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Nondenaturing solubilization of β2 microglobulin from inclusion bodies by L-arginine☆

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Abstract

Expression of β2 microglobulin (β2m) in *Escherichia coli* resulted in formation of inclusion bodies. Attenuated total reflectance Fourier transform infrared analysis suggested a native-like secondary structure of β2m in the inclusion bodies. Nondenaturing solubilization of the native-like β2m from inclusion bodies was achieved using L-arginine solution, which enables an efficient recovery of β2m with little aggregation. Greater β2m solubilization from inclusion bodies was obtained at higher temperatures. Low-temperature solubilization yielded β2m with fluorescence properties identical to those of native β2m, but its secondary structure was slightly nonnative. Solubilization at moderate temperature gave β2m with an apparently native structure. We propose an efficient nondenaturing solubilization method combining L-arginine and moderate temperature.

Keywords: Inclusion body; Nondenaturing reagent; Oligomerization; Solubilizing reagent; Spectroscopic analysis

Expression of recombinant foreign genes in bacteria, in particular in *Escherichia* (*E.*) *coli*, has become one of the most important fundamental methods used in structural genomics and protein engineering, owing to the rapid growth and convenient handling of bacteria. However, the recombinant proteins produced in vivo are often found in insoluble particles called inclusion bodies [1,2], which are tightly packed aggregates composed of mainly the recombinant protein [3]. This aggregation is a serious obstacle to efficiently utilizing the gene resources supplied from genomic analysis: for example, 50% of the targeted proteins from the hyperthermophile Crenarchaeon *Pyrobaculum aerophilum* are found in the insoluble fraction [4,5].

The secondary structures of the recombinant proteins in inclusion bodies are not necessarily native and vary with the type of protein. For example, interleukin (IL)-2 and β lactamase have more than 50% α-helix in their native secondary structures, but acquire nonnative β-structure in inclusion bodies, along with decreased α-helix content [6,7]. In contrast, IL-1β (whose native form has only β sheets) in inclusion bodies shows the same amount of β-structure as in the native form [8],

☆ *Abbreviations:* ATR, attenuated total reflectance; β2m, β2 microglobulin; CD, circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FT-IR, Fourier transform infrared; GdnHCl, guanidine-hydrochloride; GFP, green fluorescent protein; GSH, reduced form of glutathione; IL, interleukin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SEC, size-exclusion chromatography; UV, ultraviolet.

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and green fluorescent protein (GFP) is fluorescent even in inclusion bodies [9].

The formation of inclusion bodies can be suppressed by controlling culture conditions [10] and by coexpression of chaperone molecules [11,12]. However, a comprehensive application has not been developed; instead, the renaturation of the expressed proteins from the inclusion bodies has been studied for several decades. Proteins produced as inclusion bodies are generally solubilized by using a strong denaturant, which means that native proteins must be re-formed in a subsequent complicated refolding process [13–15]. Refolding may be improved by either developing a more-effective refolding technique; for example, L-arginine or a chaperone protein has been included during refolding to achieve efficient refolding [16–20], but the yield of correctly refolded protein after the use of a strong denaturant depends strongly on the intrinsic properties of the target proteins.

It thus appears that using a strong denaturant regardless of the type of protein and its structure in inclusion bodies should not be a universal approach, but that a less-denaturing, yet efficient, solubilization technique should be developed. When a protein in inclusion bodies has considerable native-like structure, it should be feasible to solubilize it under less strongly denaturing conditions.

In this study, we found that β2 microglobulin (β2m) protein, which has an immunoglobulin-fold structure, appears to have a native-like structure when expressed by *E. coli* in inclusion bodies. Hence, we tested the use of an L-arginine solution to achieve solubilization under nondenaturing conditions. We succeeded in developing an efficient and convenient nondenaturing solubilization process, which may be broadly applicable for production of recombinant proteins.

Materials and methods

Expression of β2m in inclusion bodies. The plasmid containing DNA coding for β2m was constructed by replacing the GFP-coding sequence in a pGFPuv vector obtained from Clontech. Transformed *E. coli* JM109 cells were incubated in 2× YT medium at 28 °C, and expression of β2m under the control of the T7 promoter was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside. The cells were harvested by centrifugation and suspended in 50 mM Tris–HCl (pH 8.0) buffer containing 200 mM NaCl. After sonication, the suspension was centrifuged at 5800*g* for 30 min at 4 °C. The pellet was suspended several times in 50 mM Tris–HCl (pH 8.0) buffer containing 4% Triton X-100 and 200 mM NaCl to remove nonspecifically adsorbed proteins, and the solution was centrifuged again at 5800*g* for 30 min at 4 °C [20,21]; consequently, tightly packed aggregated β2m, which cannot be solubilized by mild detergent, accounted for 90% of insoluble precipitate. Finally, the pellet was next washed several times in water to remove Triton X-100, resulting in inclusion bodies of β2m.

