Roles of the C-terminal acidic tail of α-synuclein in the solubility, stability and chaperone-like action of protein

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Roles of the C-terminal acidic tail of α-synuclein in the solubility, stability and chaperone-like action of protein

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The Doctoral Dissertation submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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This certifies that the Doctoral Dissertation of Park, Sang Myun is approved

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The Graduate School

Yonsei University

December 2002

감사의 글

아무것도 모르고 시작한 실험실 생활 4년, 막막하기만 했던 그 세월, 때로는 너무나 힘들었고, 때론 너무나 기뻤던 시절을 다 보내고 이렇게 졸업을 할 수 있게끔 이끌어 주신 김종선 교수님께 먼저 감사의 말씀을 전합니다. 제 졸업논문을 지도해 주신 안용호 교수님, 신전수 교수님, 정광철 교수님, 그리고 백자현 교수님께도 감사의 말씀을 전합니다.

또 미생물학교실의 일원으로 여러가지 일에 부족함이 없이 도와주신 박전한 교수님께도 감사의 말씀을 전합니다. 항상 인자하신 모습을 보여주신 김주덕 교수님, 김세종 교수님, 그리고 최인홍 교수님, 한가지 길로 빠지지 않고 여러가지 생각을 하며 실험을 할 수 있게 도와주신 이원영 교수님, 항상 성실하게 생활할 수 있게 모범을 보여주신 조상래 교수님, 그리고 언제나 열심히 실험할 수 있는 모습을 보여주신 이봉기 교수님, 그리고 지금은 이곳 미생물학교실에 안 계시지만 힘들 때 도움을 주신 이미옥 선생님과 박현주 선생님께도 감사의 말씀을 전합니다.

이곳에서 가장 오랫동안 생활을 하며 많은 점을 가르쳐 주신 최용준 선생님, 힘든 점을 이해하며 격려를 아끼지 않았던 신의철 선생님께도 다시 한번 감사의 말씀을 전합니다. 그외 이재면 선생님, 김철훈 선생님, 그리고 김하일 선생님께도 감사의 말씀을 전합니다.

필요할 땐 언제나 도움을 주신, 정한영 선생님, 고시환 선생님, 장윤수 선생님, 김일휘 선생님, 그리고 최유정 선생님께도 감사의 말씀을 드립니다. 교실에서 항상 티격태격하며 지냈지만, 여러면에서 많은 도움을 준 최윤희 선생님과, 김혜미 선생님께도 감사의 말씀을 전합니다. 그외 졸업을

할 수 있게 도와주신 모든 교실원들에게도 감사의 말씀을 전합니다.

끝까지 믿음으로 절 인도해 주신, 아버님, 어머님께도 감사의 말씀을 드리며, 학위기간동안 같이 자취를 하면서 고생을 했던 나의 동생 수영이에게도 고맙다는 말을 전하고 싶습니다.

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Roles of the C-terminal acidic tail of α-synuclein in the solubility, stability and chaperone-like action of protein

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α-Synuclein, an acidic neuronal protein containing 140 amino acids, is extremely heat-stable and natively unfolded with an extended structure primarily composed of random coil. α-Synuclein consists of three distinct regions. The N-terminal region contains KTKEGV repeats, which form amphipathic α-helices that are similar to the lipid-binding domain of apolipoproteins. The central region is a very hydrophobic NAC (non Aβ-component of Alzheimer's disease) peptide, and the C-terminal region is primarily composed of acidic amino acids.

The region of α-synuclein that is responsible for the heat-resistancy was initially investigated using a series of deletion mutants, and the C-terminal acidic tail (residues 96-140) was found to be crucial for the thermosolubility of α-synuclein. Using a series of GST-synuclein deletion mutants, the C-terminal acidic tail of α-synuclein (αATS, Syn96-140) was also shown to play a critical role in conferring the heat-resistance of the fusion proteins. Furthermore, the acidic tail appeared to protect the fusion protein from pH- and metal-induced protein aggregation, suggesting that the acidic tail can increase the virtual stability of the protein by protecting it from the aggregation induced by environmental stresses. Interestingly, the acidic tail also appeared to significantly protect the GST enzyme from the thermal inactivation. The acidic tail of β- and γ-synuclein (βATS and γATS, respectively) also conferred heat-

resistance to fusion proteins like that of α-synuclein. Also, GST-αATS deletion mutants appeared to be heat-resistant, although the degree of heat-resistance was generally dependent on the length of the acidic tail. Interestingly, GST-E5 and -E10 appeared to be less heat-resistant than GST-αATS deletion mutants, suggesting that specific sequence elements of the acidic tail of synuclein (ATS), as well as the negative charges, are important for the phenomena.

Subsequently, it appeared that the C-terminal acidic tail is necessary, but insufficient for chaperone activity of α-synuclein. α-Synuclein protect substrate proteins by forming HMW (High Molecular Weight) complexes like other sHSPs. The N-terminal region of α-synuclein plays a role in substrate protein binding, and the C-terminal region of α-synuclein plays a role in solubilizing the HMW complex. The C-terminal acidic tail of α-synuclein also confers chaperone activities to fusion proteins, and the efficiency and specificity of chaperone function appeared to depend on the respective fusion partner proteins.

In conclusion, the acidic tail of synuclein is a peptide that confers the environmental stress-resistance to fusion proteins by increasing the stability and solubility. Thus, the acidic tail of α-synuclein can be utilized to increase protein solubility and to protect proteins from environmental stresses. In addition, the C-terminal acidic tail of α-synuclein can be utilized to engineer synthetic chaperones for specific purposes simply by fusing the acidic tail with other proteins or peptides. Such specifically designed chaperone proteins would be useful for stabilizing target proteins both *in vitro* and *in vivo*.

Key words : α-synuclein, thermostability, solubility, chaperone

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I. INTRODUCTION

α-Synuclein, an acidic neuronal protein containing 140 amino acids[1,2], is extremely heat-stable and natively unfolded with an extended structure primarily composed of random coil[3-5]. α-Synuclein was originally identified in the electric organ of the electric eel *Torpedo californica* using antiserum against cholinergic vesicles[6]. α-Synuclein is highly expressed in brain tissues and is primarily localized at the presynaptic terminals of neurons[7]. In addition to its expression in neuronal cells, α-synuclein is expressed in other tissues, such as the heart, skeletal muscle, pancreas, and placenta, but it is less abundant than in the brain[1,2]. α-Synuclein consists of three distinct regions[7-9]. The N-terminal region contains KTKEGV repeats, which form amphipathic α-helices that are similar to the lipid-binding domain of apolipoproteins. The central region is a very hydrophobic NAC (non Aβ-component of Alzheimer's disease) peptide, and the C-terminal region is primarily composed of acidic amino acids. Moreover, the amphipathic N-terminal and the hydrophobic NAC regions are highly conserved between species, while the C-terminal region is highly variable in size and in sequence[7-9]. In addition to α-synuclein, β- and γ- synucleins, and synoretin, which belong to the synuclein family have been identified in humans[1,6,10,11].

α-Synuclein has been identified as a major component of intracellular fibrillar protein deposits (Lewy bodies) in several neurodegenerative diseases, including Parkinson's disease[12,13], diffuse Lewy body disease[14] and multiple systemic atrophy[15]. Interest in the pathological role of α-synuclein was enhanced when two different mutations, A30P and A53T, were found in some early onset familial Parkinson's disease patients[16,17]. Recently, two transgenic animal models have been described. Interestingly, α-synuclein-transgenic mice and *Drosophila* appear to have similar abnormalities that found in patients with Parkinson's disease[18,19]. Many lines of biochemical evidence also suggest that α-synuclein is closely related to the pathogenesis of Parkinson's disease[20-24]. Although significant progress has been made in understanding the pathological roles of α-synuclein in neurodegenerative disease, the biological function of α-synuclein remains to be clarified. Recently, it was reported that α-synuclein may be related with neuronal plasticity[25] and may be have a role in vesicle transporter system[8].

Previously, the thermal behavior of proteins was systematically investigated by purifying and characterizing some HRPs (Heat-Resistant Proteins) from Jurkat T cells and human serum that are not aggregated by heat treatment[26]. Many proteins in both Jurkat cell lysates and human serum appeared to be heat-resistant, and a systematic investigation of the effect of heat on the purified HRPs revealed four major types of thermal behavior of HRPs, indicating that protein heat resistance can be achieved in several different ways. Group I HRPs are represented by unstructured proteins such as α-synuclein and α_s-casein. These proteins have a semi-unfolded conformation regardless of temperature, although they are known to undergo some structural change at high temperature as shown by CD[26,27]. Group II HRPs represented by human serum fetuin and albumin are characterized by an irreversible conformational change upon heat treatment, while group III HRPs represented by transthyretin and bovine serum fetuin are characterized by a reversible conformational change. Group IV HRPs represented by conventional heat stable proteins such as hyperthermophilic proteins are characterized by the absence of heat induced conformational changes.

In solution, purified α-synuclein is natively unfolded because it has no stable secondary structure[4]. α-Synuclein does not aggrgate even upon boiling, which account for its heat stability[9]. However, conditions of elevated temperature or low pH induce transient conformational changes that have been interpreted as folding intermediates of the aggregation pathway[28]. α-Synuclein was induced to aggregate under experimental conditions by varing the time, temperature and pH[3,5,28]. Furthermore, it was reported that mutants with truncation of C-terminus aggregated more readily than wild type α-synuclein[29]. Thus, it is likely that C-terminal acidic tail of α-synuclein may play an important role in these processes.

Recently, the chaperone activity of α-synuclein *in vivo* and *in vitro* has been demonstrated[28,30,31]. Like other small heat shock proteins (sHSPs), such as HSP25, HSP16 and α-crystallin[32-36], α-synuclein is able to prevent the thermally- and chemically-induced aggregation of substrate proteins[28]. The other synuclein family members, the β- and γ-synucleins, also appear to have this chaperone activity[31]. However, the detailed mechanism of the chaperone action of α-synuclein remains unknown. Interestingly, the chaperone activity of α-synuclein is lost upon removing its C-terminal acidic tail[31], suggesting that the acidic tail plays an important role in the molecular chaperone function.

The mechanism of the chaperone action of sHSPs is comparatively well understood. sHSPs protect substrate proteins from stress (e.g. heat, chemicals, etc) by forming HMW complexes with partially unfolded substrate proteins[33,37-45]. However, alone sHSPs do not have the ability to protect enzymes from thermal inactivation or to promote their functional refolding after denaturation[36,44,46,47], although a few exceptional cases with marginal effects have been reported[34,38,48-51]. sHSPs have, therefore, been classified as 'junior chaperones'[52]. sHSPs share many properties, for example, they have extensive amino acid sequence similarity, and are found as large, aggregated complexes of average mass 200-800 kDa[53,54]. The charged C-terminal domain (also called the α-crystallin domain) is well conserved in all members of the sHSP family, whereas the hydrophobic N-terminal domain is variable in length and

sequence[54]. The N-terminal domain is known to play a crucial role in self-assembly, and thus contributes to chaperone activity[43,55], whereas the C-terminal domain is known to be crucial for substrate protein binding and stabilization[56]. In particular, a 19 amino acid peptide derived from the C-terminal domain has been shown to posses substantial chaperone activity[57]. The extended polar C-terminal tail (10-18 amino acid residues) is also important in the chaperone action of sHSPs, and appears to fulfill many roles that are not completely understood yet. Firstly, the extended C-terminal tail of sHSPs is believed to function as a solubilizer[32,58-61]. Moreover, truncation of the C-terminal tail results in a significant decrease in the chaperone function and stability of sHSPs[60,62]. In some sHSPs, the flexible C-terminal tail also appears to interact directly with substrate proteins[35,59]. Furthermore, the crystal structure of HSP16 reveals that the C-terminal tail is also involved in the organization of the HSP oligomer[33,63].

The C-terminal acidic tail of α-synuclein may play an important role in these features of α-synuclein: thermostability, aggregation property and chaperone function. To prove this hypothesis, (1) stress-induced aggregation profiles of GST-α-synuclein fusion proteins made of GST and a series of deletion fragments of α-synuclein have been investigated, focusing on the role of the C-terminal acidic tail of synucleins in protein solubility and stability; (2) the molecular mechanism of the chaperone action of α-synuclein has been investigated by analyzing the roles of the N-terminal and C-terminal domains of α-synuclein in the molecular chaperone function.

II. MATERIALS AND METHODS

1. Construction of GST-synuclein deletion mutants

cDNA of α-synuclein in pRK172 was a kind gift from Dr. R. Jakes[64], GST-Synuclein fusion constructs (pGST-Syn1-140, pGST-Syn1-60, pGST-Syn61-95, pGST-Syn61-140, pGST-Syn96-140) were a kind gift from Dr. H. Rhim[65]. pGST-βATS and -γATS were generated by PCR amplification of the β- and γ-synuclein gene with the specific primer sets (Table I). pGST-Syn103-115, pGST-Syn114-126, pGST-Syn119-140, pGST-Syn130-140, pGST-E5, pGST-E10 were generated by oligonucleotide ligation method. Briefly, the synthetic oligonucleotides containing BamHI and EcoRI restriction enzyme sites (Table I) were annealed, then ligated into the pGEX2T vector that had been digested with BamHI/EcoRI. All constructs were verified by DNA sequencing.

2. Purification of α-synuclein, its deletion mutants and GST-synuclein deletion mutants

α-Synuclein was overexpressed in *E. coli* and the recombinant protein was purified to apparent homogeneity by taking advantage of the thermosolubility of the protein and by using conventional column chromatography, as described previously[3,66]. The GST, GST-synuclein fusion proteins, GST-E5 and GST-E10 expressed in the *E.coli* strain, Bl21 (DE3) were purified by affinity chromatography using glutathione-Sepharose 4B beads, and further purified on an FPLC gel-filtration column. The α-synuclein deletion mutants, Syn61-140 and Syn96-140, were prepared from GST-Syn61-140 and GST-Syn96-140, respectively, by thrombin digestion of the fusion proteins.

3. Heat-induced protein aggregation assay

The heat-induced aggregation of α-synuclein, GST and GST-α-synuclein fusion proteins was qualitatively assayed by SDS-PAGE after heat-treating the protein samples. Each protein in PBS (0.6 mg/ml) was heated in a boiling water bath for 10

Table 1. Primer sequences for the construction of recombinant plasmids

Peptide		Sequence
βATS	S	5' - agctaaggatccaagagggaggaattcc – 3'
	AS	5' - aagtaactcgagctacgcctctggctcata – 3'
γATS	S	5' - aagaatggatcccgcaaggaggacttga – 3'
	AS	5' - aatagcgaattcctagtctcccccactct – 3'
Syn103-115	S	5' - gatccaatgaagaaggagccccacaggaaggcattctggaagattaag – 3'
	AS	5' - aattctta atcttccagaatgccttcctgtggggctccttcttcattg – 3
Syn114-126	S	5' - gatccgaagatatgcctgtagatcctgacaatgaggcttatgaataag – 3'
	AS	5' - aattcttattcataagcctcattgtcaggatctacaggcatatcttcg – 3'
Syn119-140	S	5' - gatccgatcctgacaatgaggcttatgaaatgccttctgaggaagggtatcaagactacgaacctgaag cctaag – 3'
	AS	5' - aattcttaggcttcaggttcgtagtcttgataccttcctcagaaggcatttcataagcctcattgtcag gatcg – 3'
Syn130-140	S	5' - gatccgaggaagggtatcaagactacgaacctgaagcctaag – 3'
	AS	5' – aattcttaggcttcaggttcgtagtcttgataccttcctcg – 3'
E5	S	5' - gatccgaagaagaagaagaataag – 3'
	AS	5' - aattcttattcttcttcttcttcg – 3'
E10	S	5' - gatccgaagaagaagaagaagaagaagaagaagaataag – 3'
	AS	5' - aattctta ttcttcttcttcttcttcttcttcttc g – 3'

* S - sense, AS – antisense

min, and cooled in the air. The protein samples were centrifuged at 15,000 rpm for 10 min, and the supernatants were analyzed on a 12% SDS polyacrylamide gel. The protein bands were stained with Coomassie Brillinant Blue R250. Heat-induced aggregation of GST-α-synuclein fusion proteins were also quantitatively measured by monitoring the apparent absorbance (scattering) at 360 nm as a function of time at 65ºC[28,31]. Each protein was diluted to a final concentration of 0.2 mg/ml in the PBS or Tris buffer (20mM Tris-HCl, pH 7.4). The protein sample in the spectrophotometric cuvette was placed in a thermostatic cell holder, and the apparent absorbance was monitored in a Beckman spectrophotometer (Beckman DU650).

