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Research Paper

Stabilizing Peptide Fusion for Solving the Stability and Solubility Problems of Therapeutic Proteins

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Purpose. Protein aggregation is a major stability problem of therapeutic proteins. We investigated whether a novel stabilizing peptide [acidic tail of synuclein (ATS) peptide] could be generally used to make a more stable and soluble form of therapeutic proteins, particularly those having solubility or aggregation problems.
Methods. We produced ATS fusion proteins by fusing the stabilizing peptide to three representative therapeutic proteins, and then compared the stress-induced aggregation profiles, thermostability, and solubility of them. We also compared the *in vivo* stability of these ATS fusion proteins by studying their pharmacokinetics in rats.
Results. The human growth hormone–ATS (hGH–ATS) and granulocyte colony-stimulating factor–ATS (G-CSF–ATS) fusion proteins were fully functional as determined by cell proliferation assay, and the ATS fusion proteins seemed to be very resistant to agitation, freeze/thaw, and heat stresses. The introduction of the ATS peptide significantly increased the storage and thermal stabilities of hGH and G-CSF. The human leptin–ATS fusion protein also seemed to be very resistant to aggregation induced by agitation, freeze/thaw, and heat stresses. Furthermore, the ATS peptide greatly increased the solubility of the fusion proteins. Finally, pharmacokinetic studies in rats revealed that the ATS fusion proteins are also more stable *in vivo*.
Conclusion. Our data demonstrate that a more stable and soluble form of therapeutic proteins can be produced by fusing the ATS peptide.

KEY WORDS: protein aggregation; protein solubility; protein stability; stabilizing peptide; therapeutic proteins.

INTRODUCTION

Aggregation of proteins, which is encountered routinely during the preparation, sterilization, shipping, and storage processes, is a major problem, particularly in the biomedical and biopharmaceutical fields [reviewed in Refs. (1–3)]. In addition to eliminating or reducing a protein's solubility, therapeutic efficacy, and shelf life, aggregates of therapeutic proteins can cause an unexpected immune response, hypersensitization, or even anaphylactic shock in patients who have had aggregated proteins administrated parenterally (4–8). Therefore, it is very important to completely prevent aggregate formation of therapeutic proteins during all stages of product handling. For this purpose, therapeutic proteins are usually protected against potential stresses by the addition of proper excipients or additives (1,3,9–15). In addition, chemical modification or site-directed mutagenesis is often adopted to solve the aggregation problem of each therapeutic protein (16,17). However, both finding a proper formulation for the stabilization of therapeutic proteins and creating a more stable form by chemical modification or site-directed mutagenesis are usually time consuming because the processes are based on the trial and error method for each protein. Moreover, the outcomes are not always satisfactory.

We have previously demonstrated that the introduction of peptides derived from the C-terminal acidic tail of synuclein (ATS) into a heat-labile protein, glutathione S-transferase (GST), protects the fusion proteins from heat-, pH-, and metal-induced protein aggregation (18,19). In this study, we investigated whether one of those stabilizing peptides (ATS peptide hereafter) could be generally used to make a more stable and soluble form of therapeutic

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ABBREVIATIONS: ATS, acidic tail of synuclein; CD, circular dichroism; G-CSF, granulocyte colony-stimulating factor; GST, glutathione S-transferase; hGH, human growth hormone; Tm, melting temperature; Tu, temperature for the onset of unfolding.

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proteins, particularly those having solubility or aggregation problems. For these purposes, we produced ATS fusion proteins by fusing the stabilizing peptide to three representative therapeutic proteins, human growth hormone (hGH), granulocyte colony-stimulating factor (G-CSF), and human leptin (hLeptin), and then compared the stress-induced aggregation profiles, thermostability, and solubility of these fusion proteins. We also compared the *in vivo* stability of these ATS fusion proteins by studying their pharmacokinetics in rats. Our data demonstrate that many therapeutic proteins can be greatly stabilized by simply fusing the ATS peptide.

MATERIALS AND METHODS

Preparation of hGH, hGH–ATS, and ATS–hGH Proteins

A hGH expression vector was constructed by subcloning the hGH gene into the pRSETA vector (Invitrogen, San Diego, CA, USA). The protein coding region of hGH was amplified by PCR with the 5′-oligonucleotide primer *GCGCTCGAGCC**CATATG**TTCCCAACTA TACCA* containing the underlined *Nde*I restriction site and the 3′-oligonucleotide primer *GCGCAAGCTT**AAGCTT** TTAGAAGCCACAGCTGCC* containing the underlined *Hin*dIII restriction site, respectively. The amplified DNAs were gel purified, digested with *Nde*I and *Hin*dIII, and ligated into the pRSETA vector that had been digested with the appropriate restriction enzymes and gel purified.

Human growth hormone–ATS and ATS–hGH fusion constructs were produced by consecutively subcloning the protein coding regions of hGH and the C-terminal part of the acidic tail (ATS, amino acids 119–140) into the pRSETA vector. The amino acid sequence of the ATS peptide is DPDNEAYEMPSEEGYQDYEPEA. Briefly, the DNAs encoding the ATS were chemically synthesized, as described previously (19). Using these synthetic cDNAs, ATS fusion constructs were first generated by ligating the ATS gene into the pRSETA vector using the *Nde*I and *Bam*HI restriction sites for N-terminal fusion (pATS-N) and the *Bam*HI and *Hin*dIII restriction sites for C-terminal fusion (pATS-C), respectively. The ATS–hGH fusion construct was generated by subcloning the protein coding region of hGH into the pATS-N vector using the *Bam*HI and *Hin*dIII restriction sites. The hGH–ATS fusion construct was generated by subcloning the protein coding region of hGH into the pATS-C vector using the *Nde*I and *Bam*HI restriction sites. All constructs were verified by DNA sequencing.

The recombinant plasmids were transformed into the *Escherichia coli* strain BL21 (DE3) pLysS for expression. Inclusion body proteins of hGH, hGH–ATS, and ATS–hGH were purified and refolded as previously described (20,21). After refolding, hGH, hGH–ATS, and ATS–hGH proteins were purified by ion-exchange and gel-filtration chromatography.

Preparation of Other ATS Fusion Proteins

Granulocyte colony-stimulating factor and G-CSF–ATS expression vectors were similarly constructed as in the case of hGH. Recombinant plasmids were transformed into the *E. coli* strain BL21 (DE3) pLysS for expression. Inclusion body proteins of G-CSF and G-CSF–ATS were purified and refolded as previously described (41). After refolding, G-CSF and G-CSF–ATS proteins were purified by ion-exchange and gel-filtration chromatography.

Human leptin and hLeptin–ATS expression vectors were also similarly constructed as in the case of hGH. The recombinant plasmids were expressed in *E. coli* as inclusion bodies, and the inclusion body proteins were purified as previously described (20). The inclusion body proteins were refolded as previously described (42). After refolding, hLeptin and hLeptin–ATS proteins were purified by ion-exchange and gel-filtration chromatography.

Agitation-Induced Protein Aggregation Assay

Agitation-induced aggregations of wild-type and ATS fusion proteins that were continuously shaken were examined over time. One milliliter of each protein at 1 mg/ml in phosphate-buffered saline (PBS; pH 7.4) was filtered through a 0.2-μm syringe filter to remove any clumps that could act as a nucleation center for protein aggregation. The proteins were then incubated at room temperature with continuous shaking at 150 rpm on an orbital shaker (Superteck, Seourin Science Inc.). The extent of aggregation at each time point was examined by measuring the turbidity at 405 nm.