Solubilization and renaturation of β2m by means of L-arginine. A 300-mg sample (wet weight) of the β2m inclusion bodies was soaked in 10 mL of 2 M L-arginine solution (all the arginine solutions also contained 50 mM Tris–HCl, pH 8.0, 200 mM NaCl, and 1 mM EDTA) for 20 h at various temperatures, and then centrifuged. Each supernatant was analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) [22].

The soluble β2m present in each supernatant was then buffer-exchanged at 4 °C by dialysis against a 50-fold volume (relative to the volume of the supernatant) of a denaturant-free solution (50 mM Tris–HCl, pH 8.0, 200 mM NaCl, and 1 mM EDTA). The dialysis was carried out four times to completely remove L-arginine.

Purification of solubilized β2m. The L-arginine-free β2m was partly purified by means of a cation-exchange chromatography column (HiTrapQXL, Amersham Bioscience, Tokyo, Japan) using a linear gradient of NaCl (in 50 mM Tris–HCl, pH 8.0) from 0 to 1 M and was then fractionated on a size-exclusion chromatography (SEC) column (HiLoad Superdex 75, Amersham Bioscience, Tokyo, Japan) equilibrated with 50 mM Tris–HCl, pH 8.0, 200 mM NaCl.

Spectroscopic experiments. Attenuated total reflectance (ATR) Fourier transform infrared (FT-IR) spectra were determined for the purified inclusion bodies of β2m with a FT-IR-680plus spectrometer (Jasco, Tokyo, Japan) at a resolution of 2 cm^{-1}. The FT-IR absorption spectra were deconvolved using the nonlinear curve-fitting program GRAMS/32 version 5.0 (Galactic, Salem, USA), and the fitting was continued until χ^2 (the sum of squares of the deviations normalized by the variance of the count) was <3.0.

Fluorescence spectra were recorded with an RF-5300PC spectrofluorophotometer (Shimadzu, Japan) at an excitation wavelength of 280 nm. Tryptophan fluorescence emission spectra were measured for the solubilized and SEC-purified β2m at a concentration of 1 μM in a 1-cm quartz cuvette.

Circular dichroism (CD) spectra were measured with an AVIV circular dichroism spectrometer (Proterion, New Jersey, USA): path length, 1.0 mm; resolution, 0.2 nm; and average time, 4 s. The SEC-purified β2m used for CD measurements was at 10 μM in 50 mM Tris–HCl (pH 8.0) containing 200 mM NaCl and 1 mM EDTA.

Analysis of thiol groups. β2m solution was denatured by dialysis against 6 M guanidine-hydrochloride (GdnHCl) solution (containing 100 mM potassium phosphate/HCl, pH 5, 1 mM EDTA). The β2m–GdnHCl solution was diluted to a β2m concentration of 1.5 μM with a Tris–HCl buffer containing 6 M GdnHCl, and then 50 μL of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) solution (50 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA) was added to 1.25 mL of the diluted β2m solution. The absorbance at 412 nm was monitored after the addition of DTNB, and the number of free thiol groups in β2m was calculated from the extinction coefficient of nitrothiophenol, which is generated by the reaction of DTNB with a thiol group [23]. The exact extinction coefficient of nitrothiophenol was estimated by reaction with the reduced form of glutathione (GSH).

Results

Structural analysis of β2m in inclusion bodies

Fig. 1 shows the amide I region of the FT-IR spectrum for the inclusion bodies containing β2m. The IR spectrum shows a maximum at 1627 cm^{-1}, consistent with a secondary structure rich in β-strands [8,24,25]. The FT-IR spectrum for the lyophilized native β2m was also measured. The spectrum was identical to that for the β2m inclusion bodies (data not shown). The FT-IR spectrum for the β2m inclusion bodies was, further, deconvolved with mixed Gaussian–Lorentzian functions in order to compare the β-structure content to the native form obtained from X-ray crystallography

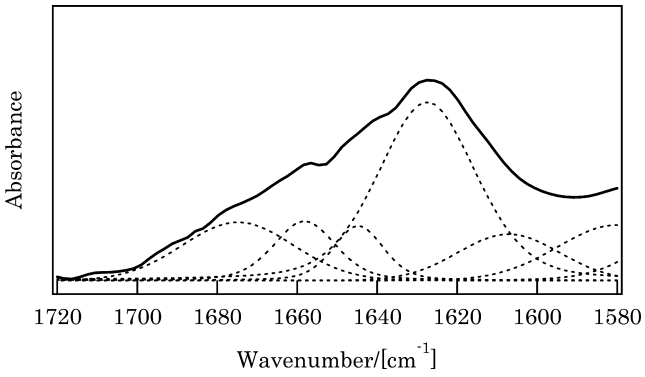

Fig. 1. Amide I region of the FT-IR spectrum (solid line) of β2m in inclusion bodies and its deconvolved components (dotted lines).