4. pH- and metal-induced protein aggregation assay

The pH-induced aggregation of GST and GST-Syn96-140 was similarly measured by monitoring the apparent absorbance (scattering) at 360 nm as a function of pH. Each protein was diluted to a final concentration of 0.2 mg/ml in buffers with different pH values. The buffers used were 0.1M acetate (pH 4.0 and 5.0), 0.1 M citrate (pH 6.0), and 0.1 M Tris-HCl (pH 7.4). The protein solutions were incubated for 1 hr at room temperature and the apparent absorbance was monitored in a Beckman spectrophotometer. The metal-induced aggregation of GST and GST-Syn96-140 was similarly measured. Each protein was diluted to a final concentration of 0.2 mg/ml in 20 mM Tris-HCl buffers containing 0 to 1.0 mM of Zn^{2+} or Cu^{2+}. The protein solutions were incubated for 30 min at room temperature and the apparent absorbance at 360 nm was measured.

5. CD measurements

The CD spectra were recorded on a Jasco-J715 spectropolarimeter (Jasco, Japan) equipped with a temperature control system in a continuous mode. The far-UV CD measurements were carried out over the wavelength range of 190 to 250 nm with 0.5 nm bandwidth, a one second response time and a 10 nm/minute scan speed at 25ºC and 100ºC. The spectra shown are an average of five scans that were corrected by subtraction of the buffer signal. The CD data were expressed in terms of the mean residue ellipticity, [θ], in $deg.cm^2.dmol^{-1}$. The protein samples for CD measurements were prepared in 10 mM sodium phosphate buffer (pH 7.5) unless otherwise specified, and all spectra were measured in a cuvette with a path length of 0.1cm. The protein concentration was 0.1mg/ml.

Thermal denaturation experiments were performed using a heating rate of 1ºC/min and a response time of 1 sec. The thermal scan data were collected from 25 to 100ºC in 0.1 cm path length cuvettes with a protein concentration of 0.1 mg/ml of GST, 0.3 mg/ml of GST-Syn96-140 and 0.4 mg/ml of GST-αATS deletion mutants. The CD spectra were measured every 0.5ºC at a wavelength of 222 nm, unless otherwise specified. The reversibility of the thermal transition was examined by

recording a new scan by decreasing the temperature and by another scan after cooling the thermally unfolded protein sample.

6. Chaperone-like activity assay

The ability of chaperone proteins to prevent heat-induced aggregation of substrate proteins (GST and aldolase) was monitored as described previously[28]. Briefly, substrate proteins (0.2 mg/ml as a final concentration) in PBS (pH 7.4) were incubated with each chaperone protein at 65ºC for specified times (see figures) in a cuvette. Light scattering was then monitored at 360 nm as a function of time, using a spectrophotometer. The ability of the chaperone proteins to prevent chemically-induced substrate protein (insulin and lysozyme) aggregation was monitored as described previously[28,31]. Substrate proteins (0.5 mg/ml as a final concentration) in 10 mM phosphate buffer (pH 7.4) were incubated with the indicated amounts of each chaperone protein at room temperature (see figures). DTT was added, to a final concentration of 20 mM, to commence the denaturation and precipitation of substrate proteins. Light scattering was then monitored at 360 nm, using a spectrophotometer. In addition, luciferase (0.1 mg/ml in PBS as a final concentration) was incubated with each chaperone protein for 10 min at 65°C in a cuvette, and light scattering was monitored at 360nm (Fig. 18B). For the insulin aggregation assay in figure 18A, insulin alone (0.5 mg/ml as a final concentration in 10 mM of phosphate buffer, pH 7.4) or a mixture of insulin and GST-Syn96-140 (0.5 mg/ml each as a final concentration) was preincubated for 5 min at 59°C in a thermostatic cell holder, and this temperature was maintained during the chaperone assay. After adding 2 mM of DTT as a final concentration, the absorbance was measured at 360 nm as a function of time.

7. Gel-filtration chromatographic analysis of the high molecular weight complexes

Individual solutions of each chaperone protein and substrate protein (GST or aldolase, final concentration of 0.1-0.2 mg/ml in PBS), or mixtures of chaperone and substrate

proteins (final concentration of 0.2-0.5 mg/ml of chaperone protein with 0.1-0.2 mg/ml of substrate protein in PBS) were either heat treated (65ºC for 10 min) or not heat-treated, and then centrifuged for 10 min at 13,000 rpm to remove precipitated proteins. 500 μl of each supernatant was loaded onto the Superdex 75 HR 10/30 column (Pharmacia, Uppsala, Sweden) equilibrated in PBS (pH 7.4), and the proteins were eluted at a flow rate of 1 ml/min at room temperature. Fractions corresponding to each protein peak were collected and analyzed in 12% SDS polyacrylamide gels. To detect α-synuclein in the HMW complex, 25 μl of each fraction was loaded into a 12% SDS polyacrylamide gel, transferred onto a PVDF membrane, and Western blotted with rabbit polyclonal anti-α-synuclein antibodies.

8. GST activity assay

The enzymatic activity of GST was assayed using a chromogenic substrate, 1-chloro-2,4-dinitro benzene (CDNB), as described previously[67]. The GST enzyme was added to the substrate solution (1 mM GSH and 2 mM CDNB in 0.1 M phosphate buffer, pH 7.4) to a final concentration of 20 μg/ml and incubated at 37°C for 10 min. Enzyme activity was measured as an increased absorbance at 350 nm, corresponding to the maximum absorbance of 1-S-glutathionyl-2,4-dinitrobenzene, using the Spectramax 250 microplate reader (Molecular Devices, CA, USA).

9. Phosphatase activity assay

The catalytic activity of protein tyrosine phosphatase-1B (PTP-1B) was assayed at 37ºC for 60 min in a reaction mixture (0.2 ml) containing 10 mM p-nitrophenyl phosphate (pNPP) as substrate. The buffer used was 20 mM Tris-HCl buffer (pH 7.4) containing 0.15M NaCl, 1 mM EDTA, and 2 mM DTT. The reaction was initiated by adding enzyme and quenched after 60 min by the addition of 1 ml of 1 N NaOH. The amount of p-nitrophenol released was determined by measuring the absorbance at 405 nm.

III. RESULTS

I. Stress-induced Aggregation Profiles of GST-α-Synuclein Fusion Proteins : Role of the C-Terminal Acidic Tail of α-Synuclein in Protein Thermosolubility and Stability

1. Thermal behavior of α-synuclein deletion mutants

α-Synuclein is an 'intrinsically unstructured protein' which almost lacks a regular secondary structure and contains a very high portion of random-coil[3,4]. Previous studies have shown that intrinsically unstructured proteins, such as α-synuclein and α_s-casein, are heat-resistant since the proteins have a similar unfolded conformation regardless of the temperature and their unfolded conformation is stable at high temperatures as well as at room temperature[26]. To investigate which region is responsible for its thermostability, the thermal stability of the α-synuclein deletion mutants obtained by thrombin digestion of GST-synuclein fusion proteins was examined. The GST-α-synuclein fusion proteins were treated with thrombin and the cleaved products were boiled in a boiling water bath. The protein solutions were centrifuged and the supernatants were analyzed on a SDS polyacrylamide gel. As shown in figure 2, wild type (Syn1-140) and two deletion mutants containing the acidic tail (Syn61-140 and Syn96-140) were found to be heat-resistant. In contrast, the N-terminal part of α-synuclein (Syn1-60) and the NAC peptide (Syn61-95) appeared to precipitate upon heat treatment. Interestingly, only the deletion mutants containing the C-terminal acidic tail were heat-resistant, indicating that the C-terminal acidic tail is responsible for the heat resistance. α-Synuclein has the ability to aggregate over time, or upon incubating at 37°C to form amyloid fibril[8,9,68,69]. Consistent with our data, previous studies have shown that C-terminally truncated α-synuclein proteins and the

NAC peptide assembled into filaments much more readily than the wild type protein[29,70-72]. Overall, it appears likely that C-terminally truncated α-synuclein mutant proteins are less stable at room temperature and higher temperatures than both the wild type and mutant proteins containing the C-terminal acidic tail.

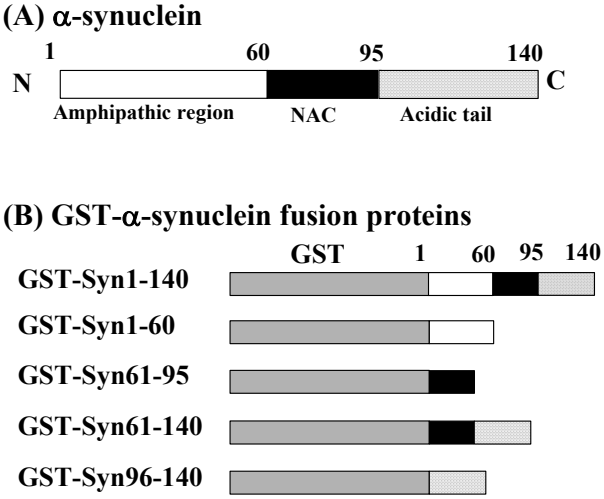

Figure 1. α-synuclein and the GST-α-synuclein fusion constructs.

(A) A schematic diagram of α-synuclein. α-synuclein consists of three distinct regions, the N-terminal amphipathic region (residues 1-60), the hydrophobic NAC region (residues 61-95), and the C-terminal acidic tail (residues 96-140). (B) GST-synuclein fusion constructs. Five GST-synuclein fusion constructs encoding the full length α-synuclein (GST-Syn1-140), the amphipathic region (GST-Syn1-60), the NAC region (GST-Syn61-95), the NAC and acidic tail regions (GST-Syn61-140), and the acidic tail region (GST-Syn96-140) were used in this study.

2. *Thermal behavior of GST-synuclein fusion proteins*

The thermal behaviors of GST-synuclein fusion proteins were similarly investigated. As shown in figure 3A, GST-Syn1-140, GST-Syn61-140, and GST-Syn96-140 did not precipitate regardless of the heat treatment indicating that these proteins are heat-resistant. Whereas, GST-Syn1-60 and GST-Syn61-95 appeared to be heat-labile and

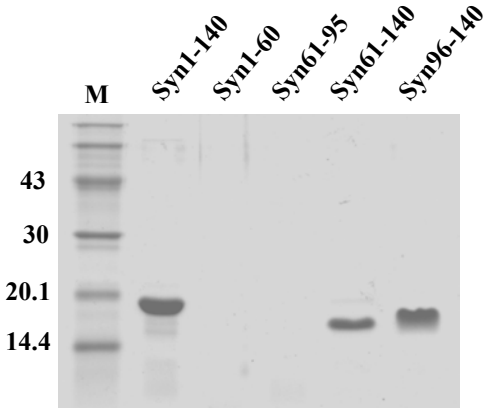

Figure 2. Thermal behavior of α-synuclein deletion mutants.

The GST-α-synuclein fusion proteins were treated with thrombin and the cleaved products were boiled in a boiling water bath. The protein solutions were centrifuged and the supernatants were analyzed on a 12% SDS polyacrylamide gel.

the proteins had completely precipitated upon heat treatment. Unlike the cases of GST-α-synuclein fusion proteins, α-synuclein did not protect GST from heat-induced aggregation when the two proteins were mixed and incubated in a boiling water bath (Fig. 3B). Consistent with previous reports[7,31], however, α-synuclein appeared to have chaperone-like activity to protect GST from heat-induced aggregation at a relatively mild temperature (60°C). These results suggest that the covalent bond between GST and α-synuclein is critical for extreme thermosolubility of the fusion protein.

The heat-induced aggregation of the GST-synuclein fusion proteins was quantitatively assessed by measuring the turbidity at 65ºC as a function of time. As shown in figure 3C, the OD_{360} of the GST protein drastically increased 2 min after heat treatment, and most of the protein had aggregated by 3 min. GST-Syn61-95 behaved similarly to the GST protein, and resulted in complete aggregation. GST-Syn1-60 also resulted in complete aggregation after heat treatment, although aggregation of this protein was relatively delayed. Consistent with the results in figure 3A, there was no evidence of any protein aggregation for GST-Syn1-140, GST-Syn61-

140, and GST-Syn96-140 even after 30 min's heat treatment. Interestingly, these three heat-resistant GST-synuclein fusion proteins all contain the acidic tail of α-synuclein. This suggests that a heat-labile protein can be transformed into a heat-resistant protein by introducing the α-synuclein acidic tail.

Previously, many of the heat-resistant proteins from Jurkat T cell lysates and human serum were reported to be highly acidic proteins[26], suggesting that the pI value may be related to the protein's heat-resistance. The solubility of proteins may play an important role in determining the heat-resistance, since highly charged proteins would be more soluble even at higher temperatures. To confirm this hypothesis, the pI and hydropathy values of α-synuclein with its deletion mutants, and those of GST and GST-synuclein fusion proteins were compared (Table 2). Table 2 clearly shows that heat-resistant proteins, such as α-synuclein, Syn61-140, Syn96-140, GST-Syn1-140, GST-Syn61-140 and GST-Syn96-140, have abnormally low pI and hydropathy values. Whereas the heat labile proteins (Syn1-60, GST, GST-Syn1-60, and GST-Syn61-95) with the exception of Syn61-95 have much higher values. Interestingly, a heat-labile peptide Syn61-95 has a very low pI value but it has an extremely high hydropathy value (Table 2). Therefore, it is possible that highly charged proteins with a low hydropathy value possesses an advantage in resisting heat-induced protein aggregation.

3. Effect of divalent cation binding

Some divalent cations, such as Cu^{2+} and Ca^{2+}, are known to bind specifically to the C-terminal acidic tail of α-synuclein with a dissociation constant of the micromolar ranges [73,74]. Zn^{2+} and other metal ions also appear to bind specifically to α-synuclein, although the binding sites are yet to be identified[28,73,75]. Since the C-terminal acidic tail of α-synuclein is important for protein heat-resistance, the effect of the divalent cation binding on the heat-induced aggregation of GST-synuclein fusion proteins containing the C-terminal acidic tail was investigated. Figure 4 shows that low concentrations of the divalent cations do not affect the heat-induced aggregation of the fusion proteins.

Table 2. Isoelectric point (pI) and Hydropathy Values of α-Synuclein and Its Deletion mutants, as Well as GST and GST-Synuclein Fusion Proteins.

Protein	Thermal behavior	pI vaule	Hydropathy vaule
α-synuclein	HR	4.67	-0.403
Syn1-60	HL	9.52	-0.188
Syn61-95	HL	4.53	0.726
Syn61-140	HR	3.85	-0.564
Syn96-140	HR	3.76	-1.567
GST	HL	6.18	-0.390
GST-Syn1-140	HR	5.25	-0.378
GST-Syn1-60	HL	7.64	-0.349
GST-Syn61-95	HL	6.01	-0.244
GST-Syn61-140	HR	4.95	-0.435
GST-Syn96-140	HR	4.85	-0.560

The pI vaules and hydropathy vaules were calculated by using the ProtParam program (www.expasy.ch).