Freeze/Thawing-Induced Protein Aggregation Assay

Wild-type and ATS fusion protein solutions were prepared at 1.0 mg/ml in PBS (pH 7.4), and the freeze/thawing-induced aggregation of these protein samples was assessed by repeated cycles of freezing in liquid nitrogen and thawing in a 37°C water bath. The extent of aggregation was examined by monitoring the absorbance at 405 nm every five cycles of freeze/thawing. Freeze/drying (lyophilization)-induced aggregation was similarly assessed after one cycle of freezing and drying.

Heat-Induced Protein Aggregation Assay

The heat-induced aggregation of wild-type and ATS fusion proteins was qualitatively assayed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) after heat treating the protein samples as previously described (18,19). The level of heat-induced aggregation of hGH, hGH–ATS, and ATS–hGH proteins was also quantitatively measured by monitoring the apparent absorbance at 405 nm as a function of time at 80°C (18,19).

Circular Dichroism Measurements

The circular dichroism (CD) spectra were recorded on a Jasco-J810 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a temperature control system in a continuous mode as previously described (18,43). Thermal denaturation experiments were performed using a heating rate of 1°C/min and a response time of 1 s. The thermal scan data were collected from 25 to 95°C in 0.1-cm path length cuvettes with a protein concentration of 0.5 mg/ml. The CD spectra were measured every 0.5°C at a wavelength of 222 nm.

Determination of Protein Solubility

Each protein sample was concentrated using a Centriprep and Centricon concentrator (Amicon, Beverley, MA, USA), and the protein concentration was determined by Bradford's dye binding method (44). Solubility of each protein was determined by repeating the centrifugation and protein quantification cycles until the protein sample was no longer concentrated.

Biological Activity of hGH, hGH–ATS, and ATS–hGH Fusion Proteins

Nb2-11 rat lymphoma cells (26) were obtained from the European Collection of Cell Cultures, and they were maintained and manipulated as recommended. Biological activities of hGH, hGH–ATS, and ATS–hGH proteins were measured as reported previously (25,27). Briefly, hGH stocks were diluted in assay media consisting of RPMI 1640, 2 mM mercaptoethanol, 50 U/ml penicillin, 50 μg/ml streptomycin, 2×10^{-3} M L-glutamine, and 10% horse serum. This mixture was then added to triplicate wells (96-well plate, Costar, Cambridge, MA, USA), each of which contained 2×10^4 Nb2 cells/well in the same assay media. The final volume was 100 μl, with various hGH concentrations. The cells were incubated at 37°C in a 5% CO_2 and 95% air atmosphere for 48 h. Their reducing activity was then assayed by adding 20 μl of MTS solution (Promega, Madison, WI, USA).

Granulocyte Colony-Forming Unit Assay

Granulocyte colony formation by murine bone marrow cells was performed as described previously (45). Briefly, 5×10^4 bone marrow cells per 0.5 ml of RPMI 1640 containing 20% fetal bovine serum and different amounts of G-CSF or G-CSF–ATS proteins were seeded into 0.5 ml of 0.6% low melting agar (Sigma, St. Louis, MO, USA) on a 24-well microplate (Nunc, Roskidle, Denmark). After incubating for 7 days at 37°C in a 5% CO_2 and 95% air atmosphere, colonies of more than 40 cells were enumerated on a microscope.

Pharmacokinetic Studies

Male Sprague–Dawley rats were administered with a single subcutaneous injection of hGH (96 mg/kg) or hGH–ATS (110 mg/kg), and the resulting plasma concentrations were determined as a function of time (46). Blood samples were collected at 0 (predose), 30 min, 1 hr, and every subsequent hour after injection in ethylenediaminetetraacetic acid-containing microtubes and mixed with an equal volume of PBS. All samples were immediately placed on ice and centrifuged at room temperature. The resultant plasma samples were then frozen and stored at −20°C. Plasma concentrations were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Roche, Lewes, UK).

Pharmacokinetic studies of G-CSF and G-CSF–ATS were performed similarly as in the case of hGH (31). Plasma concentrations were determined at 0, 30 min, 1 hr, 2 hr, and every 2 hr after injection using a commercial ELISA kit (IBL Co., Gumma, Japan).

RESULTS

Preparation and Characterization of hGH, hGH–ATS, and ATS–hGH Proteins

Human growth hormone, hGH–ATS (hGH fused with the ATS peptide at the C-terminus), and ATS–hGH (hGH fused with the ATS peptide at the N-terminus) proteins (Fig. 1a) were all overexpressed in *E. coli* as insoluble protein aggregates. The inclusion body proteins were isolated by a method previously described (20) and refolded by an alkaline method (21). The purified proteins were analyzed on a SDS–polyacrylamide gel (Fig. 1b). Consistent with previous reports (21,22), purified hGH appeared as a 22-kDa band on the SDS gel, whereas the purified hGH–ATS and ATS–hGH fusion proteins both appeared as a 24-kDa band (Fig. 1b). To obtain the secondary structural features of hGH, hGH–ATS, and ATS–hGH proteins, CD spectra were measured with a spectropolarimeter. Consistent with previous reports (17,23–25), the CD spectrum of hGH at 25°C shows the 208- and 222-nm absorption bands characteristic of α-helical proteins (Fig. 1c, solid line). The CD spectra of hGH–ATS (Fig. 1c, dashed line) and ATS–hGH fusion proteins (Fig. 1c, dotted line) seem to be very similar in shape to that of wild-type hGH, indicating that the fusion proteins are correctly folded.

Biological activities of hGH, hGH–ATS, and ATS–hGH proteins were determined by Nb2 cell proliferation assay (25–27). As shown in Fig. 1d, the Nb2 cell proliferative activities of both hGH–ATS and ATS–hGH fusion proteins were comparable to that of wild-type hGH. The hGH–ATS and ATS–hGH fusion proteins also seemed to effectively phosphorylate STAT-5 in Nb2 cells (data not shown). These results indicate that the hGH–ATS and ATS–hGH fusion proteins are fully functional.

Agitation-, Freeze/Thawing-, and Freeze/Drying-Induced Aggregation of hGH, hGH–ATS, and ATS–hGH Proteins

Therapeutic proteins are generally exposed to various types of physical, chemical, and thermal stresses during the preparation, storage, shipping, and handling processes. To investigate the effects of the ATS peptide on the stability of fusion proteins against various types of stresses, we first compared the agitation-induced aggregation profiles of hGH, hGH–ATS, and ATS–hGH proteins. Agitation is a kind of stress that occurs at the air–solution interface during the preparation, shipping, and handling steps of therapeutic proteins. As shown in Fig. 2a, hGH–ATS and ATS–hGH fusion proteins did not significantly aggregate even after a 90-h agitation, whereas the hGH aggregated rapidly within a few hours. Consequently, the biological activity of hGH after agitation greatly decreased, whereas those of hGH–ATS and ATS–hGH fusion proteins were not significantly changed (Fig. 2b).

