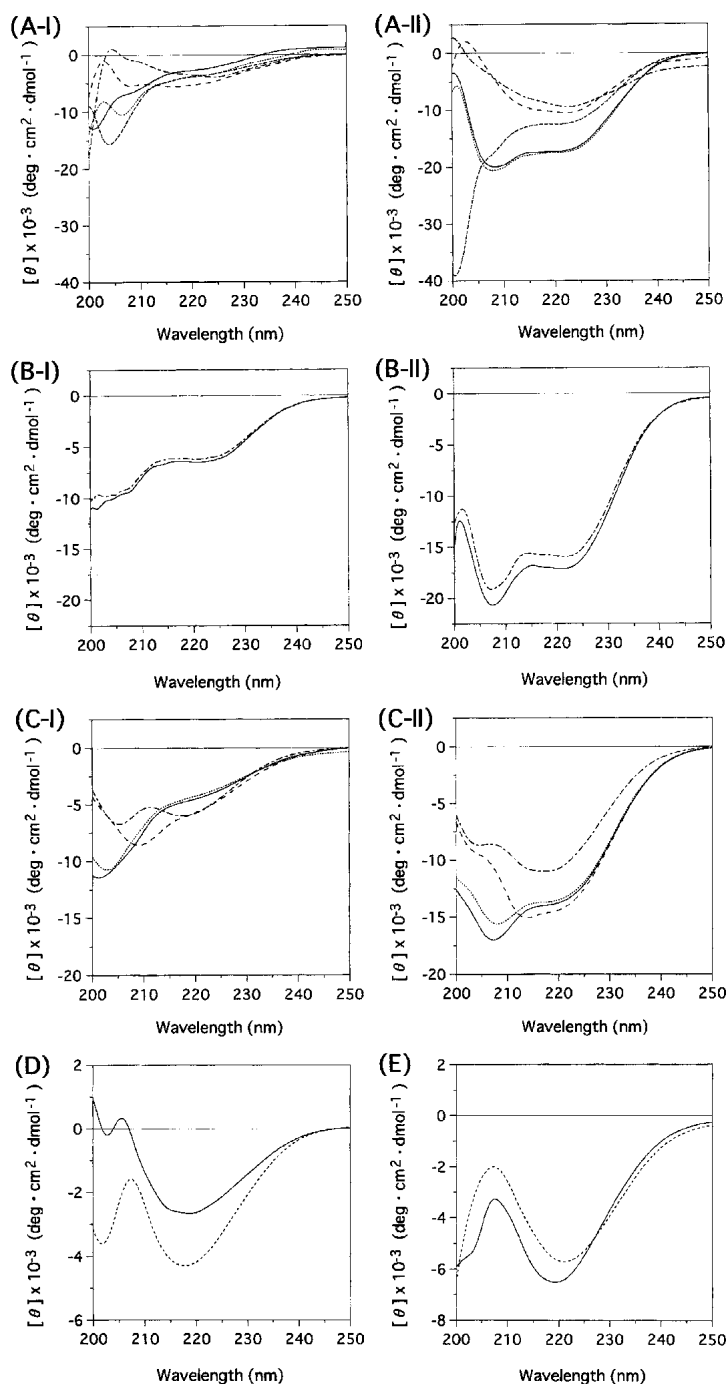
We also tested the effect of aging and concentration on the conformational change for other therapeutic peptides, porcine secretin and salmon calcitonin. Porcine secretin has high sequential homology to glucagon and the CD spectra of both non-aged peptides exhibited high similarity (Figs. 1A and 1B). On the contrary to glucagon, the CD spectrum of aged secretin at the concentration of 75 mg/ml was almost identical to that of non-aged secretin (Figs. 1B-I and 1B-II), suggesting no conformational changes to peptide aggregate of  $\beta$ -sheet-rich structures. Salmon calcitonin, without aging at concentrations as high as 75 mg/ml or with aging at a low concentration of 1 mg/ml, showed the presence of a random coil structure in the hydrophilic environment (Fig. 1C-I) and a typical  $\alpha$ -helical structure in the hydrophobic environment (Fig. 1C-II). However, as in the case of glucagon, a conformational transition from an  $\alpha$ -helical structure to  $\beta$ -sheet structure was observed after aging at high concentrations of 50 and 75 mg/ml (Fig. 1C). It was thus shown that salmon calcitonin requires about a 10-fold higher concentration than glucagon for structural changes to occur, suggesting that glucagon has a high propensity to yield  $\beta$ -sheet structure.