HR, heat-resistant, HL, heat-labile

However, high concentrations significantly increased the protein aggregation, although the fusion proteins do not result in complete precipitation. Particularly, Zn^{2+} appeared to be most effective for enhancing the heat-induced protein aggregation.

Considering that the dissociation constants between α-synuclein and the divalent cations are considerably low[73,74], and that most proteins are affected by a high concentration of metal ions, the results suggest that the specific binding of the divalent cations at the C-terminal acidic tail of α-synuclein does not affect the thermal behavior of the fusion proteins. However, nonspecific binding of the metal ions at a high concentration appears to induce more protein aggregation during heat treatment.

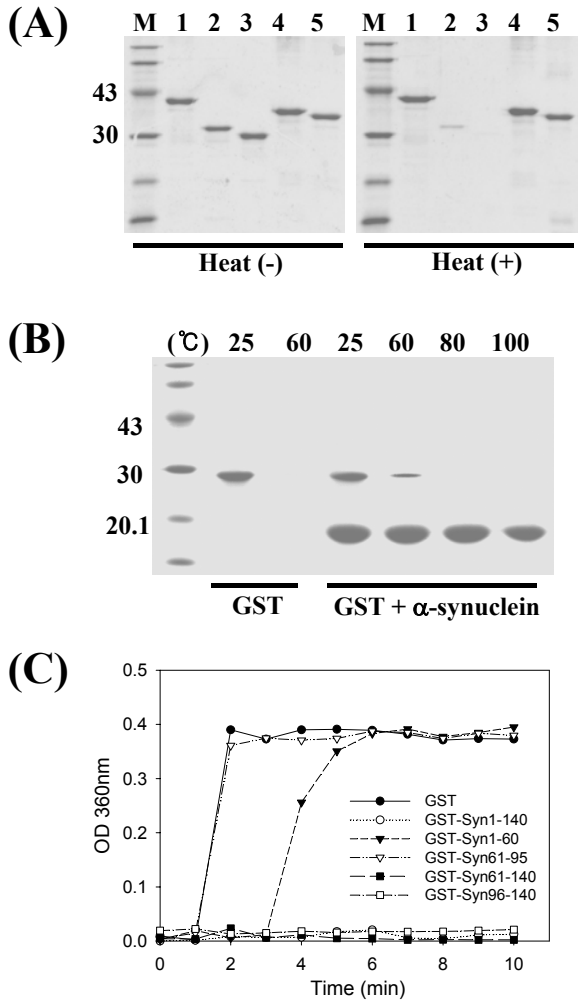

Figure 3. Thermal behavior of GST-α-synuclein fusion proteins.

(A) SDS-PAGE analysis of the GST-α-synucelin fusion proteins before (left panel) and after (right panel) boiling. Lanes: lane 1, GST-Syn1-140; lane 2, GST-Syn1-60; lane 3, GST-Syn61-95; lane 4, GST-Syn61-140; and lane 5, GST-Syn96-140. (B) α-synuclein did not protect GST from heat-induced aggregation when the two proteins were mixed and incubated in a boiling water bath. α-synuclein and GST were mixed (2:1 w/w ratio) and incubated at indicated temperatures for 10 min. After centrifugation, supernatants were analyzed on a SDS gel. (C) Heat-induced aggregation of the GST-α-synucelin fusion proteins. Heat-induced aggregation of the GST and the GST-synuclein fusion proteins was quantitatively assessed by monitoring the light scattering (OD_{360}) as a function of time at 65ºC.

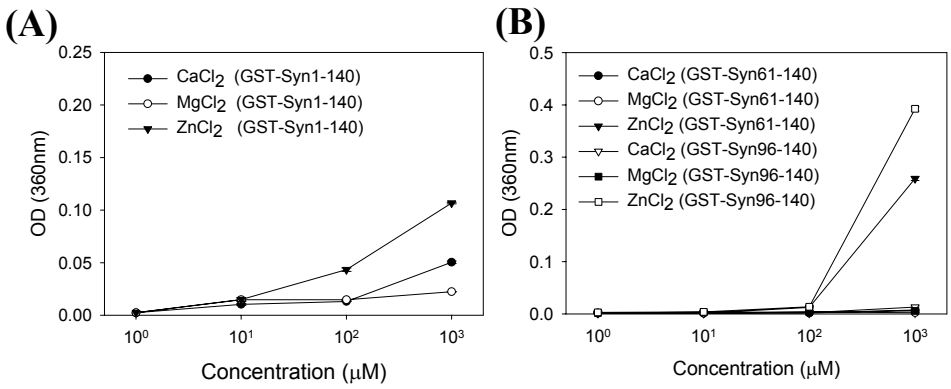

Figure 4. Effect of divalent cations on the thermal behavior of the GST-synuclein fusion proteins.

Heat-induced aggregation of GST-Syn1-140 (A), and the GST-Syn61-140 and GST-Syn96-140 (B) at 65ºC was measured as in figure 3, in the presence of the indicated cation concentrations.

4. *Heat-induced secondary structural changes of GST-Syn96-140*

Previously, heat-induced secondary structural changes of α-synuclein assessed by CD analysis were reported[26]. The CD spectrum of α-synuclein indicated that the protein almost completely lacks secondary structural elements. The CD spectrum of α-synuclein at 100ºC was slightly different from that at 25ºC, but it also represented the characteristics of random-coiled polypeptides. Consistent with these results, a linear temperature-dependence of the CD signal, often seen with unfolded peptides, was observed.

The CD spectrum of GST at 25ºC (Fig. 5A) indicates that the protein contains well-ordered secondary structural elements. However, at 100ºC, the far-UV CD spectrum was greatly diminished due to protein precipitation (data not shown). The temperature-induced change in the ellipticity of the GST at 222 nm indicates that the Tm of GST is approximately 70ºC. The GST had completely precipitated at 100ºC and the repetition temperature scan showed the absence of any CD signal at 222 nm

indicating that GST had irreversibly precipitated (data not shown). These results confirm that the GST protein is a typical heat-labile protein that unfolds and precipitates as the temperature is increased.

The far-UV CD spectra of GST-Syn96-140 are shown in figure 5B. The Far-UV CD spectrum of GST-Syn96-140 at room temperature (solid line) indicates that the protein contains well-ordered secondary structural elements. The CD spectrum showed a decrease in these elements at 100ºC but the overall shape was unchanged (dotted line), suggesting that heating does not lead to complete unfolding. Interestingly, a new absorption band at 195 nm appears, which is characteristic of random-coiled polypeptides. After cooling, the far-UV CD spectrum (dashed line) remains distinguishable from the initial one, suggesting that the conformation of GST-Syn96-140 may be irreversibly changed. The CD spectrum of the heat-treated GST-Syn96-140 at room temperature rather resembles that obtained at 100ºC, and indicates that the protein consists of two distinct domains: one with regular secondary structural elements and the other with a random-coil like conformation. To confirm the conformational changes induced by heating, the GST-Syn96-140 melting curves were measured as a function of temperature. The temperature-induced changes in the ellipticity at 222 nm are presented in the figure 5B inset. Interestingly, the heat-induced unfolding of GST-Syn96-140 appeared to take place in two stages (solid line). The transition started at 55ºC for the first transition and at 90ºC for the second. As expected, the temperature course for GST-Syn96-140 appeared to be irreversible (dotted line).

GST is a heat-labile protein, while GST-Syn96-140 is a heat-resistant protein. To compare the stability of the two proteins, it would be useful to determine the melting temperature (Tm) of both proteins. However, it is difficult to compare the Tm values of GST-Syn96-140 and GST directly since the proteins contain a different number of peptide domains. Interestingly, the Tm value of GST-Syn96-140 (62ºC for the first transition) appeared to be slightly lower than that of GST (70ºC). Since the Tm of a given protein is related to the change in the free energy between the native and thermally denatured state of a protein, the Tm has been used as a thermodynamic

parameter of the conformational stability of the protein. Therefore, it is highly likely that introducing the acidic tail to the C-terminus of GST is favorable for protein solubility and consequently for heat-resistancy, but unfavorable for intrinsic stability of the protein.

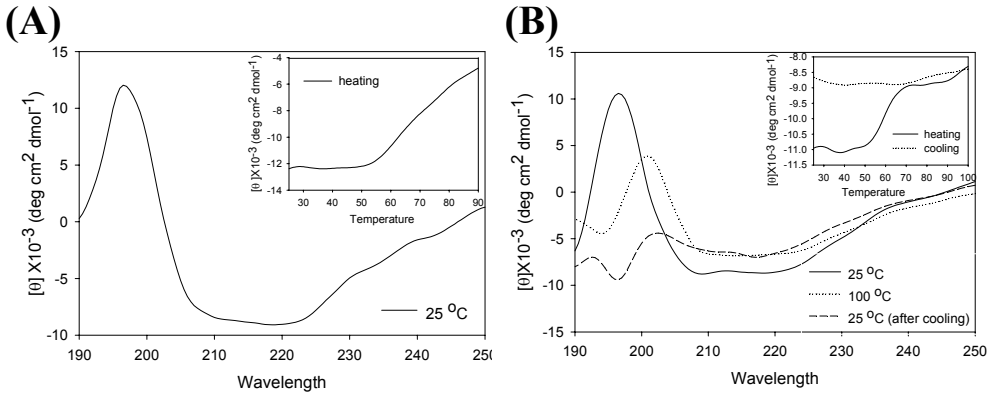

Figure 5. Far-UV CD spectra and the melting curves of (A) GST and (B) GST-Syn96-140.
Far UV-CD spectra measured at 20ºC and 100ºC are drawn as solid lines and dotted lines, respectively. The dashed lines represent the spectra measured just after cooling of the protein solution from 100ºC to 20ºC. The insets present the mean molar ellipticity per residue of each protein at 222 nm as a function of temperature. The solid and dotted lines represent temperature scans from 20ºC to 100ºC (heating mode) and from 100ºC to 20ºC (cooling mode), respectively.

5. *Enzyme activity of GST-synuclein fusion proteins after heat treatment*

Unlike the wild type GST protein, GST-fusion proteins containing the acidic tail of α-synuclein were shown to be heat resistant. This suggests that the heat-labile protein could be transformed into a heat-resistant protein simply by introducing the acidic tail of α-synuclein. Whether the heat-resistant GST-fusion proteins could keep the enzymatic activity after heat treatment was next investigated. The GST and GST-synuclein fusion proteins were boiled in a water bath for 10 min and cooled in the air to room temperature. The catalytic activities of these heat-treated proteins were then

compared. As shown in figure 6A, all the GST and GST-fusion proteins completely lost their enzymatic activity under these conditions. Subsequently, the thermostability of GST and GST-Syn96-140 was quantitatively measured by thermal inactivation curves (Fig. 6B), which were used to determine the T_{50} values, the temperatures at which 50% of initial enzymatic activity is lost after heat treatment. As shown in figure 6B, the T_{50} of GST-Syn96-140 is about 2ºC higher than that of GST. Interestingly, the thermal inactivation of GST is well correlated with the thermal aggregation of the protein. This suggests that the introduced acidic tail is able to protect the enzyme from the thermal inactivation by preventing the thermal aggregation of the fusion protein.

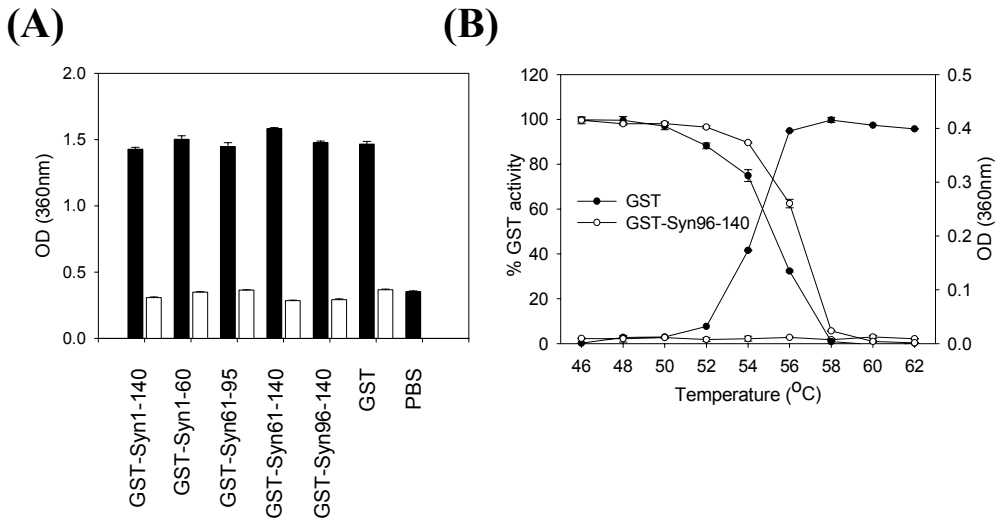

Figure 6. GST activity of the GST-synuclein fusion proteins after heat treatment.

(A) GST and the GST-fusion proteins completely lost their enzymatic activity after boiling in a water bath for 10 min (white bars). (B) Thermal inactivation and aggregation of GST and GST-Syn96-140. Activity is expressed as a percentage of initial activity (left). Values are the means of three independent experiments with the standard deviation shown as bars. Heat-induced aggregation was quantitatively assessed by monitoring the light scattering (OD_{360}) as a function of temperature (right). The protein samples were incubated for 5 min at indicated temperatures.

6. pH- and metal-induced protein aggregation

It was shown that highly charged proteins with a low hydropathy value possesse an advantage in resisting heat-induced protein aggregation (Table 2). To investigate the highly charged proteins with a low hydropathy vaule also possess any advantages against other environmental stresses, the pH-induced aggregation of GST and GST-Syn96-140 was next investigated by measuring the turbidity at 25°C as a function of pH. As shown in figure 7A, the OD_{360} of the GST protein steadily increased from pH 7.4 to pH5.0 and reached maximum value at pH 4.0. Whereas, the OD_{360} of GST-Syn96-140 was unchanged until pH 5.0, but drastically increased at pH 4.0 perhaps due to the neutralization of the acidic tail. This suggests that the C-terminal acidic tail is able to protect GST from pH-induced aggregation, though the protection effect is not sufficient under very acidic conditions. The C-terminal acidic tail also appeared to protect GST from metal-induced aggregation (Fig. 7B). The OD_{360} of the GST protein steadily increased when it was treated with 0.2 to 1.0 mM Zn^{2+}, while the OD_{360} of GST-Syn96-140 was always much lower than that of GST. Particularly, Cu^{2+} induced protein aggregation was completely blocked by the acidic tail. These results indicate that the C-terminal acidic tail can also protect GST from metal-induced aggregation.

7. Heat resistance of DHFR-αATS fusion protein

It was demonstrated that introducing the acidic tail of α-synuclein (αATS) into GST protects the fusion protein from environmental stresses, such as heat, pH, and metal ions. To investigate whether αATS can be utilized to stabilize other proteins, a DHFR-αATS fusion protein was produced and the thermal behavior of DHFR and DHFR-αATS fusion protein was compared using a qualitative heat-induced protein aggregation assay. Each protein was heat treated in a water bath at 65°C or 100°C, and the protein solution was centrifuged to remove the precipitates. Subsequently, the supernatant was analyzed on a SDS polyacrylamide gel (Fig. 8). As expected, DHFR-

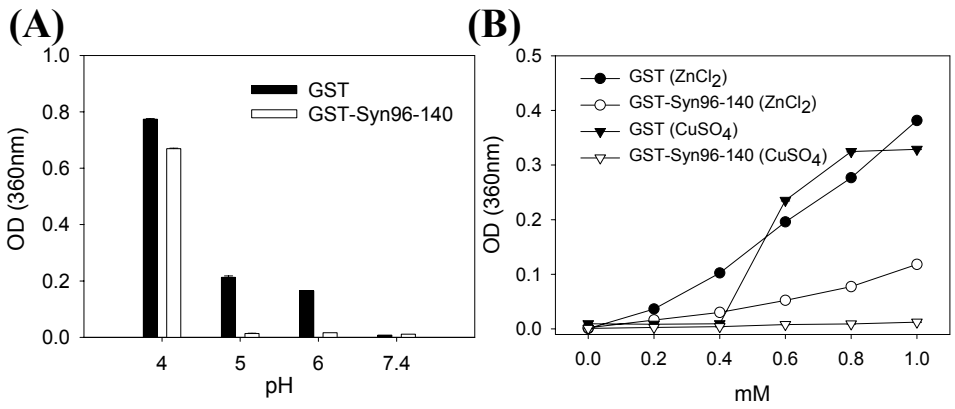

Figure 7. Effects of the acidic tail on the stress-induced protein aggregation.