We next compared the stability of hGH, hGH–ATS, and ATS–hGH proteins against freeze/thawing stress. As shown in Fig. 3a, wild-type hGH rapidly aggregated as the number of freeze/thawing cycles increased. Unlike the wild-type hGH, however, hGH–ATS and ATS–hGH fusion proteins were very resistant to freeze/thawing stress (Fig. 3a). Consequently, the biological activity of hGH after freeze/

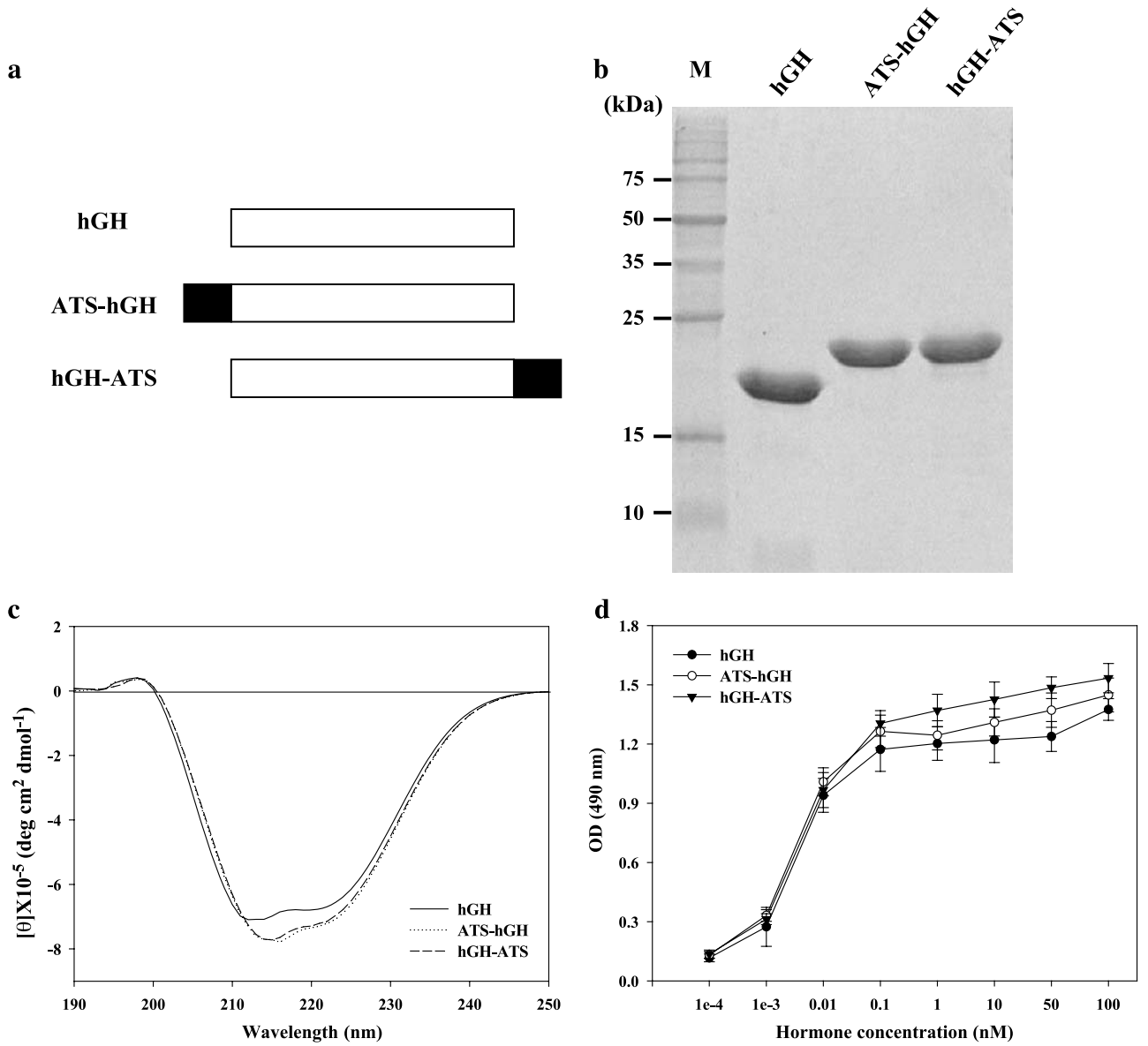

Fig. 1. Preparation of recombinant human growth hormone (hGH), hGH–acidic tail of synuclein (ATS), and ATS–hGH. (a) Schematic diagram of the hGH, hGH–ATS, and ATS–hGH constructs. hGH is denoted by the white bar and the ATS peptide by the black bar. The amino acid sequence of the ATS peptide is DPDNEAYEMPSEEGYQDYEPEA. Methionine (M) is at the N-terminus, and GS is inserted at the junction (*Bam*HI restriction site). (b) Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) analysis of the purified hGH, hGH–ATS, and ATS–hGH proteins. The purified proteins were analyzed on a 12% SDS–polyacrylamide gel, and the protein bands were stained with Coomassie Brilliant Blue R250. (c) Far-ultraviolet circular dichroism (CD) spectra of hGH, hGH–ATS, and ATS–hGH. Far-UV CD spectra of hGH, hGH–ATS, and ATS–hGH proteins measured at room temperature are drawn as a solid line, dashed line, and dotted line, respectively. (d) Biological activity of hGH, hGH–ATS, and ATS–hGH proteins were measured by Nb2 cell proliferation assay. The biological activity assay was performed as described in Materials and Methods. The results are expressed as optical densities at 490 nm, and they are the means ± SD obtained from triplicate wells. All preparations were tested on the same microtiter plate.

thawing cycles was almost completely abolished, whereas those of hGH–ATS and ATS–hGH fusion proteins decreased much less (Fig. 3b).

We also compared the stability of hGH, hGH–ATS, and ATS–hGH proteins against freeze/drying stress. As shown in Fig. 3c, hGH–ATS and ATS–hGH fusion proteins did not aggregate after one cycle of freezing, drying, and resuspending in PBS buffer, whereas significant part of hGH aggregated. Protein quantification analysis indicated that about 10% of wild-type hGH became insoluble in PBS buffer after one cycle of freezing and drying, whereas most of hGH–ATS and ATS–hGH fusion proteins seemed to be soluble (Fig. 3d). These results clearly indicate that the introduction of the ATS peptide into hGH protects the fusion proteins from agitation-, freeze/thawing-, and freeze-drying-induced aggregation and inactivation.