Table 1
Peak positions and relative areas[a] of the fitted components in the amide I region of FT-IR spectra of inclusion bodies of β2m

IR band assignment	Wave number (cm^{-1})	Relative area (%)
Turn	1675	21
α-helix or irregular/loop	1658	13
Random coil	1645	10
β-structure	1628	56
Side chains	1607	—

[a] Relative areas were estimated only for the bands derived from the backbone structure.

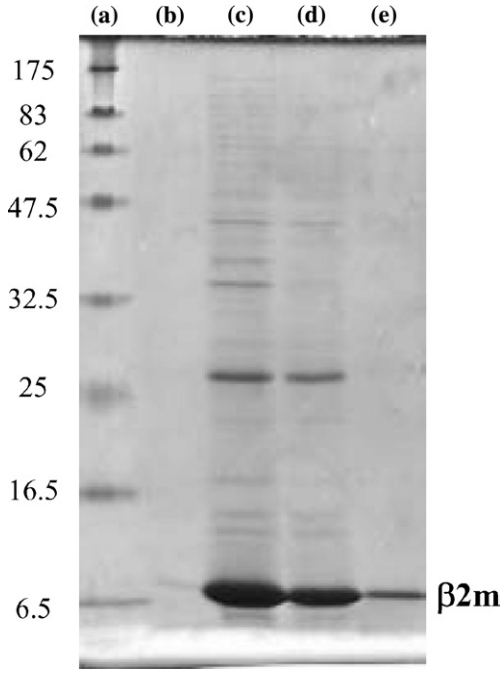

Fig. 2. SDS–PAGE (15% acrylamide) analysis of β2m solubilized from inclusion bodies by soaking for 24 h at 28 °C in Triton X-100, GdnHCl, or L-arginine (see Materials and methods). Lane: (a) molecular mass markers (numbers indicate kDa), (b) 4% Triton X-100, (c) 6 M GdnHCl, (d) 2 M GdnHCl, and (e) 2 M L-arginine.

(dotted lines in Fig. 1). The deconvolution showed that the spectrum in the amide I region was composed of five Gaussian–Lorentzian functions (one of the five is derived from the amino acid side chains), and the relative area of the major peak at 1627 cm^{-1}, assigned to β-strand structure, indicated that the β-structure content of β2m in the inclusion bodies was comparable to that of native β2m as determined by X-ray crystallography (Table 1) [26]. We can hypothesize from the deconvolution results that β2m in inclusion bodies has a native-like secondary structure.

Aggregation of β2m in inclusion bodies was due to noncovalent association, since nonreducing SDS–PAGE analysis indicated no intermolecular disulfide linkages (data not shown). These results suggest that β2m aggregates in vivo due to association of native-like structures, and hence opens the possibility of developing a method for nondenaturing solubilization.

Solubilization of β2m from inclusion bodies

Fig. 2 shows the results of SDS–PAGE analysis of β2m solubilized from inclusion bodies with various extractants at 28 °C. A 4% Triton X-100 solution resulted in no detectable solubilization of β2m (Fig. 2, lane b). To our surprise, 2 M L-arginine solution solubilized β2m from inclusion bodies without solubilizing detectable amounts of other proteins, resulting in a nearly homogeneous β2m preparation, although the amount of β2m solubilized was small (Fig. 2, lane e). For comparison, β2m was solubilized with GdnHCl, resulting in greater yield, especially with 6 M GdnHCl, than by extraction with 2 M L-arginine (Fig. 2, lanes c and d); however, GdnHCl solubilized other proteins of *E. coli* origin as well, resulting in an impure preparation of β2m. These results suggest that L-arginine specifically dissociates native-like β2m present within inclusion bodies, without releasing any *E. coli* proteins trapped in inclusion bodies.

The effect of temperature on solubilization of β2m by L-arginine was examined (Figs. 3 and 4). Higher temperatures clearly led to greater solubilization of β2m, again without cosolubilization of *E. coli*-origin proteins. The amount of solubilized β2m did not increase linearly with respect to the temperature (Fig. 4); the solubilization changed little between 37 and 60 °C.

Structural analysis of β2m solubilized from inclusion bodies

Tryptophan fluorescence emission spectra were scanned for the β2m solubilized in L-arginine solution (Fig. 5, the 4 °C solubilization was omitted due to low recovery of β2m). The emission from β2m solubilized by 2 M L-arginine at 28 °C was blue-shifted to 338 nm (solid line in Fig. 5), relative to the emission from solvent-exposed tryptophan (β2m in 6 M GdnHCl: 351 nm, data not shown), indicating that they were in a hydrophobic environment. The observed 338-nm emis-