(A) pH-induced aggregation of GST and GST-Syn96-140 at room temperature. (B) Metal-induced aggregation of GST and GST-Syn96-140 at room temperature. Protein aggregation was monitored by light scattering analysis at 360 nm.

αATS did not precipitate upon heat treatment up to 100°C, whereas the DHFR protein completely precipitated at 65°C. This indicates that αATS is a novel peptide conferring heat resistance to the fusion proteins.

8. Thermal behavior of GST-βATS, -γATS (Acidic Tail of Synucleins)

It was next investigated whether GST-ATSβ and GST-ATS ˛ fusion proteins containing the acidic tail of -synuclein (ATS) and that of -synuclein (ATS), respectively (Fig. 9A), are resistant to heat-induced aggregation. GST-βATS and GST-γATS fusion proteins were qualitatively examined for heat-resistance by SDS-PAGE. As shown in figure 9B, GST-βATS and GST-γ ATS as well as GST-αATS show protein bands after heat treatment, which indicates that they are not precipitated. Therefore, it is clear that the GST-βATS and GST-γATS fusion proteins have a high heat-resistance. Also, the thermal behaviors of the above GST-ATS fusion proteins were quantitatively assayed by monitoring absorbance at 360 nm according to time

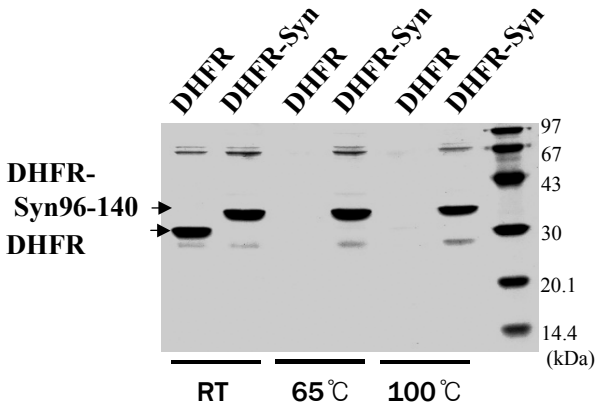

Figure 8. Thermal behavior of the DHFR and DHFR-αATS fusion proteins.

The DHFR and DHFR-ATS fusion proteins (0.6mg/ml) were incubated for 10 min at room temperature, 65°C, and 100°C. The protein solutions were centrifuged and the supernatants were analyzed on a 12% SDS polyacrylamide gel.

while setting the concentration of each protein at 0.2 mg/ml at 65℃[36,76]. In the experiment, as shown in figure 3C, the GST protein had almost aggregated after 2 to 3 minutes. In contrast, the above GST-ATS fusion proteins did not aggregate at all even 10 minutes after heat treatment. Next, the above GST-ATS fusion proteins were qualitatively assayed by monitoring the absorbance at 360 nm while varying the concentration from 0.2 mg/ml to 1.0 mg/ml after heat treatment at 80℃ for 10 minutes. As shown in figure 9D, the above GST-ATS fusion proteins did not precipitate at all after heat treatment regardless of the protein concentration, while the GST protein is completely precipitated even at a low concentration. Thus, it is demonstrated that, in addition to αATS, the βATS and γATS are peptides capable of providing heat resistance to other proteins and they can be used in preparation of fusion proteins having resistance to environmental stresses. Also, it is presumed that since the amino acid sequence of synoretin is very similar to that of γ-synuclein[11], the acidic tail of synoretin may be similarly used.

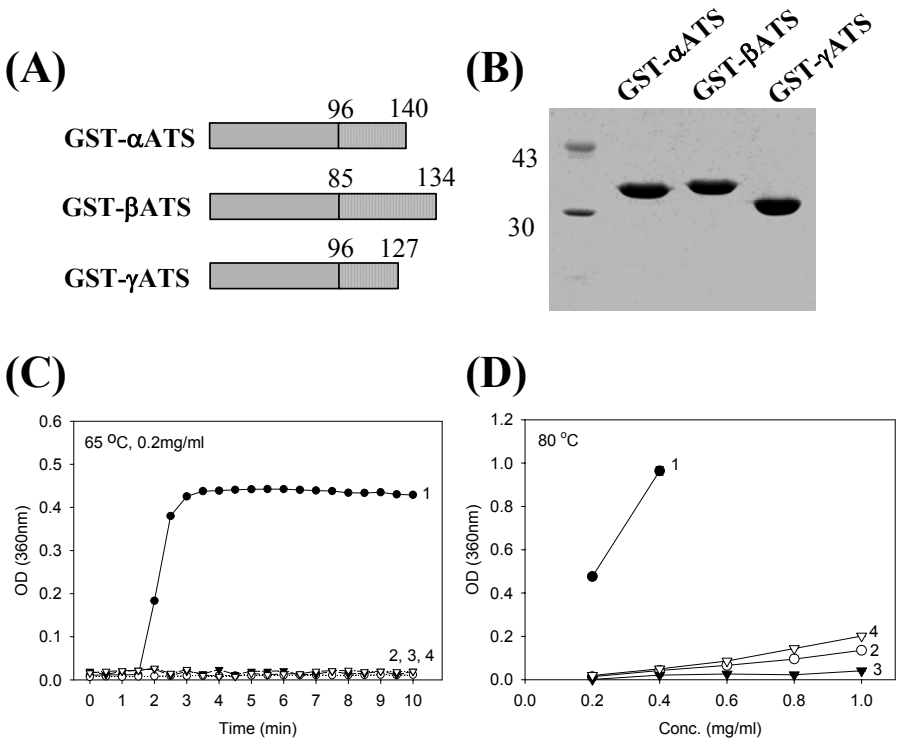

Figure 9. Thermal behavior of GST-βATS and -γATS fusion proteins.

(A) Schematic diagram of GST-α, -β, and -γATS. (B) SDS-PAGE analysis of GST-α, -β, and -γATS after boiling for 10 min. (C) Heat-induced aggregation of the GST-α, -β, and -γATS (0.2 mg/ml) were quantitatively assessed by monitoring the light scattering (OD360) as a function of time at 65°C. (D) Heat-induced aggregation of GST-α, -β, and -γATS were quantitatively assessed by monitoring the light scattering as a indicated concentration for 5 min at 80 °C. (C, D) 1. GST, 2. GST-αATS, 3, GST-βATS, 4. GST-γATS.

9. Thermal behavior of GST-αATS deletion mutants

The C-terminal acidic tail of α-synuclein (αATS) is composed of 45 amino acids (residues 96-140), and 15 Glu/Asp residues are scattered through the αATS. Next, it was investigated whether deletion mutants of GST-αATS fusion protein have heat-resistance. For this purpose, a series of GST-αATS deletion mutants were produced.

Table 3. Isoelectric point (pI) and Hydropathy Values of GST-βATS and -γATS, GST-αATS deletion mutants, GST-E5 and -E10

Proteins	Glu or Asp / total a.a.	pI vaule	Hydropathy vaule
GST-βATS	19/50	4.79	-0.571
GST-γATS	9/32	5.52	-0.511
GST-Syn103115	5/13	5.35	-0.389
GST-Syn114126	6/13	5.26	-0.431
GST-Syn119140	9/22	5.03	-0.503
GST-Syn130140	5/11	5.35	-0.446
GST-E5	5/5	5.38	-0.430
GST-E10	10/10	5.02	-0.495

The pI vaules and hydropathy vaules were calculated by using the ProtParam program (www.expasy.ch).

GST-Syn103-115 contains 13 amino acids of αATS (residues 103-115); GST-Syn114-126 contains 13 amino acids of αATS (residues 114-126); GST-Syn119-140 contains 22 amino acids of αATS (residues 119-140); and GST-Syn130-140 contains 11 amino acids of αATS (residues 130-140). As shown in figure 10B, when these deletion mutants of the GST-αATS fusion proteins were thermally treated at a high concentration (0.8 mg/ml), GST-Syn96-140 containing the entire region of αATS and GST-Syn119-140 containing 22 amino acids of αATS did not precipitate at all, while GST-Syn103-115, GST-Syn114-126 and GST-Syn130-140 containing 11-13 amino acids partially precipitated. On the other hand, when these deletion mutants of the GST-αATS fusion proteins were thermally treated at a low concentration (0.2 mg/ml), all the proteins did not aggregate at all (data not shown).

The thermal behaviors of GST-αATS deletion mutants were quantitatively analyzed by monitoring absorbance at 360 nm according to time while setting the concentration of each protein at 0.2 mg/ml at 65℃. As shown in figure 10C, the OD_{360} of the GST protein drastically increased 2 minutes after heat treatment, and most of the protein had aggregated by 3 minutes. In contrast, the GST-αATS

deletion mutants did not aggregate at all even 10 minutes after heat treatment. Next, the GST-αATS deletion mutants were quantitatively assayed by monitoring the absorbance at 360 nm while varying the concentration from 0.2 mg/ml to 1.0 mg/ml after heat treatment at 80℃ for 10 minutes. As shown in figure 10D, GST-Syn96-140 containing the entire region of αATS and GST-Syn119-140 containing 22 amino acids of αATS did not precipitate at all after heat treatment regardless of the concentration, while GST-Syn103-115, GST-Syn114-126 and GST-Syn130-140 containing 11-13 amino acids did not precipitate at all at a low concentration but increasingly aggregated as the concentration rose. It is well known that the aggregation of protein is proportional to the concentration. Thus, it is demonstrated that the deletion mutants of the GST-αATS fusion protein have heat resistance superior to that of wild type GST, and interestingly, the heat resistance varies according to the length of αATS. Therefore, optimum effects can be achieved by suitably selecting the length of αATS according to the size and property of a target protein.

10. Thermal behavior of GST-poly E (E5, E10) fusion proteins

In the C-terminal acidic tail region of synuclein, a number of negatively charged amino acid residues such as Glu/Asp residues are characteristically scattered therethrough. Next, it was examined whether GST-polyglutamate fusion proteins with genuinely negatively charged peptide fragments such as polyglutamate have heat-resistance. For this, a series of GST-polyglutamate fusion proteins were constructed by ligating the gene part of polyglutamate into pGEX vector (Fig. 11A). GST-E5 (containing 5 glutamate residues) and GST-E10 (containing 10 glutamate residues) were prepared and examined for their heat-resistance. Each protein suspended in PBS (0.6 mg/ml) was heated in a boiling water bath for 10 minutes and cooled in the air. The protein samples were centrifuged at 13,000 rpm for 10 minutes and the supernatants were analyzed on a 12% SDS polyacrylamide gel. Both

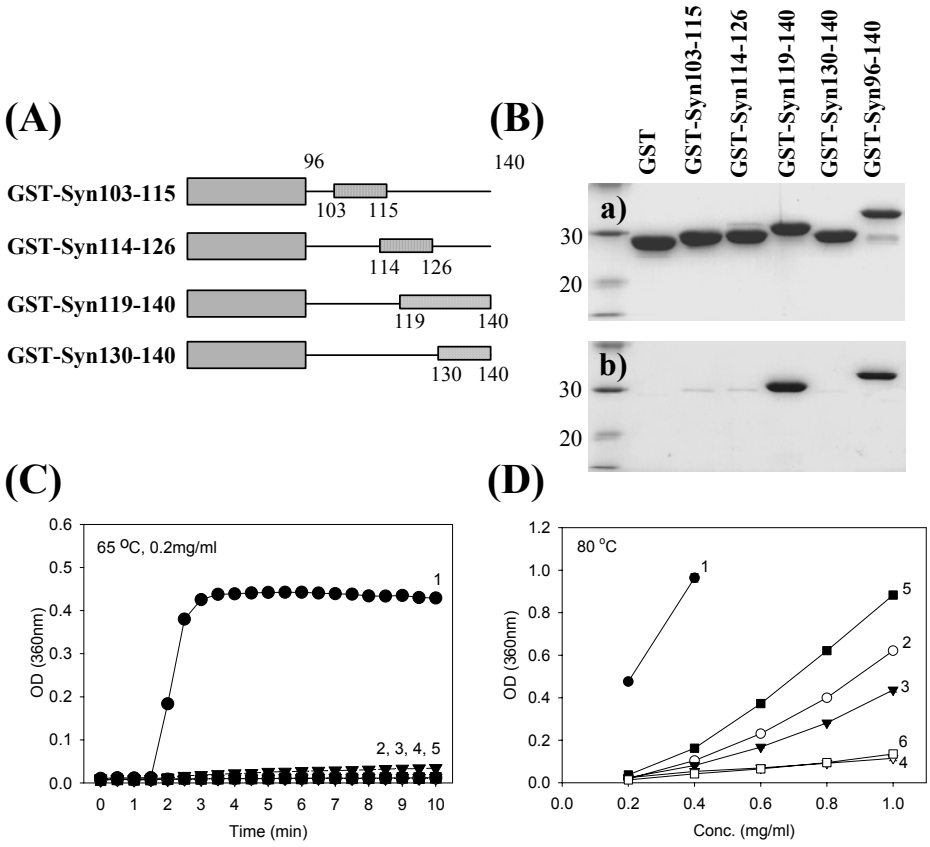

Figure 10. Thermal behavior of GST-αATS deletion mutants.

(A) Schematic diagram of GST-αATS deletion mutants. (B) SDS-PAGE analysis of GST-αATS deletion mutants before and after boiling for 10 min. (C) Heat-induced aggregation of the GST-αATS deletion mutants (0.2 mg/ml) was quantitatively assessed by monitoring the light scattering (OD_{360}) as a function of time at 65°C. (D) Heat-induced aggregation of GST-αATS deletion mutants was quantitatively assessed by monitoring the light scattering as a indicated concentration for 5 min at 80°C. (C, D) 1. GST, 2. GST-Syn103-115, 3. GST-Syn114-126, 4. GST-Syn119-140, 5. GST-Syn130-140, 6. GST-Syn96-140

GST-E5 and GST-E10 did not show protein bands after heat treatment, which indicates that they had been completely precipitated by heat treatment (data not shown). This indicates that the GST-E5 and GST-E10 do not have heat resistance at such stringent conditions.