### Electron Microscopic Studies on Peptide Aggregates

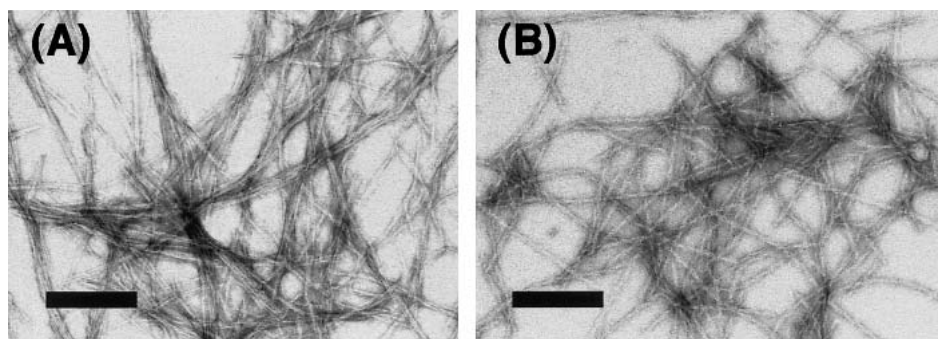
Initially, human glucagon was incubated at a concentration of 10 mg/ml in 0.01 M HCl for 24 h at 37°C and diluted to a final concentration of 1.0 mg/ml prior to applying on transmission electron microscopy (TEM). TEM showed well-defined fibrils (Fig. 2A), which morphologically resembled the classic amyloid fibrils such as A $\beta$ <sub>1–42</sub> and PrP<sub>106–126</sub> (28,29). The fibril morphologies included largely disordered, rigid, and branching fibrils stacked together edge to edge with a width of 10–50 nm and various lengths. Similar fibril structures were observed for salmon calcitonin aged at the concentration of 60 mg/ml for 24 h (Fig. 2B).

### Physicochemical Properties of Peptide Aggregates

TEM analysis and CD spectral analyses on aged and concentrated glucagon and salmon calcitonin revealed the presence of amyloidogenic fibrils and conformational alterations



**Fig. 1.** CD spectra representative of glucagon, salmon calcitonin, PrP<sub>106-126</sub>, and A $\beta$ <sub>1-42</sub>. (A) CD spectra of non-aged or aged glucagon in 20 mM Tris-HCl buffer, pH 7.4 (A-I) or 50% MeOH/20 mM Tris-HCl buffer, pH 7.4 (A-II). Solid line, non-aged glucagon; dotted line, glucagon aged at 1 mg/ml; dashed line, aged at 2.5 mg/ml; broken line, aged at 5 mg/ml; and chain line, aged at 10 mg/ml. (B) CD spectra of non-aged or aged secretin in 20mM Tris-HCl buffer, pH 7.4 (B-I) or 50% MeOH/20 mM Tris-HCl buffer, pH 7.4 (B-II). Solid line, non-aged porcine secretin; and chain line, aged at 75 mg/ml. (C) CD spectra of non-aged or aged salmon calcitonin in 20mM Tris-HCl buffer, pH 7.4 (C-I) or 50% MeOH/20 mM Tris-HCl buffer, pH 7.4 (C-II). Solid line, non-aged salmon calcitonin; dotted line, salmon calcitonin aged at 1 mg/ml; dashed line, aged at 50 mg/ml; and chain line, aged at 75 mg/ml. (D) CD spectra of non-aged or aged PrP<sub>106-126</sub> in 20 mM Tris-HCl buffer, pH 7.4 (solid line) or 50% MeOH/20 mM Tris-HCl buffer, pH 7.4 (dashed line). (E) CD spectra of normal or aged A $\beta$ <sub>1-42</sub> in 20 mM Tris-HCl buffer, pH 7.4 (solid line) or 50% MeOH/20 mM Tris-HCl buffer, pH 7.4 (dashed line).



**Fig. 2.** Electron micrographs of negatively stained fibrils formed by aged (A) glucagon and (B) salmon calcitonin. Fibrils of glucagon and salmon calcitonin were prepared by aging at the concentration of 10 and 60 mg/ml, respectively, in 0.01 M HCl for 24 h. Prior to TEM analysis, the samples were diluted to the final concentration of 1 mg/ml. The bars represent 0.2  $\mu\text{m}$ .

to the  $\beta$ -sheet structures, respectively. Here, in order to put these observations together, we performed physicochemical analyses including the binding assay using Congo Red and ThT, and turbidity assays. Congo Red selectively binds to amyloid-like aggregates having a  $\beta$ -sheet rich conformation, and is used to detect amyloid fibrils in pathological specimens (30). When glucagon was incubated at various concentrations ranging from 1 to 10 mg/ml at 37°C, bathochromic effect was observed upon staining with Congo Red, indicating the formation of aggregate at 5 mg/ml or higher (Fig. 3A). The rate of aggregation was in a time-dependent manner; it reached the maximal level approximately 6 min after aging at 7.5 and 10 mg/ml and 25 min at 5 mg/ml. Glucagon at 1 and 2.5 mg/ml did not show any signs of fibrillar formation within 24 h aging.