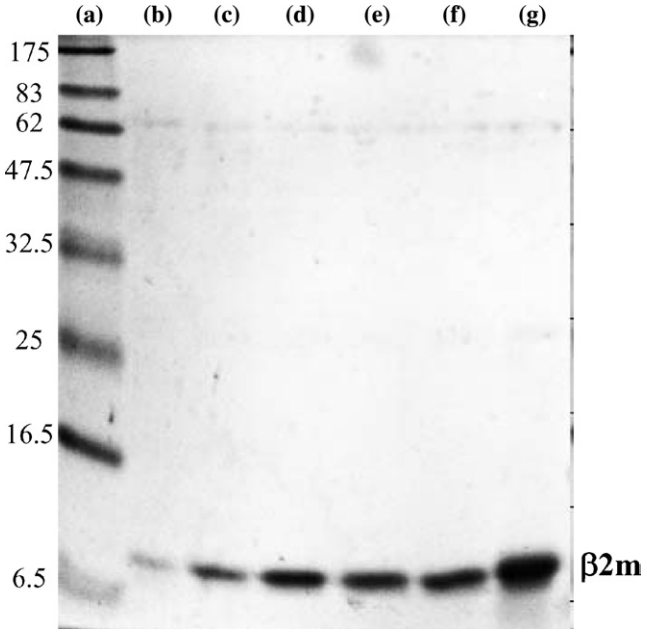

Fig. 3. SDS–PAGE (15% acrylamide) analysis of β2m solubilized from inclusion bodies by soaking for 24 h at various temperatures in 2 M L-arginine. Lane: (a) molecular mass markers, (b) 4 °C, (c) 28 °C, (d) 37 °C, (e) 50 °C, (f) 60 °C, and (g) 80 °C.

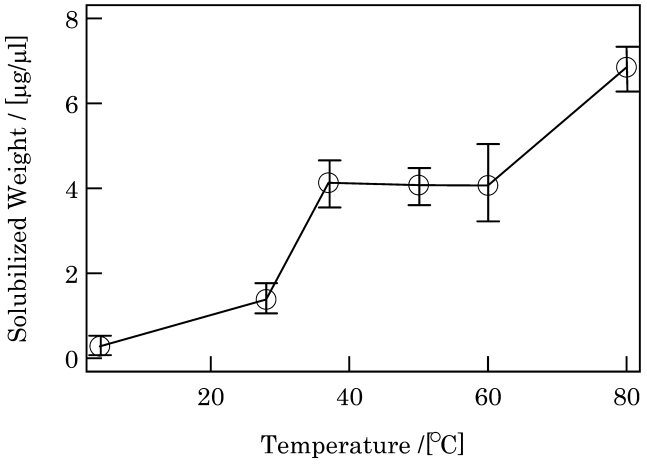

Fig. 4. Amount of β2m solubilized by L-arginine at various temperatures. The amount of solubilized β2m was estimated from the band intensities in Fig. 3 using the calibration curve of bovine serum albumin. The error bars were determined from five same experiments.

sion maximum is identical to that of the native protein. Solubilization in 2 M GdnHCl resulted in a blue-shifted tryptophan emission maximum at 342 nm (data not shown), indicating that tryptophan residues were in less hydrophobic environments than after solubilization with L-arginine. Solubilization in 2 M GdnHCl results in somewhat denatured β2m.

The effects of solubilization temperature on fluorescent emission were also examined. After extraction at temperatures of 37 and 50 °C (dotted and dashed lines in Fig. 5), emission bands were observed at the same

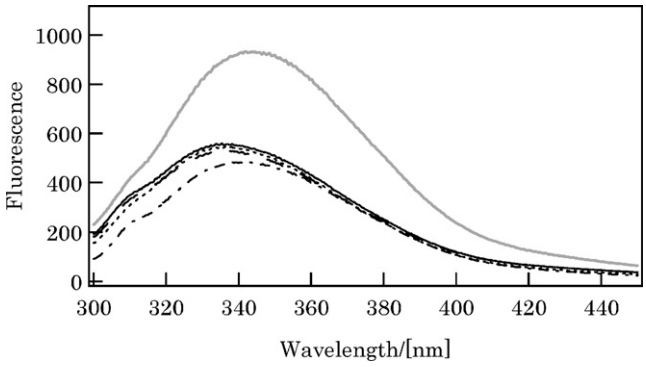

Fig. 5. Tryptophan fluorescence spectra at room temperature of β2m solubilized by 2 M L-arginine at various temperatures: 28 °C (solid black line), 37 °C (dotted line), 50 °C (dashed line), 60 °C (dash-dot line), or 80 °C (gray line). Excitation was at 280 nm and the fluorescence intensity was normalized to a β2m concentration of 1 μM.

wavelength (338 nm) and with comparable intensity. After solubilization of β2m at 60 or 80 °C, however, the emission maximum occurred at slightly higher wavelengths (Fig. 5: dash-dot line, 60 °C: 342 nm; and gray line, 80 °C: 345 nm), indicating that the fluorescent tryptophans were more exposed to water. The use of high temperature critically influences the solubilized form of β2m.