The thermal behaviors of the above GST-E5 and GST-E10 fusion proteins were quantitatively assayed by monitoring absorbance at 360 nm according to time while setting the concentration of each protein at 0.2 mg/ml at 65°C. As shown in figure 11C, the GST protein were almost aggregated after 2 to 3 minutes and the GST-E5 fusion protein were aggregated in a considerable amount under the same conditions, whereas the GST-E10 fusion protein did not aggregate at all even after heat treatment for 10 minutes at 65°C. Next, the GST-polyglutamate fusion proteins were quantitatively assayed by monitoring the absorbance at 360 nm while varying the concentration from 0.2 mg/ml to 1.0 mg/ml after heat treatment at 80℃ for 10 minutes. As shown in figure 11D, the GST protein is completely precipitated at a low concentration and most of the GST-E5 protein was precipitated at a high concentration. In contrast, the GST-E10 protein was partially precipitated after heat treatment under the same conditions and increasingly aggregated as the concentration rose. Thus, it is noted that, as the length of polyglutamate increases, the negative charge considerably increases and thereby, aggregation decreases. Interestingly, however, it is noted that the polyglutamate tail is considerably less effective to provide heat resistance, as compared to ATS peptides containing the same number of glutamate residues (Table 3). In fact, GST-Syn130-140 shows heat resistance far superior to GST-E5 containing the same number of glutamate residues and even slightly higher than that of GST-E10 containing two times more glutamate residues (compare Fig. 11D with Fig. 12D). Therefore, it is suggested that the characteristic amino acid sequence of ATS, in addition to the increased solubility of proteins due to the increase of the negative charge, plays an important role in the mechanism, by which fusion proteins with ATS show high resistance to environmental stresses. Interestingly, it was also observed that a GST fusion protein containing a positively charged peptide such as polyarginine does not show heat resistance at all (data not shown), which supports that the characteristic amino acid sequence of ATS plays a very important role in providing resistance to environmental stresses.

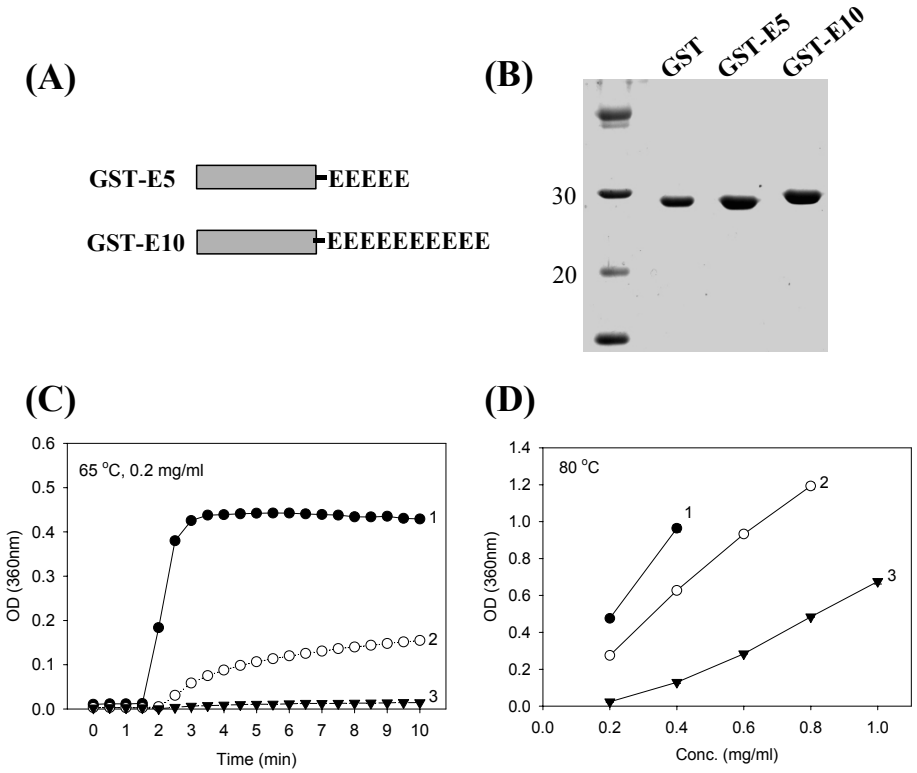

Figure 11. Thermal behavior of GST-E5 and GST-E10 fusion proteins.

(A) Schematic diagram of GST-poly E (E5, E10), (B) SDS-PAGE analysis of GST-E5 and -E10, (C) Heat-induced aggregation of the GST-E5 and -E10 (0.2 mg/ml) was quantitatively assessed by monitoring the light scattering (OD_{360}) as a function of time at 65°C, (D) Heat-induced aggregation of GST-E5 and -E10 was quantitatively assessed by monitoring the light scattering as a indicated concentration for 5 min at 80°C. (C, D) 1. GST, 2. GST-E5, 3. GST-E10

11. Structural Analysis of GST-αATS deletion mutants and GST-poly E fusion proteins

The temperature-induced changes in the ellipticity at 222 nm of GST-Syn96-140 show that the heat-induced unfolding of GST-Syn96-140 appeared to take place in two stages (Fig. 5B). To investigate the temperature induced structural changes of GST-αATS deletion mutants and GST-poly E fusion proteins, melting curves of these proteins were analyzed. Interestingly, the heat-induced unfolding of all of GST-αATS

deletion mutants appeared to take place in two stages like that of GST-Syn96-140 described above (Fig. 12). The transition initial point of GST-αATS deletion mutants started at about 55°C for the first transition and about 90°C for the second, though the first transition initial point of GST-Syn114-126 started at a relatively higher temperature (about 60°C). However, the heat-induced unfolding of GST-poly E fusion proteins appeared to take place in one stage like that of wild-type GST. The correlation between the melting temperature and thermal behavior of GST-αATS deletion mutants was not found in this experiment.

12. Enzyme activity of GST-αATS deletion mutants after heat-treatment

GST-αATS deletion mutant fusion proteins were shown to be heat-resistant like GST-Syn96-140. Next, it was investigated whether the GST-αATS deletion mutants fusion proteins could keep the enzymatic activity after heat treatment. As shown in figure 13, the T_{50} of GST-αATS deletion mutants are between that of GST and that of GST-Syn96-140. This suggests that the introduced αATS deletion fragments is not able to protect the enzyme more efficiently than wild-type ATS, though it can protect thermal aggregation of fusion proteins similar to αATS peptides in low concentration.

II. Distinct Roles of the N-terminal-binding Domain and the C-terminal-solubilizing Domain of α-Synuclein, a Molecular Chaperone

13. The C-terminal acidic tail is necessary, but insufficient for the chaperone activity of α-synuclein

Previous studies have shown that α-synuclein has chaperone activity *in vitro* [28,31]. Like

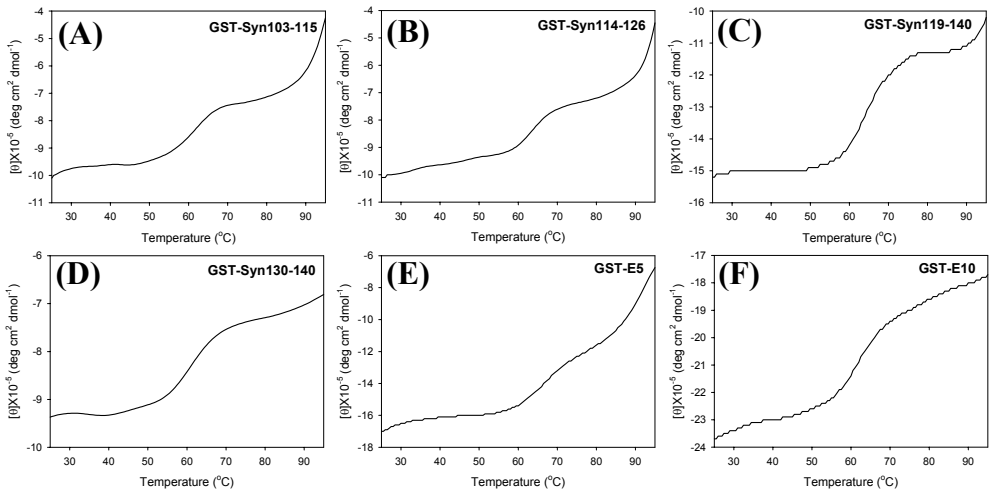

Figure 12. The melting curves of GST-αATS deletion mutants, GST-E5 and GST-E10.

The mean molar ellipticity per residue of each protein at 222 nm was measured a function of temperature respectively.

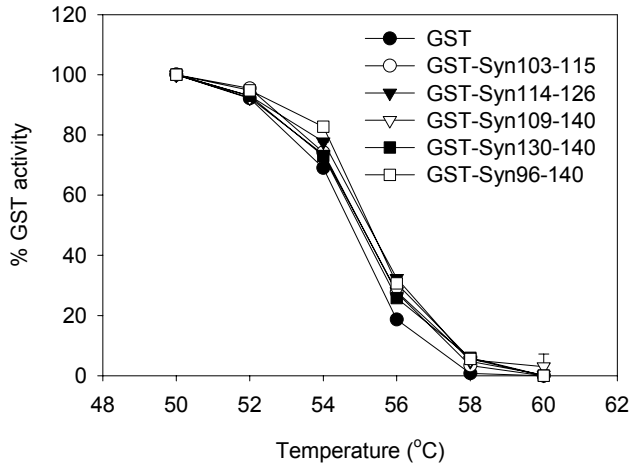

Figure 13. GST activity of the GST-αATS deletion mutants after heat treatment.

Thermal inactivation of GST-αATS deletion mutants was assessed by GST activity assay as a function of temperature. The protein samples were incubated for 5 min at indicated temperatures.

other small molecular chaperone proteins, α-synuclein is able to protect a variety of

proteins from stress-induced aggregation. Interestingly, the chaperone activity of α-synuclein is completely lost upon removing its C-terminal acidic tail [31], which suggests that the acidic tail is critical for its chaperone activity. It was first asked whether the acidic tail is sufficient to have the chaperone activity. Two deletion mutants encoding either the acidic tail only (residues 96-140, Syn96-140) or the NAC region plus the acidic tail (residues 61-140, Syn61-140) were used to address this question and their chaperone activities were compared with that of wild type α-synuclein. The effect of the deletion mutants on the heat-induced precipitation of substrate proteins was first investigated by using the conventional chaperone activity assay. As shown in figure 14. the C-terminal acidic tail alone (Syn96-140) did not protect GST from heat-induced precipitation (Fig. 14A, line 4), whereas Syn61-140 containing the NAC region and the acidic tail appeared to protect GST from heat-induced precipitation almost as effectively as the wild-type α-synuclein (Fig. 14A, lines 3 and 2, respectively). This result suggests that Syn61-140 has all the units necessary for chaperone activity. Similar results were obtained when aldolase was used as the substrate for the chaperone activity assay (Fig. 14B).

The chaperone activity of the α-synuclein deletion mutants were compared with each other by measuring the chemically-induced aggregation of insulin and lysozyme (Figs. 14C and D). Consistent with the results obtained from the heat-induced precipitation assay, Syn96-140 did not protect the substrate proteins from DTT-induced precipitation (Fig. 14C, line 6). On the other hand, Syn61-140 effectively protected the substrate proteins from DTT-induced precipitation (Fig. 14C, lines 3-5; Fig. 14D, lines 3-4). Interestingly, Syn61-140 appeared to be much more efficient than wild type α-synuclein (Figs. 14C and D, line2) in terms of protecting the substrate proteins from DTT-induced precipitation. This aggregation was almost completely suppressed at an insulin to chaperone weight ratio of 1:0.5, corresponding to a stoichiometric ratio of 1:0.3 (Fig. 14C, line 4). Syn61-140 also appeared to protect lysozyme from DTT-induced aggregation at a substoichiometric ratio (Fig. 14D, line 4). Syn61-140, Syn96-140, and wild type α-synuclein alone did not precipitate under these conditions (data not shown). These results indicate that the C-terminal acidic tail

of α-synuclein is necessary, but not sufficient for the chaperone activity of α-synuclein, and also suggest that the N-terminal region (residues1-95) of α-synuclein may determine the efficiency of the chaperone function.

Small molecular chaperones, such as small heat shock proteins, α-crystallin, tubulin and clusterin, prevent protein precipitation by forming soluble HMW complexes [33,37-42,44,45]. It was investigated whether α-synuclein also acts in this way to prevent protein precipitation, and then which region of α-synuclein is critical for substrate protein binding (Fig. 15). The substrate protein (GST) was incubated with wild-type α-synuclein or its C-terminal fragment (Syn96-140) for 10 min at 65°C and the protein mixtures were purified on an FPLC gel-filtration column. Each peak fraction was then analyzed on SDS-polyacrylamide gel. As expected, α-synuclein formed a soluble HMW complex with the substrate protein (Fig. 15A). Syn61-140 also appeared to form a HMW complex with GST (data not shown). In contrast, the C-terminal acidic tail of α-synuclein (Syn96-140) did not form a HMW complex (Fig. 15B), which suggested that the substrate protein binding may be mediated by the N-terminal region of α-synuclein. Unlike other small molecular chaperone proteins, however, the HMW complex formed between α-synuclein and GST contained only a trace amount of α-synuclein, which was detected by immunoblotting (Fig. 15A, f). The immunoreactivity of α-synuclein was only detected in fractions corresponding to the HMW complex, indicating that these HMW forms of α-synuclein are not contamination of the aggregates. A similar phenomenon was observed when other substrate protein was used (data not shown). Previous studies have shown that Syn1-97 precipitates upon heat treatment and does not have the chaperone activity[31]. In addition, NAC and the C-terminally truncated α-synuclein have been shown to aggregate faster than the full-length α-synuclein[29,70-72]. Taken together, it is highly likely that the N-terminal region of α-synuclein functions as a binding domain for substrate proteins and that the C-terminal acidic tail functions as a solubilizing domain for the HMW complexes and for α-synuclein itself.

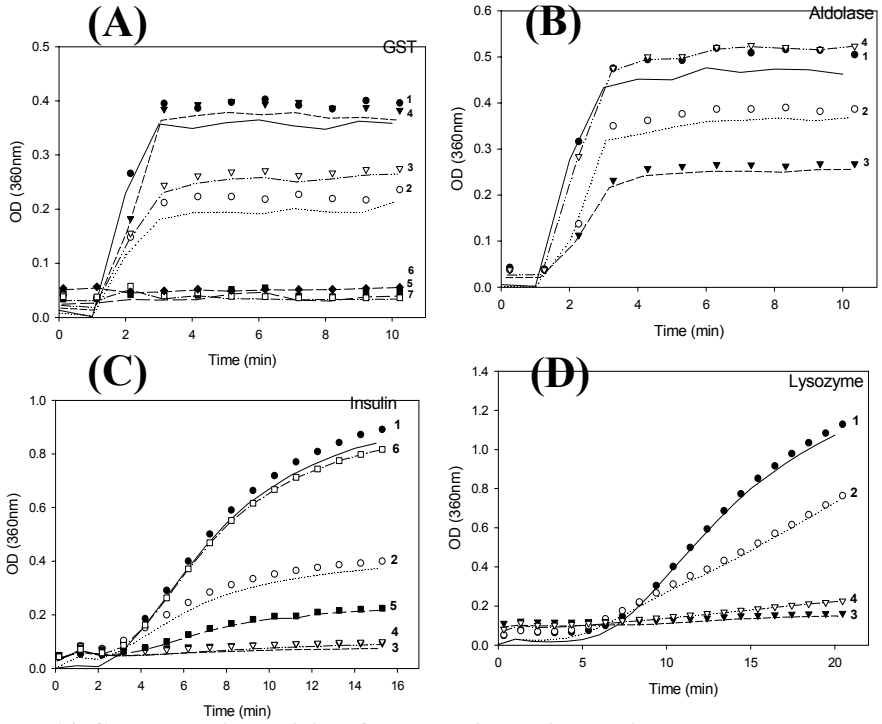

Figure 14. Chaperone-like activity of α-synuclein and its deletion mutants.