Thermostability of hGH, hGH–ATS, and ATS–hGH Proteins

We next investigated whether hGH–ATS and ATS–hGH fusion proteins are resistant to heat-induced aggregation

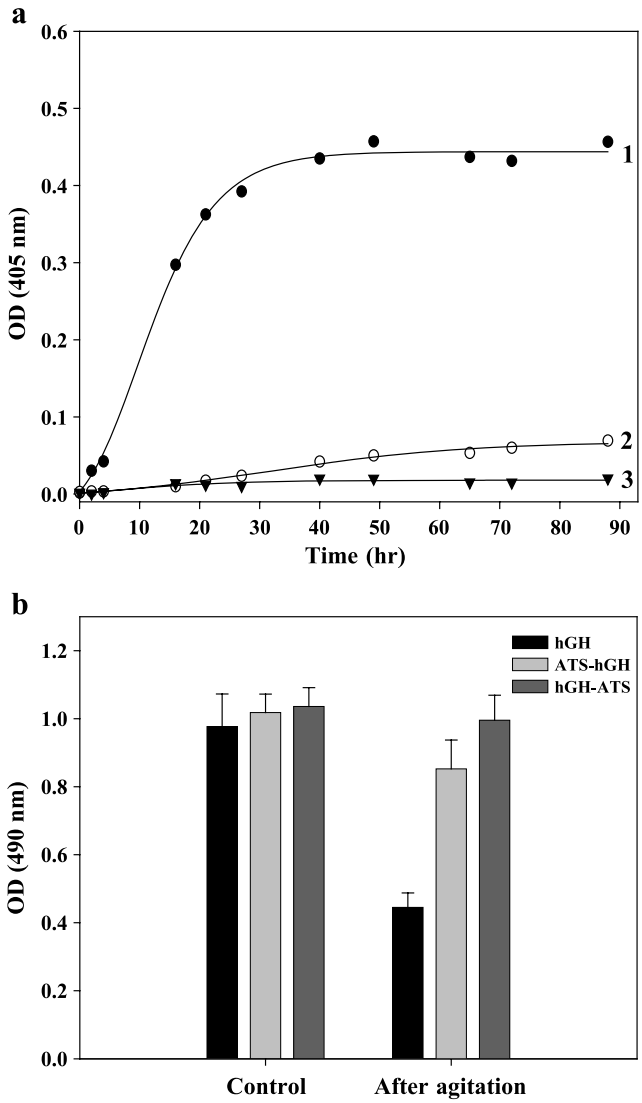

Fig. 2. Agitation-induced aggregation profiles of hGH, hGH–ATS, and ATS–hGH. (a) Agitation-induced aggregation kinetics of hGH, hGH–ATS, and ATS–hGH proteins. Agitation-induced aggregation was quantitatively assessed by monitoring light scattering (turbidity) at 405 nm as a function of agitation time at room temperature. Graphs: 1, hGH; 2, hGH–ATS; and 3, ATS–hGH. (b) Biological activity of hGH, hGH–ATS, and ATS–hGH samples after 90-h agitation. Biological activity was measured as in Fig. 1d.

(Fig. 4). For this purpose, hGH–ATS and ATS–hGH fusion proteins were first qualitatively examined for heat resistance by SDS-PAGE after heat treatment as described previously (18). As shown in Fig. 4a, hGH–ATS and ATS–hGH fusion proteins did not precipitate at all after heat treatment at 80 or 100°C for 10 min, whereas the hGH completely precipitated under these conditions. Subsequently, the thermal behaviors of the hGH–ATS and ATS–hGH fusion proteins were quantitatively compared by monitoring their absorbance at 405 nm over time, while setting the concentration of each protein at 0.5 mg/ml at 80°C. In this experiment, as shown in Fig. 4b, the hGH protein had almost completely aggregated after 2–3 min. In contrast, the hGH–ATS and ATS–hGH fusion proteins did not aggregate at all after 10 min of heat treatment. These results indicate that the ATS peptide is also capable of providing heat resistance and thermosolubility to hGH.

The secondary structural changes of hGH, hGH–ATS, and ATS–hGH proteins induced by the increase of temperature were next investigated by CD spectroscopy. To specifically compare the thermal stabilities of these proteins, the thermal unfolding of each protein was monitored at 222 nm as a function of temperature (Fig. 4c). Consistent with previous reports (16,17), the temperature-induced unfolding of hGH started at around 78°C with a Tm of 80°C being noted (Fig. 4c, solid line). Unlike wild-type hGH, the temperature-induced unfolding of hGH–ATS and ATS–hGH fusion proteins took place at much higher temperatures (Fig. 4c, dashed line and dotted line, respectively). The temperature-