We also quantified the free thiol groups in solubilized β2m by means of Ellman's assay (native β2m has a typical immunoglobulin fold with a single intramolecular disulfide bond [26]). The assay showed one free thiol group per β2m molecule for all preparations of β2m (in both GdnHCl and L-arginine solutions at all temperatures). Considering that only one band, equivalent to the β2m monomer, was observed in nonreducing SDS–PAGE analyses (data not shown), we can conclude that half of the solubilized β2m molecules most likely have an intramolecular disulfide linkage.

Buffer exchange of solubilized β2m

We tested the use of dialysis against a denaturant-free solution at 4 °C for removal of GdnHCl or L-arginine from the solubilized β2m solutions. Removal of GdnHCl after solubilization with 6 and 2 M GdnHCl solutions resulted in a low yield of soluble β2m (6 M GdnHCl; 68%, 2 M GdnHCl; 44%), owing to the formation of insoluble aggregates. However, it should be noted that precipitation of β2m did not occur in any of the samples prepared with L-arginine. Free thiol groups were quantified for the β2m preparations, so that few free thiol groups were observed for all the β2ms (data not shown). This indicates that the residual free thiol groups in the solubilized forms are naturally oxidized during the buffer-exchange procedure.

After removal of L-arginine, the β2m preparations were examined further for evidence of aggregation, by SEC analysis (Fig. 6). The amount of β2m loaded onto

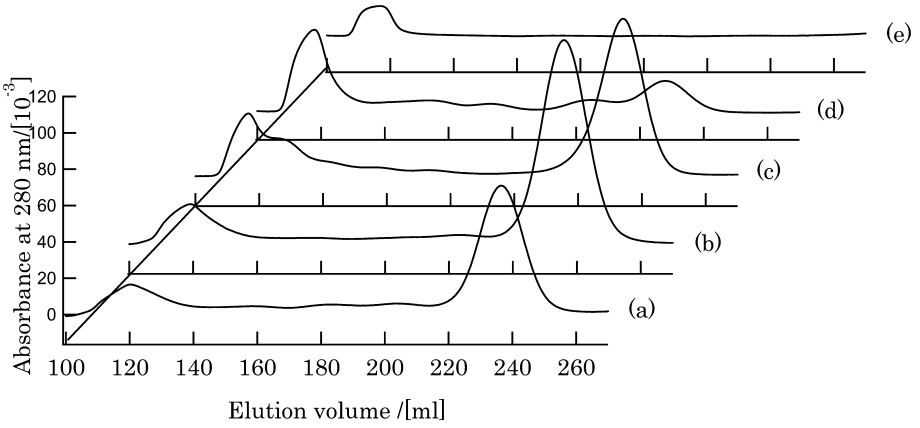

Fig. 6. Size-exclusion chromatography of β2m after removal of L-arginine by dialysis. β2ms were solubilized from inclusion bodies by soaking for 24 h in 2 M L-arginine at (a) 28 °C, (b) 37 °C, (c) 50 °C, (d) 60 °C, or (e) 80 °C. A 10-mL sample of each dialyzed β2m sample was applied to a column of HiLoad Superdex 75; the absorbance of the eluant was monitored at 280 nm.

the SEC columns varied, because of differences in solubilization efficiency at different extraction temperatures; consequently, the signal intensity was normalized with respect to the amount of inclusion bodies used (Fig. 4). The results for β2m that had been solubilized at 28–50 °C (Fig. 6, a–c) revealed that it was present mainly in a monomeric form and that the extracts contained only a small amount of oligomers eluting in earlier fractions. The amount of the monomeric form decreased with increasing oligomers for extraction temperatures greater than 60 °C (Fig. 6, d and e). The total peak area for β2m solubilized at 80 °C seems to be much lower for other samples. This might indicate more heterogeneous aggregation of β2m.

The monomeric form of β2m fractionated by SEC was analyzed by measuring tryptophan fluorescence emission and CD (Table 2 and Fig. 7). A striking observation was the lack of any apparent difference in the emission wavelengths of β2m monomer solubilized at any of the temperatures used, indicating that 2 M L-arginine does not affect the structure of β2m (Fig. 5 and Table 2). The tryptophan fluorescence from the β2m monomer solubilized at 28–50 °C showed an identical emission maximum to that of the native protein.