(A, B) Thermally induced aggregation assay. (A) Aggregation curves of GST (0.2 mg/ml in PBS, pH 7.4) at 65°C in the absence and in the presence of α-synuclein deletion mutants. Lines: 1, GST alone; 2, GST + α-synuclein (0.2 mg/ml); 3. GST + Syn61-140 (0.2 mg/ml); 4, GST + Syn96-140 (0.2 mg/ml); 5, α-synuclein (0.2mg/ml) alone; 6, Syn61-140 (0.2mg/ml) alone; 7, Syn96-140 (0.2 mg/ml) alone. (B) Aggregation curves of aldorase (0.2 mg/ml in PBS, pH 7.4) at 65°C in the absence and in the presence of α-synuclein deletion mutants. Lines: 1, aldolase alone; 2, aldolase + α-synuclein (0.2 mg/ml); 3, aldolase + Syn61-140 (0.2 mg/ml); 4, aldolase + Syn96-140 (0.2 mg/ml). (C, D) DTT-induced aggregation assay. (C) Aggregation curves of insulin (0.5 mg/ml in 10 mM phosphate buffer, pH7.4) induced with 20 mM DTT in the absence and presence of α-synuclein deletion mutants. Lines: 1, insulin alone; 2, insulin + α-synuclein (0.5 mg/ml); 3, 4, and 5, insulin + Syn61-140 (1:1, 1:0.5, 1:0.1 w/w, respectively); 6, insulin + Syn96-140 (0.5 mg/ml). (D) Aggregation curves of lysozyme (0.5 mg/ml in 10mM phosphate buffer, pH7.4) induced with 20 mM DTT in the absence and presence of a-synuclein deletion mutants. Lines: 1, lysozyme alone; 2, lysozyme + α-synuclein (1:1 w/w); 3 and 4, lysozyme + Syn61-140 (1:1 and 1:0.2 w/w, respectively)

14. The chaperone-like activity of GST and DHFR-αATS fusion protein (The binding domain can be substituted by other proteins)

This study shows that the binding domain and solubilizing domain are structurally distinct in α-synuclein. Based on this finding, it was hypothesized that the binding domain could be substituted by other proteins, since the binding domain of molecular chaperone proteins is not likely to be specific for individual substrate proteins. The chaperone activity of Syn61-140 (Fig. 14) supports this idea. The GST-synuclein fusion protein, GST-Syn96-140, containing the acidic tail of α-synuclein at the C-terminus of GST, was used to prove this hypothesis further. Surprisingly, GST-Syn96-140 prevented GST and aldolase from heat-induced precipitation (Figs. 16A and B). Furthermore, GST-Syn96-140 appeared to be a more efficient chaperone protein than wild type α-synuclein and Syn61-140 (Figs. 14A and B; Figs. 16A and B), and almost completely prevented GST and aldolase from heat-induced precipitation when they were incubated at a ratio of 1:1 w/w (Fig. 16A, line 4; Fig. 16B, line 4). However, GST-Syn96-140 was not as effective as α-synuclein at protecting proteins from chemically-induced precipitation (Figs. 16C and D). In Particular, GST-Syn96-140 did not prevent lysozyme from DTT-induced precipitation, although it slightly alleviated the DTT-induced precipitation of insulin.

Like other small molecular chaperone proteins, GST-Syn96-140 formed a HMW complex when incubated with aldolase, a substrate protein, for 10 min at 65°C (Fig. 16E). GST-Syn96-140 also formed a HMW complex with GST when the proteins were co-incubated at 65°C (data not shown). Unlike α-synuclein, GST-Syn96-140 appeared to form HMW complexes with a stoichiometric ratio (Fig. 16Ed; Fig. 16Ef, lanes 3-6), which suggests that it interacts with substrate proteins in a similar way to the sHSPs. These results indicate that the binding domain of α-synuclein can be substituted with another protein or peptide and the resultant engineered protein functions as a molecular chaperone with a different efficiency and specificity.

To address whether any proteins containing the acidic tail of α-synuclein

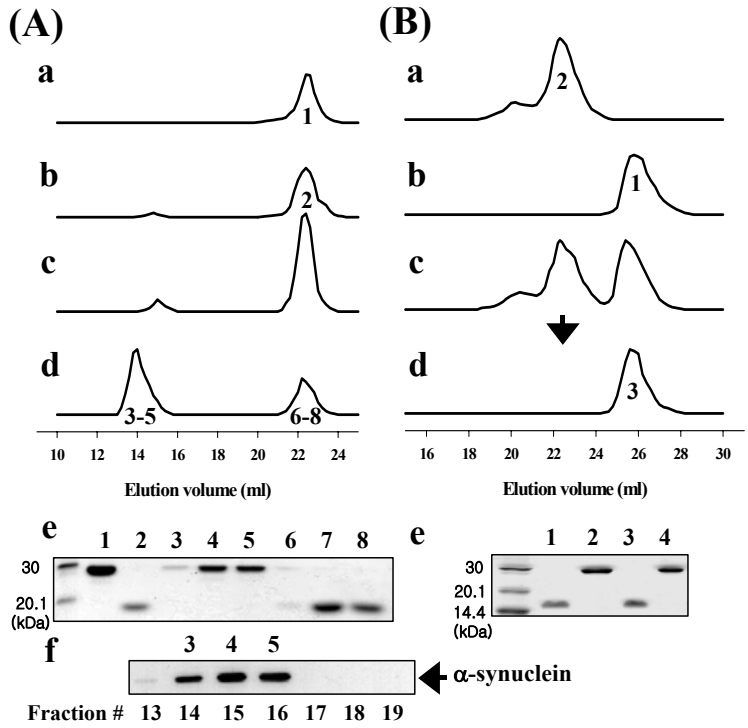

Figure 15. The N-terminal region of α-synuclein mediates substrate protein binding.

(A) FPLC gel-filtration chromatography (a-d) and SDS-PAGE (e,f) analysis of the HMW complex of wild type α-synuclein and GST. Protein samples (500 μl) were loaded onto the Superdex 75 HR column (Pharmacia) equilibrated in PBS, and eluted at a flow rate of 1ml/min at room temperature. a. GST (0.1 mg/ml). b. α-synuclein (0.5 mg/ml). α-synuclein incubated at 65°C for 10 min was eluted at the same position (data not shown) c. GST (0.1 mg/ml) + α-synuclein (0.5 mg/ml) were mixed and loaded onto the column. d. GST (0.1 mg/ml) + α-synuclein (0.5 mg/ml) were mixed and incubated for 10 min at 65°C, then loaded onto the column. e. SDS-PAGE analysis of the α-synuclein:GST complex. The peak fractions numbered in a-d were separated on a 12% SDS-polyacrylamide gel and stained with Coomassie brilliant blue R-250. f. Western blot was performed to detect α-synuclein in the HMW complex (containing peak fractions 3-5 from d). (B) FPLC gel-filtration chromatography (a-d) and SDS-PAGE (e) analysis of the Syn96-140:GST complex a. GST (0.2 mg/ml). b. Syn96-140 (0.2 mg/ml). c. GST (0.2 mg/ml) + Syn96-140 (0.2 mg/ml) were mixed and loaded onto the column. d. GST (0.2 mg/ml) + Syn96-140 (0.2 mg/ml) were mixed and incubated for 10 min at 65°, then loaded onto the column. The first arrow indicates the expected position for GST. e. SDS-PAGE analysis of the Syn96-140:

GST complex. The peak fractions numbered in a-d were separated on a 12% SDS-polyacrylamide gel and stained with Coomassie brilliant blue R-250. Lanes: 1, Syn96-140; 2, GST; 3, peak 3 from d; 4, Pellet fraction obtained after incubating the Syn-96-140:GST mixture for 10 min at 65°C.

have chaperone activity, The DHFR-synuclein fusion protein, DHFR-Syn96-140, which contains the acidic tail of α-synuclein at the C-terminus, was constructed. Interestingly, DHFR-Syn96-140 was extremely heat-resistant, whereas DHFR was so heat-labile that it easily precipitated by thermal stress (Fig. 8). The chaperone activity of DHFR-Syn96-140 was next examined. As shown in figure 17, DHFR-Syn96-140 effectively protects aldolase from heat-induced aggregation, indicating that the fusion protein functions as a molecular chaperone. DHFR-Syn96-140 also appeared to prevent GST from heat-induced precipitation (data not shown). Therefore, it is highly likely that the C-terminal acidic tail of α-synuclein can be used to engineer synthetic chaperones.

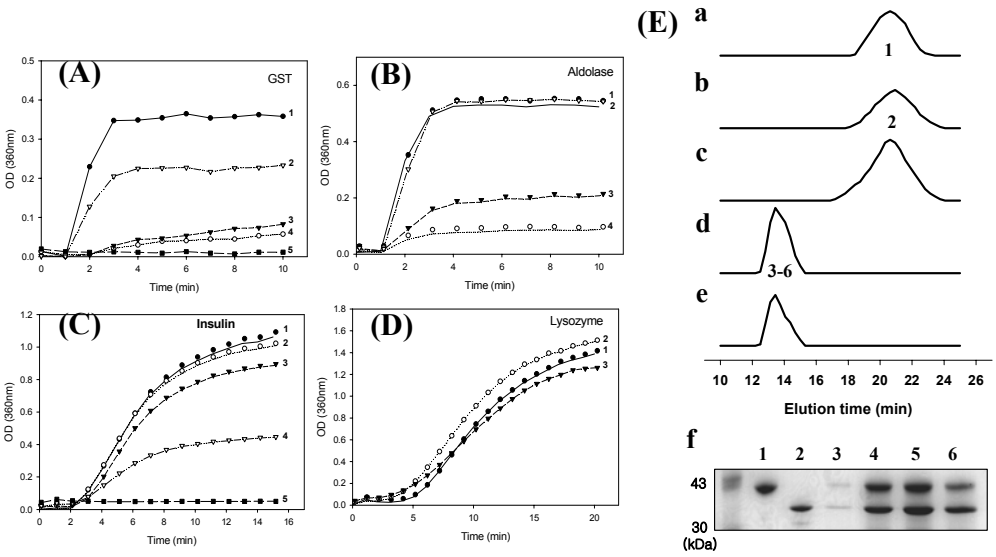

Figure 16. Chaperone-like activity of GST-Syn96-140.

(A, B) Thermally induced aggregation assay. (A) Aggregation curves of GST (0.2 mg/ml in PBS, pH 7.4) at 65°C in the absence and in the presence of GST-Syn96-140. Lines: 1, GST alone; 2, 3,

and 4, GST + GST-Syn96-140 (1:0.1, 1:0.5, and 1:1 w/w, respectively); 5, GST-Syn96-140 (0.2 mg/ml) alone. (B) Aggregation curves of aldolase (0.2 mg/ml in PBS, pH 7.4) at 65°C in the absence and in the presence of GST-Syn96-140. Lines: 1, aldolase alone; 2, 3, and 4, aldolase + GST-Syn96-140 (1:0.2, 1:0.5, and 1:1 w/w, respectively). (C, D) DTT-induced aggregation assay. (C) Aggregation curves of insulin (0.5 mg/ml in 10 mM phosphate buffer, pH7.4) induced with 20 mM DTT in the absence and in the presence of GST-Syn96-140. Lines: 1, insulin alone; 2, 3, and 4, insulin + GST-Syn96-140 (1:1, 1:3, 1:5 w/w respectively); 5, GST-Syn96-140 (0.5 mg/ml) alone. (D) Aggregation curves of lysozyme (0.5 mg/ml in 10 mM phosphate buffer, pH7.4) induced with 20 mM of DTT in the absence and in the presence of GST-Syn96-140. Lines: 1, lysozyme alone; 2 and 3, lysozyme + GST-Syn96-140 (1:1 and 1:3 w/w, respectively). (E) FPLC gel-filtration chromatography (a-d) and SDS-PAGE (e) analysis of the HMW complex between GST-Syn96-140 and aldolase. Protein samples (500 μl) were loaded onto the Superdex 75 HR column (Pharmacia) equilibrated in PBS, and eluted with a flow rate of 1 ml/min at room temperature. a. Aldolase (0.2 mg/ml). b. GST-Syn96-140 (0.2 mg/ml). c. Aldolase (0.2 mg/ml) and GST-Syn96-140 (0.2 mg/ml) were mixed and loaded onto the column. d. Aldolase (0.2 mg/ml) and GST-Syn96-140 (0.2 mg/ml) were mixed and incubated for 10 min at 65°C, then loaded onto the column. e. GST-Syn96-140 (0.2 mg/ml) were incubated for 10 min at 65°C, then loaded onto the column. f. SDS-PAGE analysis of the HMW complex. The peak fractions numbered in a-d were separated on a 12% SDS-polyacrylamide gel and stained with Coomassie brilliant blue R-250.

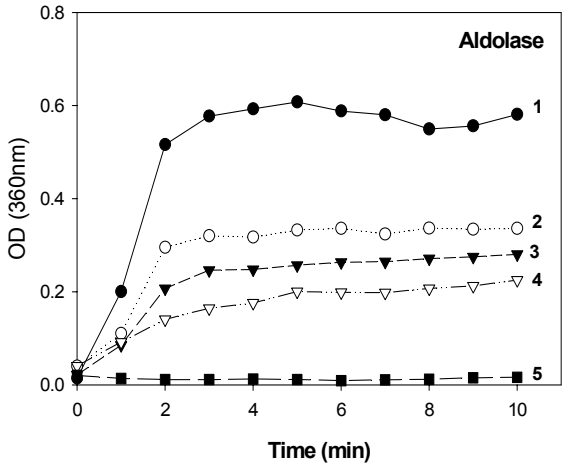

Figure 17. Chaperone-like activity of DHFR-Syn96-140.

Aggregation curves of aldolase (0.2 mg/ml in PBS, pH 7.4) at 65°C in the absence and in the

presence of DHFR-Syn96-140. Lines: 1, aldolase alone; 2, 3, and 4, aldolase + DHFR-Syn96-140 (1:0.5, 1:1, and 1:2 w/w, respectively); 5, DHFR-Syn96-140 (0.2 mg/ml) alone.

15. The binding domain determines the efficiency and specificity of the chaperone function.

This study show that engineered chaperone proteins (Syn61-140, GST-Syn96-140 and DHFR-Syn96-140) containing the acidic tail of α-synuclein as a solubilizing domain display different chaperone activities and substrate specificities, and that these differences might originate from the intrinsic properties of the binding domain. For example, Syn61-140 appeared to be a better chaperone than wild type α-synuclein at preventing the DTT-induced precipitation of substrate proteins (Figs. 14C and D). Furthermore, GST-Syn96-140 appeared to inhibit the heat-induced precipitation of substrate proteins far more so than wild-type α-synuclein or Syn61-140 (Figs. 16A and B), but to only weakly suppress the DTT-induced precipitation of substrate proteins (Figs. 16C and D). To confirm the notion that the binding domain might determine the efficiency and specificity of the chaperone function, the conformational changes in GST-Syn96-140 was induced by heating, and the changes were examined in its chaperone-like activity during the DTT-induced aggregation of insulin. The conformation of GST-Syn96-140 was irreversibly changed at high temperatures with a melting temperature (Tm) of 62ºC, but insulin alone did not precipitate at this temperature (data not shown). Interestingly, the chaperone activity of GST-Syn96-140 in the DTT-induced aggregation of insulin was significantly improved at 59ºC (Fig. 18A) compared to the case at room temperature (Fig. 16C), suggesting that the efficiency of its chaperone action is affected by conformational changes in its N-terminal substrate binding domain (GST domain in this case). In addition, it was found that GST-Syn96-140 did not protect the heat-induced aggregation of luciferase, while α-synuclein and Syn61-140 effectively prevented this aggregation (Fig. 18B). Therefore, it seems highly likely that the binding domain determines the efficiency and specificity of the chaperone function.