In addition to Congo Red, ThT has also been used commonly as an amyloid-binding dye for *in vitro* studies (23), and this fluorochrome is considered to be a potential pharmacophore for further design of amyloid-imaging agents. ThT binding activities were observed in the glucagon preparations aged at 2.5 and 5 mg/ml for 24 h, and their fluorescence intensities at each concentration were 5-fold and 20-fold higher than those of glucagon with or without aging at 1 mg/ml, respectively (Fig. 3B). There was also a significant increase in the turbidity 24 h in the glucagon preparation aged at 2.5 and 5 mg/ml for 24 h, whereas the turbidity of the preparation aged at 1 mg/ml was almost equal to that of the non-aged preparation at 1 mg/ml (Fig. 3C). Hence, these physicochemical analyses indicated that aging at higher concentrations (>5 mg/ml) accelerated the misfolding of glucagon, producing amyloidogenic fibrils readily.

Surprisingly, when glucagon was aged at 1 mg/ml for 30 days at room temperature, the presence of a  $\beta$ -sheet structure was detected by both Congo Red and ThT binding analyses (data not shown). It should also be pointed out that a concomitant formation of a fibrillar aggregate was observed in TEM analysis (data not shown).

The physicochemical properties on salmon calcitonin are summarized in Table I. Peptide aggregation was indicated by the increases in turbidity, Congo Red and ThT bindings after 24 h aging. The peptide concentration requisite for aggregation was 40 mg/ml in turbidity and ThT binding analyses and 60 mg/ml in Congo Red binding analysis, which was almost consistent with the result of the CD study.

### Size-Exclusion Chromatography of Glucagon Aggregate

We performed size exclusion chromatography (SEC) to check the size and molecular pattern of the glucagon aggregate. Each glucagon preparation aged for 0, 1, 3, and 6 h was applied to a SEC column (ZORBAX Bio Series GF-250) at 7.5 mg/ml. The SEC analysis performed with 20 mM sodium phosphate buffer containing 130 mM NaCl (pH 7.4) revealed that there was only one peak at 3.5 kDa corresponding to a molecular weight of monomeric glucagon even with the preparations of glucagon aggregate or non-aged glucagon, suggesting that the aggregated glucagon was immediately disassembled on SEC. On the other hand, when 20 mM citrate-HCl buffer containing 130 mM NaCl (pH 3.0) was used as the mobile phase, a main peak appeared at 2000 kDa for the glucagon aggregate (Fig. 4A), suggesting that glucagon fibril was much stable in the acidic condition as compared to the neutral condition. During the time course from the non-aging state to 6 h aging, the peak at 3.5 kDa of monomeric glucagon was shifted to generate a peak at 2000 kDa, suggesting that intermolecular association is likely to be involved in the generation of the glucagon aggregate.

Under the acidic condition, the retention times for molecular weight standards and non-aged glucagon were well associated with each molecular weight (MW) in the range from 1.2 kDa to 2000 kDa. The correlation coefficient between retention time and Mw was estimated to be  $r^2 = 0.95$  ( $p < 0.01$ ) according to the  $\log(\text{MW})$  vs. elution time standard curve (Fig. 4B).

### Inhibition of Glucagon Antiserum on the Formation of Glucagon Aggregate

The antisera raised against A $\beta_{1-42}$ , PrP<sub>106-126</sub>, and polyglutamine have the ability to suppress their respective aggregations *in vitro* and *in vivo* (29,31,32). Thus, we examined the effect of antiserum against glucagon on the formation of glucagon fibrils. According to X-ray analysis, glucagon adopts a mainly  $\alpha$ -helical conformation in the amino acid sequence between positions 10 and 25, which is stabilized by the hydrophobic interactions between glucagon molecules related by 3-fold symmetry (33). The glucagon antiserum used recognized the entire glucagon molecule but not a fragment of glucagon... (data not shown), indicating that the  $\alpha$ -helical

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