Table 2
Tryptophan fluorescence emission maximum for fractionated β2m after removal of L-arginine

Temperature during solubilization (°C)	Main forms present after SEC purification	Tryptophan emission peak of SEC fractions (nm)
28	Monomer	338
37	Monomer	338
50	Monomer	338
60	Monomer	340
	Multimer	341
80	Multimer	345

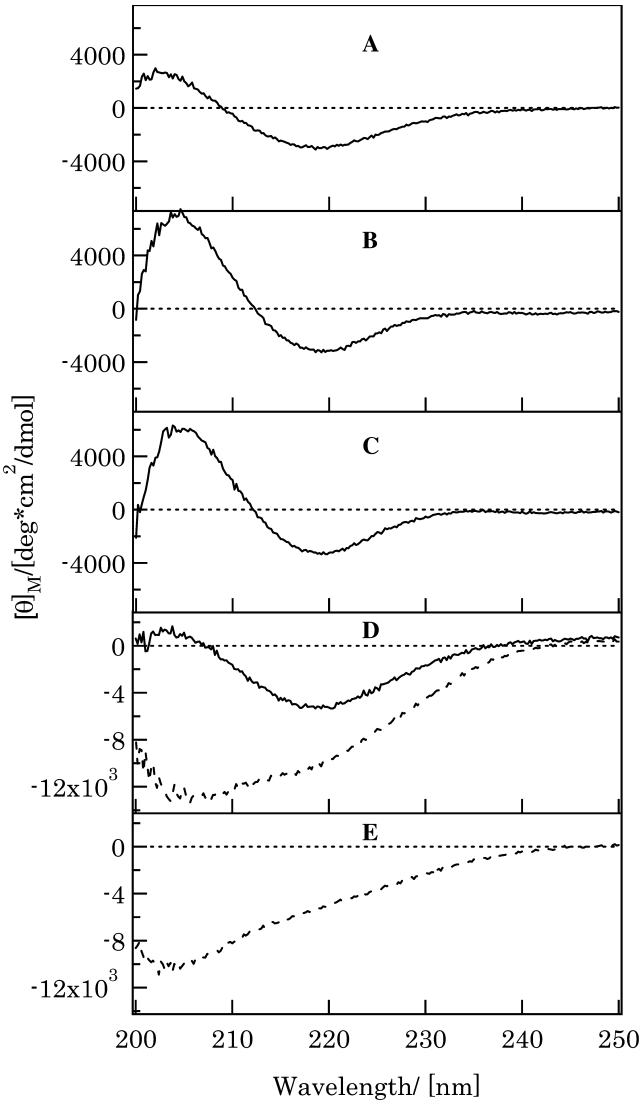

Fig. 7. CD spectra for SEC-fractionated β2m solubilized at (A) 28 °C, (B) 37 °C, (C) 50 °C, (D) 60 °C, or (E) 80 °C. The solid and dotted lines correspond to the monomeric and multimeric forms, respectively.

The CD spectra of β2m monomer solubilized at 28–50 °C showed a peak at 203–205 nm and a trough at 218 nm (Figs. 7A–C), which are characteristic of β-strand structure. However, the monomeric β2m that had been solubilized at 37 and 50 °C had CD spectra that were identical to those of the native form (Figs. 7B and C); in contrast, the monomeric β2m from the 28 °C-extraction showed a smaller peak at 205 nm than the native form (Fig. 7A). This indicates that, although the fluorescence spectrum of the 28 °C-solubilized material resembles that of the native protein, the secondary structure is nonetheless slightly different from that of the native state. Considering that the β2m sample solubilized at 50 °C contained a greater proportion of oligomers than that prepared at 37 °C (Fig. 6), we can conclude that solubilization at 37 °C generates the greatest amount of β2m having native structure.

At 60 °C and above, the amount of the monomeric form decreased with increasing oligomers (Fig. 6, d and e), and the CD spectra of the monomeric and multimeric forms were different from those of the native form (Figs. 7D and E). Considering that the fluorescence results indicate greater exposure of tryptophans to solvent in β2m extracted at 60–80 °C (Fig. 5), the monomer from 60 °C is nonnative and the increase of multimeric form suggests that partial unfolding at these high temperature solubilizations resulted in some oligomerization. This unfolded structure could not be converted to the native form by simple dialysis to remove L-arginine. The condition that enabled us to prepare β2m in its native form from inclusion bodies was solubilization by L-arginine at 37 °C.

Finally, free thiol groups in the monomeric β2m were quantified by Ellman's assay and revealed no detectable thiol groups in any monomeric and β2m preparation after buffer exchange and SEC purification (data not shown). No free thiol group was also detected in oligomeric β2m; however, nonreducing SDS–PAGE analysis for the β2m preparation from the solubilization at 60 °C demonstrated intermolecular disulfide linkages in oligomeric β2m (Fig. 8). The residual free thiol groups in β2m solubilized by L-arginine at high temperature might result in nonnative intermolecular disulfide bond formation.