16. The chaperone-like activity of GST-βATS, GST-γATS, GST-αATS deletion mutants, and GST-poly E fusion proteins

It is highly likely that the C-terminal acidic tail of α-synuclein can be used to engineer synthetic chaperones. It was next investigated whether the acidic tails of β-, and γ-synuclein have same properties like that of α-synuclein. GST-βATS and -γATS prevented GST from heat-induced precipitation comparable to GST-Syn96-140 (Fig. 19). This suggested that the acidic tails of other synuclein family can be also used to

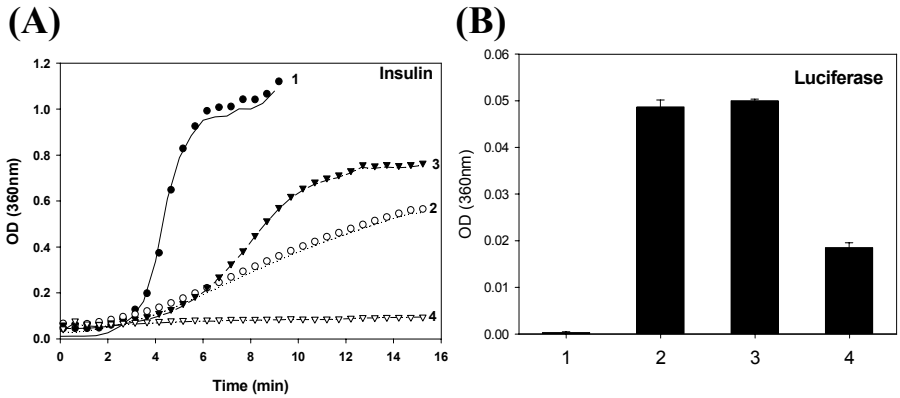

Figure 18. Substrate specificity of GST-Syn96-140 as a molecular chaperone.

(A) Temperature effect on the chaperone-like activity of GST-Syn96-140. Protein mixtures were preincubated for 5 min at 59°C, then 2 mM of DTT was added to induce the aggregation of insulin, and mixtures were then placed on a thermostatic cell holder. Lines: 1, insulin (0.5 mg/ml) alone; 2, insulin + GST-Syn96-140 (1:1 w/w); 3, insulin + GST-Syn96-140 (1:0.5 w/w); and 4, GST-Syn96-140 (0.5 mg/ml) alone. (B) GST-Syn96-140 did not protect luciferase from heat-induced aggregation. Protein mixtures were incubated for 10 min at 65°C, and light scattering was measured at 360 nm. Graphs: 1, luciferase (0.1 mg/ml) alone at room temperature; 2, luciferase (0.1 mg/ml) alone at 65°C; 3, luciferase + GST-Syn96-140 (1:1 w/w) at 65°C; and 4, luciferase + Syn61-140 (1:1 w/w) at 65°C.

engineer synthetic chaperones like that of α-synuclein. Also, the GST-αATS deletion mutants prevented GST from heat-induced precipitation. Interestingly, the efficiencies of synthetic chaperones are different from each other in some degree (Fig. 20). This

suggested that not only the acidic tail of synuclein acts a solubilizing domain but also that of α-synuclein may have an influence on the fusion protein and/or substrate proteins.

Subsequently, it was investigated whether GST-E5 and -E10 have chaperone-like activity. As shown in figure 20, GST-E5 had not chaperone-like activity. As the concentration of GST-E5 increased, the turbidity also increased. The degree of turbidity was like the sum of the turbidity of GST and GST-E5. GST-E10 appeared to have chaperone-like activity. However, the degree of turbidity increased, as the concentration of GST-E10 increased. As mentioned above, GST-E10 is less thermostable than GST-αATS deletion mutants, as the concentration increase. This also suggested that the interaction of fusion protein and acidic tail may also have an important role in chaperone-like activity in addition to the solubilizing effect of the acidic tail of synuclein.

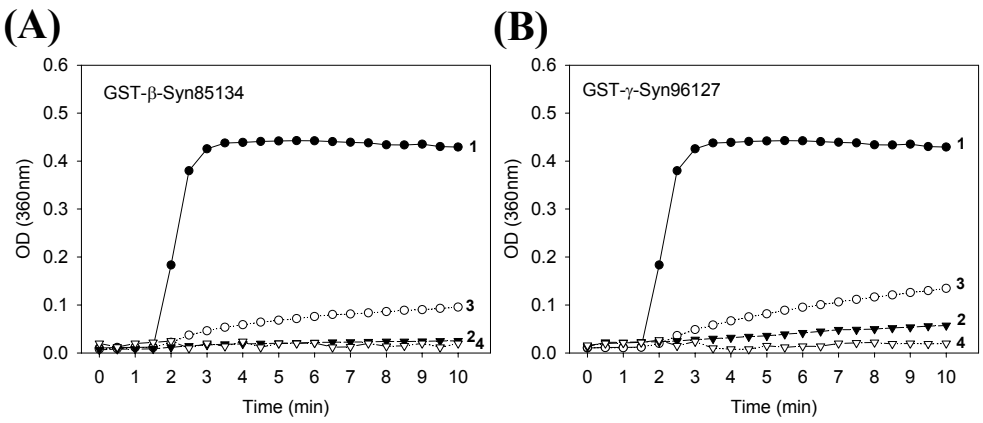

Figure 19. Chaperone-like activity of GST-βATS and -γATS

Aggregation curves of GST (0.2 mg/ml in PBS, pH 7.4) at 65°C in the absence and in the presence of GST-β and γATS (A, B) Lines: 1, GST alone; 2, and 3, GST + GST-βATS and -γATS (1:1, and 1:0.2 w/w, respectively); 4, GST-βATS and -γATS (0.2 mg/ml) alone, respectively.

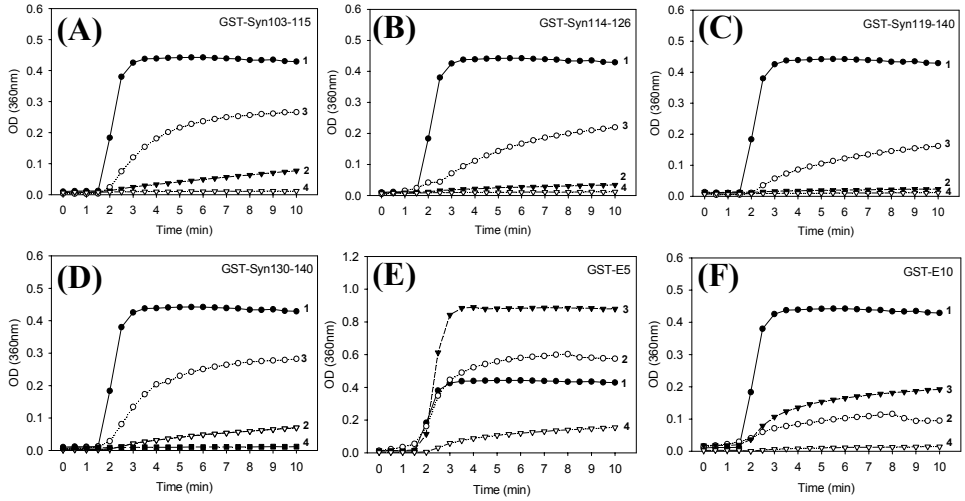

Figure 20. Chaperone-like activity of GST-αATS deletion mutants, GST-E5, and -E10

Aggregation curves of GST (0.2 mg/ml in PBS, pH 7.4) at 65°C in the absence and in the presence of GST-αATS deletion mutants (A, B, C, D) Lines: 1, GST alone; 2, and 3, GST-αATS deletion mutants (1:1, and 1:0.2 w/w, respectively); 4, GST-αATS deletion mutants (0.2 mg/ml) alone, respectively. Aggregation curves of GST (0.2 mg/ml in PBS, pH 7.4) at 65 °C in the absence and in the presence of GST-E5 and E10 (E, F) Lines: 1, GST alone; 2, and 3, GST-E5 or E10 (1:1, and 1:2 w/w, respectively); 4, GST-E5 or E10 (0.2 mg/ml) alone, respectively.

17. GST-Syn96-140 does not protect enzymes from heat-induced inactivation

Small molecular chaperone proteins are generally known to be inefficient at preventing the thermal inactivation of enzymes[36,44-46,77], although some small molecular chaperone proteins are reported to have a marginal potential to protect certain enzymes from thermal inactivation[34,38,48-51]. Previously, it was shown that α-synuclein did not protect GST enzyme from heat-induced inactivation[28]. To investigate whether GST-Syn96-140 is able to protect enzymes from thermal inactivation, the thermostabilities of GST and PTP-1B were measured using thermal inactivation curves in the presence and in the absence of the GST-Syn96-140 (Fig. 21). The thermal inactivation curves were used to determine the T_{50} values, the temperatures at

which 50% of initial enzymatic activity is lost after heat treatment. As shown in figure 21, T_{50} values of GST and PTP-1B appeared to be similar in the presence and in the absence of the chaperone protein. This suggests that GST-Syn96-140 is incapable of protecting enzymes from thermal inactivation, although it can prevent the enzymes from thermal aggregation.

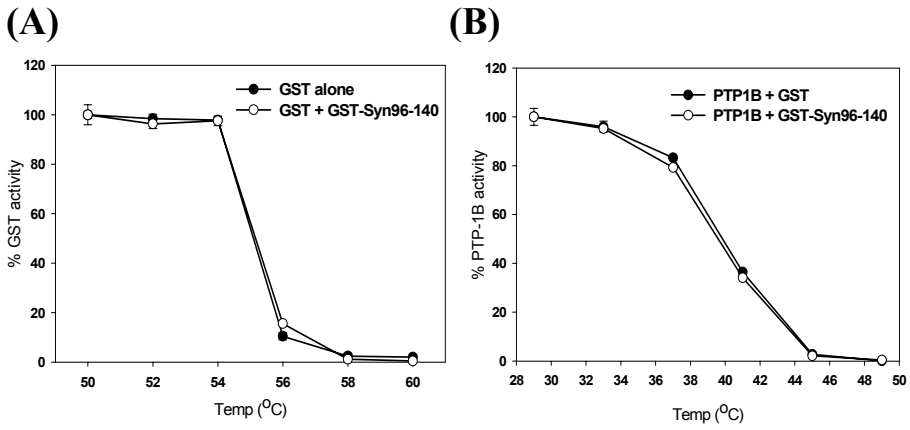

Figure 21. Thermal inactivation curves of (A) GST and (B) PTP-1B in the presence and in the absence of GST-Syn96-140.

Activity is expressed as a percentage of initial activity. Values are the means of three independent experiments, and the standard deviation is shown as bars. The protein samples were incubated for 5 min at the indicated temperatures, and the enzymatic activities measured as described in Materials and Methods at 37ºC.

IV. DISCUSSION

α-Synuclein is a well-known heat-resistant protein that does not aggregate when heat treated, whereas GST is a heat-labile protein that easily precipitates by thermal stress. In this study, it was shown that the C-terminal acidic tail of α-synuclein (residues 96-140) renders the protein heat-resistant. Furthermore, it was demonstrated that the GST-α-synuclein fusion protein is also heat-resistant. Like the α-synuclein, the fusion protein appears to be extremely thermosoluble even when it is boiled in a water bath. Using a series of GST-synuclein deletion mutants, it was shown that the C-terminal acidic tail of α-synuclein also plays a critical role in conferring the heat-resistancy of the fusion proteins. In addition, the acidic tail turns out to protect the fusion protein from the loss of activity after heat treatment. However, a systematic investigation of the heat-induced secondary structural changes of α-synuclein, GST and the GST-α-synuclein fusion protein reveals that the fusion protein is irreversibly denatured by heat treatment. Interestingly, the GST protein fused with the acidic tail appears to be more resistant to the pH- and metal-induced protein aggregation, suggesting that the acidic tail increases the virtual stability of the protein by protecting it from environmental stresses.

The C-terminal acidic tails of the synuclein family members are very diverse in size as well as in sequence[7-9,68]. Considering that the N-terminal amphipathic region is strictly conserved among the synuclein family members from the Torpedo to humans, the C-terminal acidic tail may be responsible for the specificity of each synuclein protein. In this study, the C-terminal acidic tail of α-synuclein appears to play a critical role in conferring heat-resistancy to the GST fusion protein as well as to the synuclein protein itself. Also, the C-terminal acidic tails of β- and γ-synuclein appear to play a critical role in conferring heat-resistancy to the GST fusion protein. Presumably, the abolishment of heat-induced protein aggregation in α-synuclein and the GST-synuclein fusion proteins at high temperatures results from the facts that the acidic tail increases the solubility of protein by increasing the hydrophilicity of protein, and that the acidic tail makes the intermolecular interaction unfavorable by repulsion between negatively charged residues. This idea is supported by the observation that α-

synuclein with a truncated C-terminal region and the NAC peptide lacking the C-terminal acidic tail are found to aggregate faster than the full-length α-synuclein under the same conditions[29,70-72]. It is well documented that the solubility of a protein is approximately proportional to the square of the net charge on the protein[78]. In fact, introducing the acidic tail significantly decreases the pI and hydropathy values of the fusion proteins (Table 2). Considering that all the synuclein proteins are extremely heat-resistant, all the acidic tails from the synuclein proteins may increase the solubility of the fusion proteins and consequently could make the proteins heat-resistant. Consistently, Kumar et al, reported that an increase in the proportion of charged residues and improved electrostatic interactions are among the most consistent mechanisms for increasing protein thermal stability[79].

The acidic tail appears to protect the fusion protein from the thermal inactivation to a considerable extent. Presumably, a significant increase in thermostability observed from the GST-Syn96-140 results from the fact that the acidic tail protects the protein from heat-induced aggregation. As shown in figure 6b, GST aggregates from 52ºC, which is much lower than the Tm (70ºC). Interestingly, loss of GST activity is very well correlated with the aggregation of the protein that occurs prior to complete unfolding. On the other hand, GST-Syn96-140 does not aggregate, but it unfolds at high temperature with the Tm of 62°C. For the case of GST-Syn96-140, loss of activity appears to result from the unfolding of the protein. The acidic tail also appears to protect the fusion protein from pH- and metal-induced aggregation. Therefore, it is likely that the introduced acidic tail increases the virtual stability by protecting the protein from stress-induced aggregation, although the effect on thermostability is limited due to the decrease of the Tm (from 70°C to 62ºC).

α-Synuclein has the potential to bind several divalent cations and metal ions including Fe^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+} and Ca^{2+} [66,73-75]. Metal ions (Fe^{2+}, Al^{3+}, Zn^{2+} and Cu^{2+}) bind to α-synuclein and induce self-oligomerization of the protein. Cu^{2+} and Ca^{2+} are known to specifically bind to the C-terminal acidic tail with a dissociation constant of 59 μM and an IC_{50} of 300 μM, respectively[73,74]. However, the binding sites and binding constants of Fe^{2+}, Al^{2+}, Zn^{2+} have yet to be determined. In this study, divalent cation binding does not appear to affect the thermal behavior of both α-synuclein and

the GST-α-synuclein fusion proteins, although nonspecific binding at high cation concentrations appears to induce a certain amount of protein aggregation.

Four GST-αATS deletion mutants were used to investigate the mechanism of themostability of fusion proteins in more details. All of GST-αATS deletion mutants are heat-resistant though the degree of heat-resistancy varies according to its sequence and size, respectively. All of them also lower the pI and hydropathy value of GST (Table 3). These also suggest that the effect of the lowering the pI and hydropathy value through the fusion with acidic tail have an important role in thermostability of fusion proteins. However, as shown in that of GST-E5 and -E10 fusion proteins, the dramatic heat-resistancy of GST-synuclein fusion proteins could not be explained through only the effect of lowering PI and hydropathy value of acidic tail of synuclein. Other factors of acidic tail, such as the structural changes by the interaction of fusion partner and acidic tails, in addition to the electrostatic repulsion, could also participate in their thermostability.

Subsequently, the shortening the size of the acidic tail seems not to increase the protein stability in terms of protein aggregation profiles and enzymatic activities. Interestingly, the GST-αATS deletion mutants have two transition points as shown in the temperature scan like GST-Syn96-140. However, the GST-E5 and -E10 have only one transition point like wild-type GST. These findings suggest that the acidic tail of synuclein may have an influence on the heat-induced unfolding of GST fusion proteins, and the interaction between the fusion protein and acidic tails may make themselves more heat-resistant than GST-E5 and -E10. More detailed investigation is necessary to clarify the mechanism of thermostability of ATS fusion proteins.