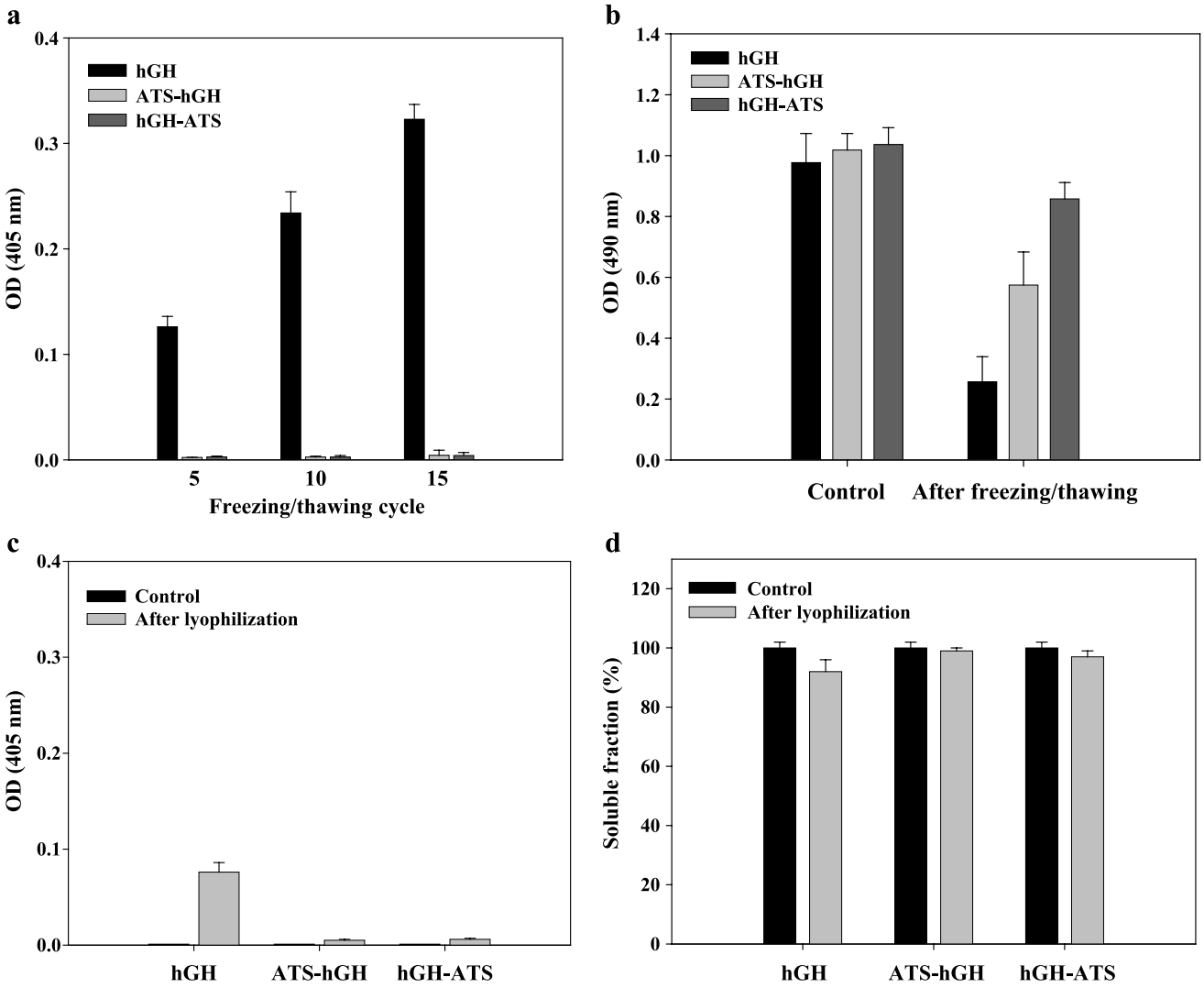

Fig. 3. Freeze/thawing- and freeze/drying-induced aggregation profiles of hGH, hGH–ATS, and ATS–hGH. (a) Freeze/thawing-induced aggregation was quantitatively assessed by monitoring light scattering (turbidity) at 405 nm after every five cycles of freeze/thawing. (b) Biological activity of hGH, hGH–ATS, and ATS–hGH samples after 15 cycles of freeze/thawing. Biological activity was measured as in Fig. 1d. (c) Freeze/drying-induced aggregation was quantitatively assessed by monitoring light scattering (turbidity) at 405 nm after one cycle of freeze/drying and resuspension in PBS buffer. (d) Solubility of freeze/dried hGH, hGH–ATS, and ATS–hGH proteins. Freeze/dried hGH, hGH–ATS, and ATS–hGH samples were resuspended, and the soluble fractions were quantified after centrifugation.

induced unfolding of hGH–ATS started at around 83°C with a Tm of 87°C, and the unfolding of hGH–ATS started at around 85°C with a Tm of 90°C. The Tm values given here are not rigorous thermodynamic parameters because the measured thermal melting was not reversible. However, these results indicate that the introduction of the ATS peptide into hGH significantly improved the thermal stability of ATS fusion proteins. Biological activity data of the heat-treated samples at various temperatures also indicated that hGH–ATS and ATS–hGH fusion proteins were more thermostable than wild-type hGH (Fig. 4d).

Stability of Granulocyte Colony-Stimulating Factor–Acidic Tail of Synuclein Fusion Protein

To investigate whether ATS could stabilize other therapeutic proteins, we next prepared G-CSF and G-CSF–ATS fusion proteins and compared their stress resistances. Purified G-CSF and G-CSF–ATS fusion protein appeared as 22- and 24-kDa bands, respectively, on a SDS gel (data not shown), and the CD spectrum of G-CSF–ATS seems to be very similar in shape to that of wild-type G-CSF (Fig. 5a), indicating that the fusion protein is correctly folded. Interestingly, G-CSF–ATS fusion protein showed about four times higher specific activity than that of wild-type G-CSF in mouse bone marrow progenitor cells by colony-forming unit assay in soft agar (Fig. 5b). This may result from the improved stability of the G-CSF–ATS fusion protein (see below).

Thermal denaturation curves of G-CSF and G-CSF–ATS proteins were first compared to investigate the effects of ATS peptide fusion on the stability of G-CSF (Fig. 5c). As in the case of hGH, the Tm of G-CSF–ATS was about 5°C higher than that of G-CSF. In addition, the

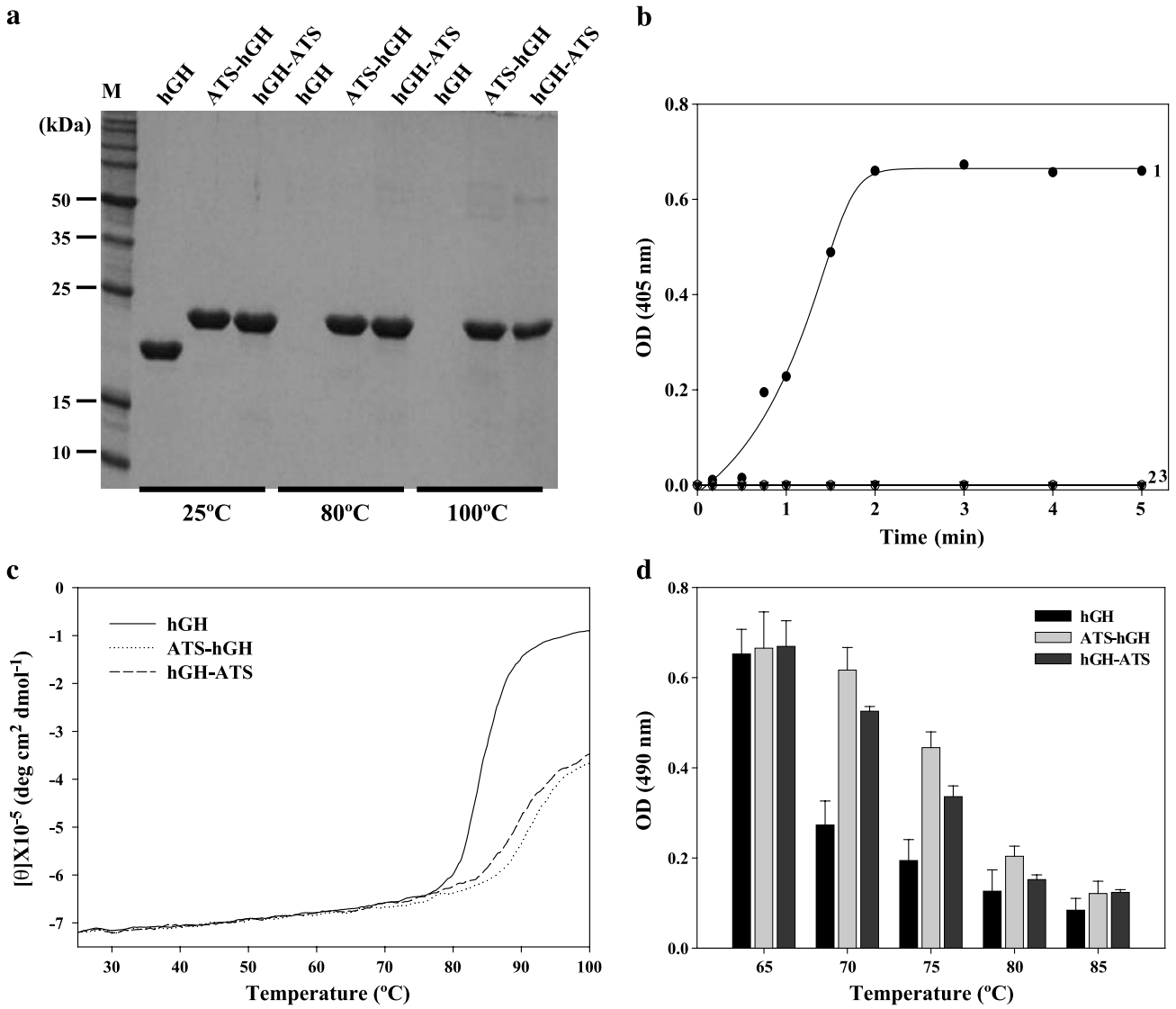

Fig. 4. Thermal stability of hGH, hGH–ATS, and ATS–hGH proteins. (a) SDS-PAGE analysis of hGH, hGH–ATS, and ATS–hGH proteins before and after heat treatment at various temperatures for 10 min. (b) Aggregation kinetics of hGH, hGH–ATS, and ATS–hGH proteins. Heat-induced aggregation was quantitatively assessed by monitoring light scattering (turbidity) at 405 nm as a function of time at 80°C. (c) CD melting curves of hGH, hGH–ATS, and ATS–hGH proteins. The graphs present the mean molar ellipticity per residue of each protein at 222 nm as a function of temperature. (d) Biological activity of hGH, hGH–ATS, and ATS–hGH samples after heat treatment at various temperatures. Biological activity was measured as in Fig. 1d.