Discussion

In vivo aggregated structure of β2m

Few techniques are available for the analysis of aggregated proteins, such as inclusion bodies, because

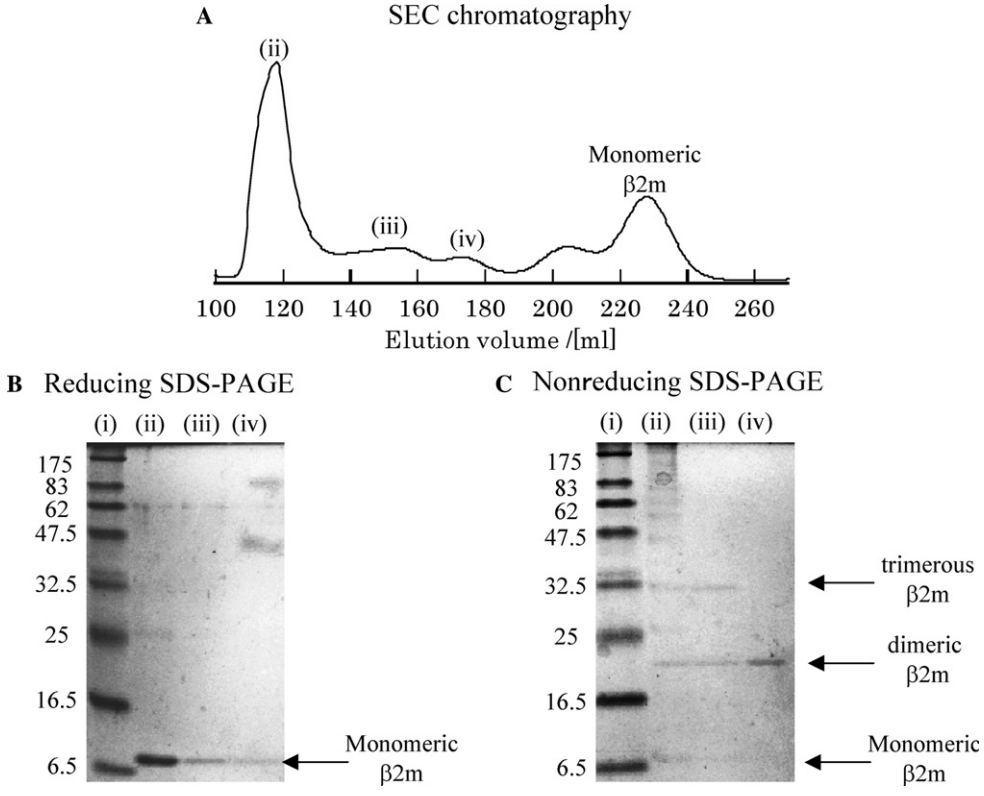

Fig. 8. Analysis of β2m preparation from solubilization at 60 °C. β2ms were solubilized from inclusion bodies in 2 M L-arginine at 60 °C, and the dialyzed β2m sample was fractionated by (A) SEC chromatography (HiLoad Superdex 75, the absorbance of the eluant was monitored at 280 nm). The fractionated β2m samples at different elute times were analyzed by (B) reducing and (C) nonreducing SDS–PAGE (15% acrylamide) analysis. Lane: (i) molecular mass markers, (ii)–(iv) fractionated β2ms at the elute times indicated in SEC chromatography.

of their heterogeneous structures. FT-IR spectroscopy is one of the few techniques used to analyze protein secondary structure in inclusion bodies, using absorption of amide-bond stretching [6,8,24]. The relative amount of each type of secondary structure can be estimated by deconvolving the FT-IR spectra. We applied this technique to the analysis of β2m in inclusion bodies, obtaining the results that the relative amount of β-structure is comparable to that in the native protein (Fig. 1 and Table 1) [26].

Our present study shows that L-arginine solution can solubilize β2m from inclusion bodies with little change in its secondary structure and that solubilization at elevated temperatures below 50 °C leads to the native structure. These results strongly suggest that β2m basically has a native-like structure in inclusion bodies, as observed by FT-IR of inclusion bodies. We previously reported that L-arginine can suppress protein aggregation with little effect on protein stability [27]; for example, hen lysozyme becomes denatured and aggregated above 70 °C in the absence of L-arginine, but adding L-arginine to the lysozyme solution resulted in no change in the melting temperature but no apparent heat-induced aggregation. This is consistent with our present observation that β2m is solubilized from inclusion bodies by L-arginine solution without destroying its secondary structure. Structural analysis of solubilized β2m and its SEC-purified monomer provided additional evidence that β2m aggregated in vivo retains a native-like structure.

L-Arginine effects on the nondenaturing solubilization of β2m from inclusion bodies

L-Arginine has been utilized as an additive in many in vitro refolding systems to enhance the yield of correctly refolded protein [16–18]. Although L-arginine has been considered to facilitate correct folding by destabilizing incorrectly folded structures [16,28,29], the mechanism of suppressing aggregation by L-arginine has not been fully elucidated. Ahn et al. [30] suggested that L-arginine supplements the role of GdnHCl during refolding, because addition of L-arginine to a refolding solution decreased the GdnHCl concentration needed for optimal refolding. We found that the presence of both L-arginine and GdnHCl during refolding results in the inhibition of hydrophobic interactions among partly folded structural elements [31]. These results, along with recent thermal unfolding studies on RNase A and lysozyme [27], indicate that L-arginine suppresses aggregation by inhibiting hydrophobic interactions without denaturing or destabilizing proteins.