α-Synuclein has been suggested to work as a chaperone protein in mammalian cells[30], and recent studies have shown that α-synuclein acts as a chaperone *in vitro*, and that the C-terminal truncated form of α-synuclein (Syn1-97) has no chaperone activity[28,31]. This study showed that the C-terminal acidic tail itself (Syn96-140) dose not interact with the substrate protein and consequently does not protect the protein from stress-induced aggregation. However, in common with wild-type α-synuclein, an N-terminal truncated form of α-synuclein (Syn61-140) binds the

substrate protein and retains the chaperone activity, albeit with a slightly different efficiency and substrate specificity. This indicates that the N-terminal region of α-synuclein (residues 1-95) plays a critical role in substrate protein binding, and that the C-terminal acidic tail might function to solubilize the HMW complex. Interestingly, the N-terminal binding domain can be substituted by other proteins or peptides, such as GST, DHFR or NAC peptide, and the resulting fusion proteins were also found to have chaperone activity. More importantly, the synthetic chaperone proteins appear to display differential chaperone activity in terms of their efficiencies and substrate specificities.

Most of chaperone proteins have a flexible, hydrophilic tail that is important for proper chaperone function. For example, GroEL is known to have flexible N- and C-terminal tails, which protrude into the central cavity of the molecule[80]. SecB, a bacterial chaperone involved in protein export, has been proposed to have a highly flexible C-terminal region that is involved in binding to non-native proteins[81]. X-ray crystallographic and NMR spectroscopic analyses have shown that sHSPs, such as HSP25, HSP16 and α-crystallin, contain a flexible C-terminal extension[33,35,63,82]. Other small molecular chaperones, such as clusterin and tubulin, are also known to have a flexible tail at the C-terminus[77,83]. These flexible, hydrophilic tails have been suggested to play a critical role in substrate and chaperone protein interactions, and to function as a solubilizer [32,58-61]. In fact, a mutation in the C-terminal end or a deletion of the C-terminal end caused a significant decrease in the chaperone activity of α-crystallin[60,62]. Tubulin also lost its chaperone-like activity when the C-terminal acidic tail was removed by protease digestion[83]. A previous study showed that the removal of the C-terminal acidic tail of α-synuclein abolished its chaperone activity[31]. This study indicate that the C-terminal acidic tail is indeed necessary, but not sufficient for the chaperone function of α-synuclein. The acidic tail itself does not have chaperone activity, and does not appear to interact with the substrate protein. It is highly likely that the role of the introduced acidic tail is to increase protein solubility by electrostatic repulsions. In fact, introducing the acidic tail greatly decreases the pI and hydropathy values of the fusion protein, and the C-terminal truncated α-synuclein

mutants are found to aggregate faster than the full-length α-synuclein under the same conditions[29,70-72]. Furthermore, fusion proteins containing the α-synuclein acidic tail (GST-Syn96-140 and DHFR-Syn96-140) has chaperone-like activity, which suggests a possible role of the acidic tail in chaperone function might involve solubilizing the substrate-chaperone complex, as well as the chaperone protein itself.

It was shown that the N-terminal region of α-synuclein binds the substrate protein forming a soluble HMW complex (Fig. 15). The GST-Syn96-140 fusion protein also appears to form such a HMW complex, suggesting that the GST domain interact with the substrate protein (Fig. 16). Interestingly, however, the efficiency of the chaperone function of GST-Syn96-140 differs from that of wild type α-synuclein. GST-Syn96-140 appeared to be more efficient than α-synuclein at preventing GST and aldolase from heat-induced aggregation, but less efficient at preventing DTT-induced aggregation of insulin and lysozyme (Fig. 16). In addition, an N-terminal truncated form of α-synuclein (Syn61-140) appeared to be more efficient than wild type α-synuclein at preventing proteins from DTT-induced aggregation (Fig. 14). These results strongly suggest that the N-terminal binding domain plays a crucial role in the efficiency of the chaperone function. This idea is further supported by the observation that GST-Syn96-140 effectively prevents insulin from DTT-induced aggregation at elevated temperatures (Fig. 18A). At the elevated temperatures, tertiary structure of GST must be changed and the perturbed structure seems to become more favorable for substrate protein binding. Furthermore, GST-Syn96-140 does not protect luciferase from heat-induced aggregation (Fig. 18B), although it effectively protects GST and aldolase (Figs. 16A and B) and α-synuclein is able to protect all these molecules from heat-induced aggregation (Figs. 14A and B. Fig. 18B). These results suggest that the chaperone function of GST-Syn96-140 is much more specific/limited than that of α-synuclein. Taken together, our data demonstrate that the N-terminal binding domain governs the efficiency and the substrate specificity of the chaperone proteins.

The chaperone action of sHSPs requires a common step of substrate protein

binding and a subsequent step of solubilizing the HMW complex of chaperone and substrate protein[33,37-45]. The present study demonstrates that α-synuclein functions in the same manner as the sHSPs; α-synuclein prevents protein aggregation by binding substrate protein and subsequently by solubilizing the HMW complex. Furthermore, our results show that the substrate binding domain and the solubilizing domain are clearly separated in α-synuclein; the N-terminal region (residues 1-95) binds the substrate protein and the C-terminal acidic tail (residues 96-140) solubilizes the HMW complex. Unlike α-synuclein, however, sHSPs do not appear to have well-separated substrate binding and solubilizing domains. In the case of sHSPs, the hydrophobic and the charged/hydrophilic regions are scattered through the N- and C-terminal domains[54]. sHSPs also have short, hydrophilic extensions at the C-terminus (10-15 residues), but these C-terminal extensions play a significant role in substrate binding, as well as in solubilizing the HMW complexes[59,61]. Furthermore, C-terminal truncated forms of sHSPs still retain the chaperone activity, although the chaperone activity is somewhat reduced and limited in some cases[57,62]. This further suggests that the amino acid residues responsible for substrate binding and solubilizing sHSP based HMW complexes are scattered through the whole of the sHSP molecules.

The chaperone activity of sHSPs towards DTT- and UV-induced protein aggregation is enhanced as the temperature increases, since the conformation of sHSPs is presumably perturbed and consequently hydrophobic surfaces are more exposed at the higher temperatures[45,84-89]. A similar temperature-dependent interaction between GroEL and substrate protein has been reported[90]. The chaperone-like activity of tubulin also becomes more pronounced as temperature increases[83]. Previously, it was reported that preheating α-synuclein, which is believed to reorganize the molecular surface of the protein, increases its chaperone activity[28]. This study demonstrated that GST-Syn96-140 more efficiently protects insulin from DTT-induced aggregation at 59ºC than at room temperature (Fig. 18A). Therefore, as has been observed for other molecular chaperone proteins, temperature-induced structural perturbation of the GST domain (substrate binding domain) seems to be responsible for the increased chaperone-like activity observed at higher temperatures.

Interestingly, GST-βATS and -γATS also have chaperone-like acitivity. Both fusion proteins protect GST from heat-induced aggregation like GST-Syn96-140. This study suggest that β- and γ-synuclein may have same properties like α-synuclein, and share the mechanism of the chaperone action with α-synuclein. Also, GST-αATS deletion mutants protect GST from heat-induced aggregation. These findings are consistent with the hypothesis that the N-terminal domain acts as the binding domain, and the acidic tail acts as the solubilizing domain. However, the GST-E5 did not protect GST from heat-induced aggregation, and the GST-E10 has minimal activity of the protection from heat-induced aggregation of GST. Also, the efficiency of chaperone activity of GST-αATS deletion mutants differed from each other in some degrees. These suggested that the acidic tail of synuclein acts not only as the solubilizing domain, but also as a modulator of fusion protein through the interaction of fusion partner and acidic tail. This idea is supported by the observation of the thermal behavior of GST-αATS deletion mutants as described above. In this study, the interaction of GST and syn96-140 was not shown. These findings mean that the acidic tail of synuclein may have influences on only fusion partner and modulate the chaperone activity of fusion partner, or/and the acidic tail of synuclein may bind not only fusion partner but also substrate protein directly, however, the interaction of acidic tail of synuclein and substrate proteins was not shown because of the low affinity. Further investigation of the detailed mechanism of the chaperone activity of acidic tail of synuclein fusion protein is necessary for the elucidation of the role of acidic tail of synuclein in chaperone activity.

The list of the new small molecular chaperones discovered is increasing quite steadily, and recently tubulin, clusterin and nucleolar protein B23 have been added as new members[77,83,91]. Tubulin also has a C-terminal acidic tail and the removal of this tail abolishes its chaperone-like activity[83]. Clusterin contains three putative amphipathic α-helical regions that might mediate interaction with hydrophobic molecules[77], and nucleolar protein B23 has been reported to have chaperone-like activity through a similar mechanism[91]. These chaperone proteins (tubulin, clusterin and B23) have no amino acid sequence similarity with either sHSPs or α-synuclein.

Therefore, it would be interesting to compare detailed the molecular mechanisms of the chaperone action mediated by these molecules with those of α-synuclein and sHSPs.

There are numerous critical applications for proteins in human health care through the protein therapeutics directly, and through the biomedical research indirectly. However, even the most promising and effective protein applications will not be beneficial if its stability cannot be maintained during storage and handling. Instability can lead to protein aggregation, which is a major problem in the biomedical and biopharmaceutical field. In addition to reducing efficacy, aggregates in parenterally administrated proteins can cause adverse patient reactions such as immune response, sensitization, or even anaphylactic shock[92,93]. Therefore, if even a small percentage of the protein molecules are aggregated, a product can be rendered unacceptable. So, it is essential that aggregate formation is completely prevented during all stages of product handling. Many researchers have made an effort to enhance protein thermostability. Two approaches have been done recently, one is to enhance the thermostability of protein itself through mutational analysis, and the other is to develop the protein stabilizers.

In this study, the researches for the acidic tail of synuclein appeared to satisfy the requisite of the two approaches. Many biologically or medically important proteins that have solubility problems or stress-induced aggregation problems might be saved by introducing the acidic tail of α-synuclein. The C-terminal acidic tail of α-synuclein can also be utilized to engineer synthetic chaperones for specific purposes simply by fusing the acidic tail with other proteins or peptides. Such specifically designed chaperone proteins would be useful for stabilizing target proteins both *in vitro* and *in vivo*. Further investigation of the acidic tail of synuclein will help the understanding of protein stability and the efforts for inhibiting the aggregation of protein against environmental stresses.

V. CONCLUSION

It has been demonstrated that the introduction of the acidic tail of α-synuclein into a heat-labile protein protects the protein from environmental stresses, such as heat, pH and metal ions. Consequently, the acidic tail greatly increases the solubility of the fusion protein and significantly improves the thermostability. Overall, these data suggest that the acidic tail contributes to the virtual stability of the protein, although it does not appear to increase its intrinsic stability. Introducing the acidic tail also contributes to the protein solubility since it will greatly increase the hydrophilicity of the protein and make intermolecular interactions unfavorable through electrostatic repulsion. Therefore, the acidic tail of α-synuclein can be utilized to increase protein solubility and to protect the protein from environmental stresses. Many biologically or medically important proteins that have solubility problems or stress-induced aggregation problems might be saved by introducing the acidic tail of α-synuclein.

It has been also demonstrated that the N-terminal region of α-synuclein binds substrate protein and forms a HMW complex, while the C-terminal acidic tail solubilizes the HMW complex during the chaperone action. Since the substrate binding domain and the solubilizing domain are well separated in α-synuclein, the N-terminal binding domain can be substituted with other proteins or peptides. Moreover, the resulting engineered chaperone proteins appear to display different efficiencies and substrate specificities in terms of the chaperone function. This implies that the C-terminal acidic tail of α-synuclein can be utilized to engineer synthetic chaperones for specific purposes simply by fusing the acidic tail with other proteins or peptides. Such specifically designed chaperone proteins would be useful for stabilizing target proteins both *in vitro* and *in vivo*.

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국문요약

단백질의 용해성, 안정성 및 샤페론 활동에 있어서 α-시누클라인의 C-말단 산성꼬리의 역할

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박상면

α-시누클라인은 아미노산 140개로 구성된 이차구조가 거의 없는 비정형구조 단백질로서, 열에 안정하며 전시냅스 말단에서 주로 발견된다. 이 단백질은 N-말단의 아포리포단백질의 지질결합부위와 유사한 양친매성 α-나선구조를 보이는 KTKEGV가 반복되는 부위, 중간의 소수성을 띠는 NAC(Non Aβ Component of Alzheimer's disease) 부분, 그리고 (-) 전위를 띤 아미노산이 상당히 많이 분포하는 C-말단 산성 꼬리 부분으로 구성되어 있다.

열에 안정성을 보이는 α-시누클라인의 영역을 여러개의 결실 돌연변이를 이용하여 측정해본 결과, C-말단 산성 꼬리(96-140번 아미노산)이 α-시누클라인의 열용해성을 유지시키는데 매우 중요하다는 사실을 알아냈고, 또한 글루타치온 에스 트랜스퍼라제(GST) 단백질과 α-시누클라인의

여러 결실 돌연변이와의 융합단백질을 제작하여 열안정성을 측정해본 결과, α-시누클라인의 C-말단 산성꼬리 부분이 GST 융합단백질도 열에 안정성을 갖게 해주는 것을 알아냈다. 더 나아가 이러한 산성 꼬리부분은 pH나 금속에 의해 유도되는 응집에도 융합단백질을 보호하는 것을 볼 수 있었으며, 이를 통해서 산성 꼬리부분이 융합단백질의 실질적인 안정성을 증가시킴으로써 환경스트레스로부터 융합단백질의 응집을 억제할 수 있다는 것을 알아냈다. 또한 산성 꼬리부분은 열로부터 GST 효소작용을 상당한 정도 보호하는 것으로 나타났다. 이러한 α-시누클라인의 산성 꼬리부분의 특성은 β- 와 γ-시누클라인의 산성 꼬리부분에서도 또한 나타나며, GST-αATS 결실돌연변이 단백질들에서도 역시 열에 안정성을 보이는 것을 확인하였다. 흥미로운 사실은 GST-E5와 -E10도 열안정성을 보이나, GST-αATS 결실돌연변이들보다 덜 열에 안정한 것을 보임으로써 산성 꼬리부분의 음전위 외에 시누클라인의 시퀀스 요소도 이러한 현상에 관련되어 있는 것을 알 수 있었다.

다음으로, α-시누클라인의 샤페론 활동에는 C-말단 산성꼬리부분이 반드시 필요하나, 그 부위만으로는 충분하지 않은 것을 알 수 있었다. α-시누클라인은 다른 열충격단백질들처럼 기질단백질과 HMW (High Molecular Weight) 복합체를 형성함으로써 기질단백질을 보호하는 것을 관찰할 수 있었다. 또한 α-시누클라인의 N-말단 영역은 결합부위로, C-말단 영역은 용해부위로 작용하는 것을 알 수 있었다. 이러한 C-말단 영역은 C-말단 산성꼬리 영역을 포함하는 여러 융합단백질로 하여금 샤페론 기능

을 하게끔 해주고, 그러한 융합단백질의 샤페론 기능은 융합단백질 각각의 특성에 따라 그 정도가 달라지는 것을 관찰할 수 있었다.

결론적으로, 시누클라인의 산성 꼬리부분은 융합단백질의 안정성과 용해성을 증가시킴으로써 환경스트레스에 저항성을 보이게끔 해줌으로써 환경스트레스에 저항적인 단백질의 제작에 이용할 수 있을 뿐만 아니라, 또한 생체외, 생체내 특이 단백질을 안정화시키는데 이용하는 합성 샤페론을 제작하는데에도 이용할 수 있을 것이다.

핵심되는 말 : α-시누클라인, 열안정성, 용해성, 샤페론