G-CSF–ATS fusion protein did not precipitate at all after heat treatment independent of temperature, whereas the G-CSF protein completely precipitated at 45°C (data not shown). Agitation-induced aggregation profiles of G-CSF and G-CSF–ATS proteins were compared next. As expected, G-CSF–ATS fusion protein did not aggregate even after 50 h of agitation, whereas the G-CSF aggregated rapidly within a few hours (Fig. 5d). Finally, the stabilities of G-CSF and G-CSF–ATS proteins against freezing/thawing stress were compared. As shown in Fig. 5e, wild-type G-CSF rapidly aggregated as the number of freeze/thawing cycles increased, whereas the G-CSF–ATS fusion protein was very resistant to freeze/thawing stress. In addition, the biological activity of G-CSF after the agitation or the freeze/thawing cycles was almost completely abolished, whereas that of G-CSF–ATS was not significantly changed (data not shown).

Stability of Human Leptin–Acidic Tail of Synuclein Fusion Protein

We next investigated the stress-induced aggregation of hLeptin and hLeptin–ATS fusion protein. Purified hLeptin appeared as a 22-kDa band on a SDS gel, whereas the purified hLeptin–ATS fusion protein appeared as a 24-kDa band (Fig. 6a). Thermal behaviors of hLeptin and hLeptin–ATS proteins were compared by monitoring their absorbance at 405 nm over a temperature range. As shown in Fig. 6b, the hLeptin–ATS fusion protein did not precipitate after heat treatment independent of temperature, whereas the hLeptin precipitated at 50°C. Human leptin–ATS fusion protein did not aggregate even after 50 h of agitation, whereas the hLeptin aggregated rapidly within a few hours (Fig. 6c). Furthermore, wild-type hLeptin rapidly aggregated as the number of freeze/

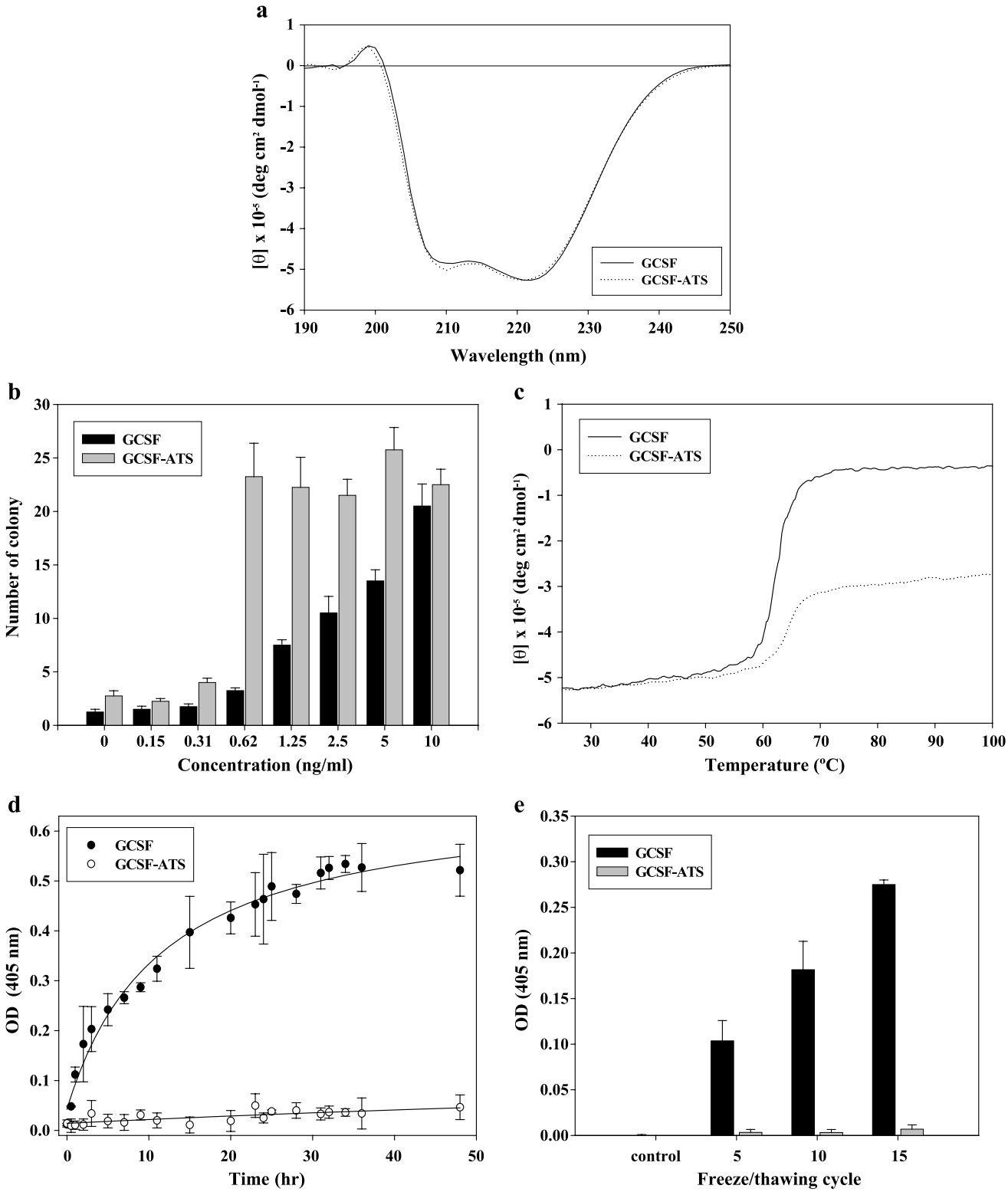

Fig. 5. Stability of granulocyte colony-stimulating factor–ATS fusion protein. (a) Far-UV CD spectra of G-CSF and G-CSF–ATS. Far-UV CD spectra of G-CSF and G-CSF–ATS proteins measured at room temperature are drawn as a solid line and dotted line, respectively. (b) Biological activity of G-CSF and G-CSF–ATS proteins was measured by granulocyte colony-forming unit assay. The biological activity assay was performed as described in Materials and Methods. The results are expressed as colony numbers and are the means ± SD obtained from triplicate experiments. (c) Thermal stability of G-CSF and G-CSF–ATS determined by CD melting curves. The graphs present the mean molar ellipticity per residue of each protein at 222 nm as a function of temperature. (d) Agitation-induced aggregation kinetics of G-CSF and G-CSF–ATS. (e) Freeze/thawing-induced aggregation profiles of G-CSF and G-CSF–ATS. (d, e) Protein aggregation was quantitatively assessed by monitoring light scattering (turbidity) at 405 nm.