In our previous study, fluorescently active GFP protein expressed in the insoluble particles, soluble with mild detergents, was solubilized by L-arginine solution. L-Arginine was readily removed by dialysis, resulting in an active protein without having to pass through a completely denatured form [9]. Here, nondenaturing solubilization procedures utilizing L-arginine were applied to inclusion bodies of β2m, which cannot be solubilized by mild detergents. The β2m inclusion bodies appear different from those of GFP in that they are tightly packed aggregates.

If we consider the L-arginine effect to be due to a decrease in hydrophobic interactions, its solubilization of β2m implies that β2m aggregates in vivo because of the formation of nonnative hydrophobic interactions between correctly folded structural elements, and that L-arginine solubilizes β2m from inclusion bodies by interfering with the nonnative hydrophobic interactions. Kita et al. have shown the contribution of free amino acids to the surface energy perturbation occurring during protein–solvent interaction [33]: glycine, proline, serine, and alanine increase the melting temperature of a protein without binding to the protein surface, but L-arginine preferentially binds to the native form of protein. Some direct interactions between L-arginine and the protein surface might thus result in solubilization of the protein from inclusion bodies [32].

Temperature effects on the nondenaturing solubilization of β2m from inclusion bodies

The structure of β2m solubilized by L-arginine from inclusion bodies was affected by the solubilization temperature. When β2m was solubilized at 28 °C, far-UV CD revealed a secondary structure slightly different from the native state (Fig. 7A), although the tryptophan fluorescence spectrum was similar to that of the native protein (Table 2). When β2m was solubilized at higher temperatures, both the tryptophan fluorescence and far-UV CD spectra closely resembled those of the native protein. Although other possibilities exist, these results suggest that β2m has a native-like structure in inclusion bodies, which is retained during solubilization at 28 °C but which can be converted to the complete native state by solubilization at moderate warm temperatures.

The red-shifted tryptophan emission peak for β2m solubilized at 60 or 80 °C shows that solubilization with L-arginine solution above 60 °C denatures the solubilized β2m (Table 2); the recovered protein by dialysis becomes refolded to a monomeric form having nonnative structure or forms soluble aggregates containing little native structure (Figs. 6 and 7). Interestingly, buffer exchange of β2m after the denaturing solubilization at 60 and 80 °C yielded solutions containing no insoluble aggregates, regardless of the initially solubilized forms. The above results are consistent with the idea that the effect of arginine is neither the destabilization of incorrectly folded structure nor the facilitation of refolding, but rather the suppression of insoluble-aggregate formation.

Formation of disulfide linkage

β2m has one immunoglobulin-fold domain, whose internal disulfide linkage has a critical influence on the protein's stability [33,34]: cleavage of the disulfide bond causes destabilization of folded structures and protein aggregation. Recently, we analyzed the off-pathway to insoluble aggregates of single-chain antibodies with the immunoglobulin fold [35]; the antibodies had the comparable amount of β-strands to that of native form even in the insoluble aggregates formed on the refolding at reducing conditions. The β2m also formed the insoluble aggregates with the native-like β-structure content at reducing conditions (data not shown). These results suggest that the disulfide linkage strongly influences the correct interaction between secondary structure fragments.

Although in this study we did not include any oxidizing reagents during L-arginine solubilization, about half of the cysteinyl residues were oxidized upon solubilization. Goto and Hamaguchi's [36] regeneration study of the reduced constant fragment of antibodies demonstrated that a short distance between two cysteinyl residues is essential for the formation of a disulfide linkage. Spontaneous oxidation implies that formation of native-like structure drives the formation of the disulfide linkage. The finding that an intramolecular disulfide bond in β2m is formed during solubilization by L-arginine at 28–50 °C suggests that the native-like structure assists in the formation of correct disulfide linkages. The increase of the temperature applied for the solubilization of β2m from inclusion bodies resulted in the formation of oligomeric β2m by intermolecular disulfide linkages (Fig. 8). Considering that the application of high temperature to solubilization denatures the solubilized β2m in L-arginine solution (Fig. 5), the denatured structure might lead to nonnative intermolecular disulfide linkages.

Conclusions

β2m protein expressed in *E. coli* has a native-like structure even though it is present in inclusion bodies, although some nonnative structures may exist in the solubilized structure. Solubilization of the native-like structure under mild conditions is an effective and convenient process, in contrast to processes that take a protein through a completely denatured form. GdnHCl may generally be the best choice as a solubilizing reagent, but it should be avoided when the proteins expressed in inclusion bodies have native-like structure. In the latter situation, L-arginine may be more appropriate, because of its ability to preferentially solubilize proteins and suppress their aggregation. The use of L-arginine as a solubilizing agent should be applicable to the recovery of many other recombinant proteins that form native-like structures in inclusion bodies.

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