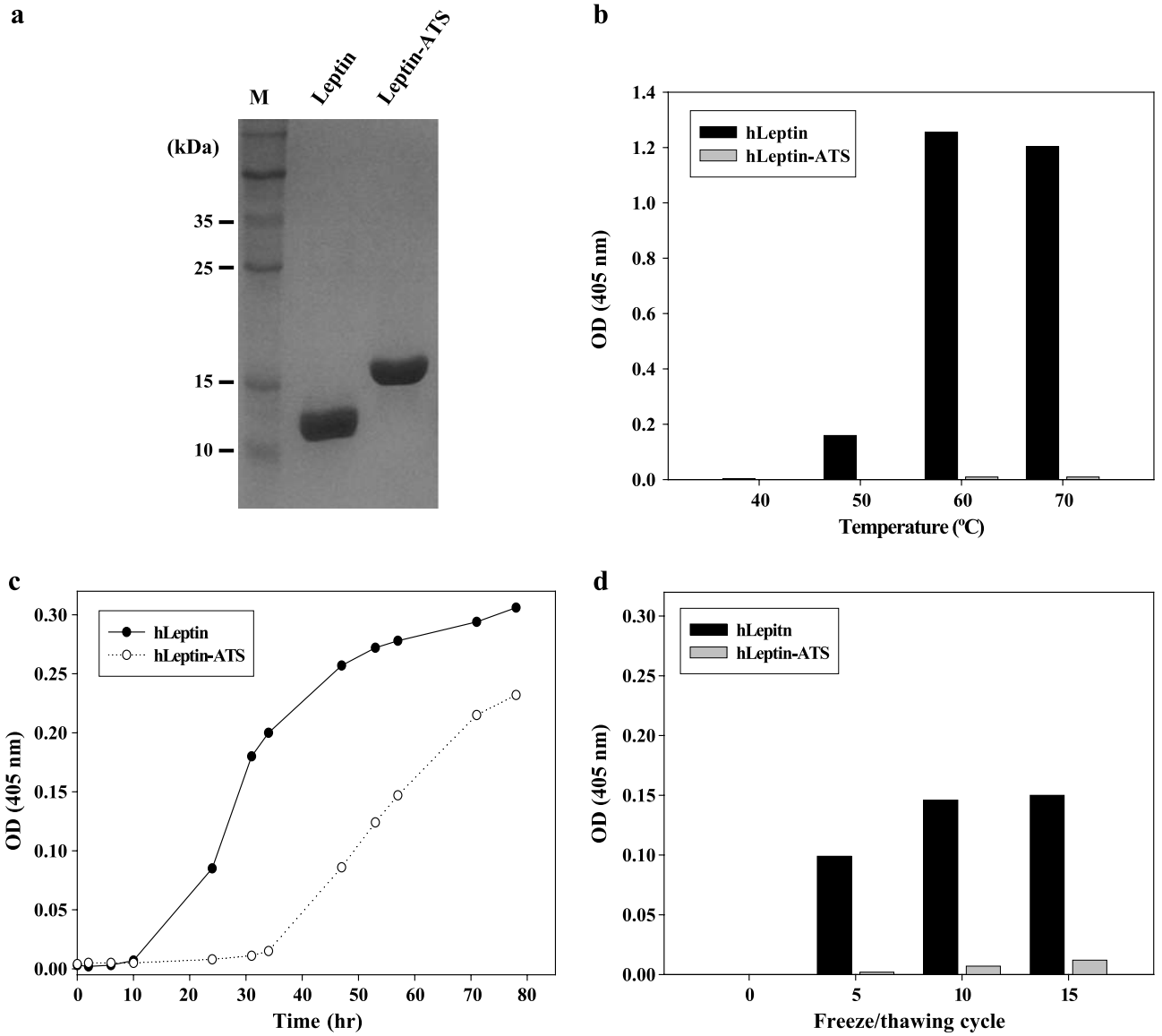

Fig. 6. Stability of hLeptin–ATS fusion protein. (a) SDS-PAGE analysis of the purified hLeptin and hLeptin–ATS proteins. The purified proteins were analyzed on a 12% SDS–polyacrylamide gel, and the protein bands were stained with Coomassie Brilliant Blue R250. (b) SDS-PAGE analysis of hLeptin and hLeptin–ATS before and after heat treatment at various temperatures for 10 min. (c) Agitation-induced aggregation kinetics of hLeptin and hLeptin–ATS. (d) Freeze/thawing-induced aggregation profiles of hLeptin and hLeptin–ATS. (c, d) Protein aggregation was quantitatively assessed by monitoring light scattering (turbidity) at 405 nm.

thawing cycles increased, whereas the hLeptin–ATS fusion protein was very resistant to freeze/thawing stress (Fig. 6d).

Improved Solubility of Acidic Tail of Synuclein Fusion Proteins

We next investigated the effect of ATS peptide fusion on protein solubility. As expected from the highly charged nature of the fusion peptide, ATS fusion proteins seemed to be much more soluble than wild-type proteins (Table I). As a result of ATS peptide fusion, solubilities of GST–ATS, hGH–ATS, and hLeptin–ATS increased about 1.2-, 2-, and 5-fold, respectively, compared to wild-type proteins. Furthermore, ATS fusion proteins did not precipitate at all during the concentration process, whereas wild-type hGH and hLeptin precipitated slightly at high concentration.

Pharmacokinetics of hGH–ATS and G-CSF–ATS Fusion Proteins

To address the *in vivo* stability of ATS fusion proteins, the pharmacokinetics of hGH, hGH–ATS, and ATS–hGH were compared in rats after a single subcutaneous injection. As shown in Fig. 7a, serum concentration of wild-type hGH reached maximum value 1 h after injection, then rapidly decreased with a half-life ($t_{1/2}$) of about 2 h, whereas that of hGH–ATS or ATS–hGH increased until 2 h after injection, then slowly decreased with a half-life of about 4 h. As a result, subcutaneous injection of the hGH–ATS or ATS–hGH fusion proteins results in greater systemic exposure (area under concentration-time curves) than wild-type hGH (Fig. 7a). Analysis of the serum concentration-time curves of G-CSF and G-CSF–ATS also shows that subcuta-

Table I. Solubility of Wild-Type and ATS Fusion Proteins

Proteins	Solubility (mg/ml)	Precipitation?
GST	~200	No
GST–ATS	>250	No
hGH	~80	Yes
hGH–ATS	~150	No
hLeptin	~20	Yes
hLeptin–ATS	~100	No

neous injection of the G-CSF–ATS fusion protein results in greater systemic exposure than wild-type G-CSF (Fig. 7b). The half-life of G-CSF in rat serum was about 4 h, whereas that of G-CSF–ATS was about 5 h. These results indicate that ATS fusion proteins are also more stable *in vivo*.

DISCUSSION

Aggregate formation of therapeutic proteins is a major problem in biopharmaceutics. For example, hGH has limited stability both for liquid and lyophilized formulations. Previous studies have demonstrated that hGH in solution is rapidly aggregated by shaking or agitation stress (11,12,15,28). Eckhardt et al. (29) have reported that hGH forms insoluble aggregates under freezing stress. High temperatures cause the irreversible aggregation of hGH and porcine GH (11,30). Granulocyte colony-stimulating factor also has limited stability and requires strict formulation and storage conditions (31–33). Human leptin also easily aggregates during the preparation and storage steps (34). Therefore, it is still necessary to develop a more stable form of these therapeutic proteins that is more resistant to various types of stresses and, consequently, that would increase its biological efficacy and shelf life, but decrease the detrimental effects caused by protein aggregation. In this study, we introduced the ATS fusion peptide into three representative therapeutic proteins to make a more stable form and showed that these ATS fusion proteins are very resistant to various types of stresses, such as agitation, freeze/thawing, and high temperatures, even in the absence of excipients. These results indicate that the ATS peptide can be utilized to protect therapeutic proteins from stress-induced aggregation.

Comparison of the storage stability of wild-type and the ATS fusion proteins showed that hGH was mostly degraded after incubation at room temperature or at 37°C for 30 days, whereas most of the hGH–ATS and ATS–hGH fusion proteins remained intact (data not shown). At 60°C, wild-type hGH rapidly aggregated and most of the protein degraded within 3 days, whereas the hGH–ATS and ATS–hGH proteins remained soluble within this time period. The G-CSF–ATS fusion protein also displayed improved storage stability (data not shown). These results demonstrate that the ATS peptide can increase the storage stability of fusion proteins in solution. Increased shelf life is important for the distribution and storage of therapeutic proteins.

The ATS peptide used in this study is highly charged with Glu and Asp representing 9 of 22 amino acid residues. Because protein solubility is known to be approximately proportional to the square of the net charge on the protein (35), the ATS peptide fusion could increase the solubility of fusion proteins. In fact, the solubilities of hGH–ATS and hLeptin–ATS seemed to increase about 2- and 5-fold, respectively, compared to wild-type proteins (Table I). These results indicate that the ATS peptide can be utilized to improve the solubility of therapeutic proteins. Development of high protein concentration formulations is necessary for the compliance of patients, but is challenging for some proteins that have limited solubility (36).

Human growth hormone, hGH–ATS, and ATS–hGH proteins were expressed at similar levels as inclusion bodies in *E. coli*. It is interesting that the refolding efficiency of hGH–ATS or ATS–hGH fusion protein by the alkaline method was about two times higher than that of wild-type hGH (data not shown). Refolding of the proteins by other methods resulted in the same result, suggesting that the ATS peptide plays a helpful role in the refolding process. Consequently, the final yield of hGH–ATS or ATS–hGH fusion protein was also about two times higher than that of wild-type hGH. Unlike wild-type human adiponectin, an adiponectin–ATS fusion protein was even expressed as a soluble protein in

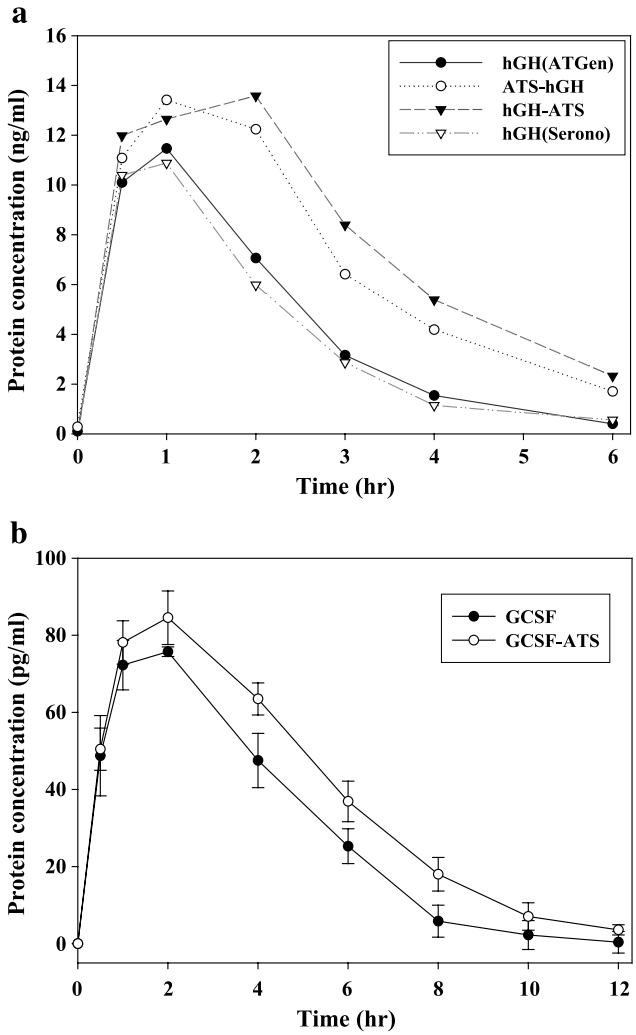

Fig. 7. Pharmacokinetic studies in rats. (a) Pharmacokinetics of hGH, hGH–ATS, and ATS–hGH fusion proteins. (b) Pharmacokinetics of G-CSF and G-CSF–ATS.

E. coli (data not shown). These results indicate that the ATS peptide can also be used to improve the refolding efficiency of inclusion proteins.

It was shown that the ATS peptide could greatly increase the solubility of many ATS fusion proteins (Table I). The ATS peptide also has a potential to interact with other proteins [reviewed in Ref. (37)] and with the fusion protein it is fused to, presumably through the action of hydrophobic residues scattered throughout the ATS (19). As a consequence, ATS has a potential to provide a chaperone-like activity for ATS fusion proteins (38). Therefore, it seems highly likely that the stabilization of the ATS fusion proteins against various types of stresses, and the improved refolding efficiency, might result from the increased solubility and self-chaperoning effect of the ATS peptide-containing fusion proteins.

Many therapeutic proteins have limited stability, particularly in solution, and so these protein drugs are commonly formulated in a lyophilized form. Unfortunately, the lyophilization process generates both freezing and drying stresses, which can denature proteins to various degrees (39). Furthermore, lyophilized formulations have disadvantages of complex processing, more manipulation, and inconvenient application for patients (40). Therefore, it is still necessary to develop a more stable form for liquid formulation. Our data show that ATS fusion proteins are very stable in solution against various types of stresses, even in the absence of excipients. These results clearly indicate that ATS fusion proteins could be used to develop a therapeutic product in liquid form. A liquid formulation of therapeutic proteins can definitely decrease the production cost to the manufacturer, while improving convenience and compliance for patients to use. In addition, the ATS fusion proteins could also be utilized to develop alternate delivery methods (such as a patch, continuous pump, and aerosol spray), or sustained-release formulations, because the proteins would be more stable under the harsh conditions that are usually associated with these delivery systems.

CONCLUSIONS

The stability of therapeutic proteins is important for their preparation, storage, shipping, and delivery. In this study, we have shown that therapeutic proteins, such as hGH, G-CSF, and hLeptin, can be greatly stabilized by fusing a novel stabilizing peptide (the ATS peptide) either at the N- or C-terminus of proteins. Acidic tail of synuclein fusion proteins seem to be very resistant to various types of stresses, such as agitation, freeze/thawing, and heat stresses. Our *in vitro* stability data clearly indicate that ATS fusion increases not only the virtual stability of therapeutic proteins by abolishing stress-mediated aggregation, but also the intrinsic stability by improving the melting temperature. Pharmacokinetic studies in rats show that ATS fusion proteins are also more stable *in vivo* than wild-type proteins. Furthermore, introduction of the ATS peptide significantly increases the solubility of the fusion proteins. These results demonstrate that the ATS peptide can be used to improve the solubility and stability of any protein of therapeutic interest. Both the improvement of solubility and stability by introducing the ATS peptide into therapeutic proteins should help make them more amenable to alternate delivery systems and formulations.

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