J. Biochem. **142,** 383–391 (2007)
doi:10.1093/jb/mvm145

Effect of Buffer Species on the Unfolding and the Aggregation of Humanized IgG

Daisuke Kameoka[1,2], Etsuro Masuzaki[1,2], Tadashi Ueda[1,†] and Taiji Imoto[2,*]

[1]Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582; and [2]Formulation Technology Research Department, Chugai Pharmaceutical Co. Ltd., Tokyo 115-8543, Japan

Received May 23, 2007; accepted June 27, 2007; published online July 23, 2007

The aggregation propensity of humanized antibody after heat treatment is evaluated in the presence of six buffer species. The comparison under equivalent pH showed high aggregation propensity on phosphate and citrate buffer. In contrast, 2-(N-Morpholino) ethane sulfonate (MES), 3-(N-Morpholino) propane sulfonate (MOPS), acetate and imidazole buffer showed lower aggregation propensity than the above two buffers. Meanwhile, unfolding temperature evaluated by differential scanning calorimetry measurement was not altered among these buffer species. The light scattering analysis suggested that heat-denatured intermediate was aggregated slightly on MES and acetate buffer. Therefore, it was found that the different aggregation propensity among buffer species was caused from the aggregation propensity of heat-denatured intermediate rather than the unfolding temperature. Furthermore, it was revealed that the aggregation dependency on buffer species is accounted for by the specific molecular interaction between buffer and IgG, rather than the ionic strength. On the contrary, on the analyses of unfolding and aggregation propensity by molecular dissection of IgG into Fab and Fc fragments, aggregation propensity of Fc fragment on MES, acetate and phosphate buffer was almost the same as whole IgG. From the above results, it was suggested that the specific interaction between buffer molecule and Fc domain of IgG was involved in the aggregation propensity of heat-denatured IgG.

Key words: buffer, DSC, humanized antibody, protein aggregation, protein stabilization.

Abbreviations: ANS, 8-Anilino-1-naphthalene sulfonate; DSC, differential scanning calorimetry; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(N-Morpholino) ethane sulfonate; MOPS, 3-(N-Morpholino) propane sulfonate; PAGE, polyacrylamide gel electrophoresis; SDS, Sodium dodecyl sulfate.

Progress in gene recombinant technology has enabled the large-scale production of many physiologically active proteins that exist in minute concentrations in the living mammalian body, which, in turn, has allowed many physiologically active protein species, such as cytokines and hormones, to be developed into pharmaceutical products. Moreover, treatment with antibodies, which specifically bind to antigen molecules resulting in the so-called antigen–antibody reaction, is of great interest, because many disease-related molecules are being discovered with continuing progress in genome-based drug discovery research. In other words, the application of antibodies specifically directed against disease-related molecules as pharmaceutical products can yield specific therapeutic effects without side effects. Many antibodies developed as pharmaceuticals are currently in use for the treatment of some intractable diseases, such as cancer and rheumatoid arthritis.

For the development of such proteins as described earlier as pharmaceuticals, establishment of a suitable manufacturing process and storage conditions is important to keep the physicochemical properties and the stability of the molecules to be maintained. Unlike the case of the general low-molecular weight pharmaceuticals, not only prevention of chemical degradation, but also maintenance of the higher-order structure in the native state is necessary to maintain the biological activity of these protein pharmaceuticals. That is, the protein activity can be lost even by partial denaturation occurring near the active centre, irrespective of any changes occurring in the primary structure. Therefore, most proteins must be protected against denaturation occurring during storage. On the other hand, aggregation can cause serious problems in quality control, especially for liquid formulations of the protein drug substances (*1*, *2*). In general, aggregated proteins are insolubilized with the growth of the molecular size, causing the formation of insoluble particles in the protein formulation. Even if such insoluble particles are not formed, aggregation of proteins causes a loss of the biological activity (*3*). Moreover, administration of such aggregated protein products could cause some critical immune reaction-related adverse effects, including

[†]To whom correspondence should be addressed. Tel: +81-92-642-6662, Fax: +81-92-642-6667,
E-mail: ueda@phar.kyushu-u.ac.jp
*Present address: Faculty of Biotechnology and Life Science, Sojo University, Kumamoto 860-0082, Japan

anaphylactic shock. In the meantime, the solution conditions, such as the pH and the salt species and concentration, can alter the physicochemical properties from the viewpoint of aggregation and chemical degradation, of proteins and reduce the protein stability. For example, recombinant factor VIII SQ is stable only in a narrow range of solution pH that is, 6.5–7.0, and its aggregation is inhibited in the presence of high concentrations of sodium chloride in the solution (*4*). Therefore, establishment of optimal solution conditions is important for suppressing chemical degradation so as to maintain the protein stability and retain the higher-order structure.

Immunoglobulin G (IgG) is a protein molecule with a molecular weight of about 150 kDa, and is composed of two H chains and two L chains connected by disulfide bonds and non-covalent interactions. This molecule also has the features of multi-domain proteins, and consists of two Fab domains and one Fc domain, and a secondary structure with a high content of β-sheets. Aggregation of IgGs in high-concentration solutions poses some serious problems in their pharmaceutical application because the administration dose of IgGs is usually more than 100 mg/kg-bodyweight, and the IgG concentrations in drug products become rather high, often on the order of several tens of mg/ml. The influence of heat treatment and pH on the denaturation and aggregation of IgGs in solution has been a subject of investigation for a long time. However, there have been few reports on the systematic research of denaturation and aggregation using humanized monoclonal antibody (mAb). Therefore, the optimal solution conditions under which the formation of aggregates or denatured intermediates of IgG can be suppressed need to be investigated. On the other hand, the thermodynamic properties of immunoglobulins have been examined in detail using differential scanning calorimetry (DSC). There are two or more transitions during their heat denaturation phenomenon, and it has been reported that these transitions are attributable to denaturation of each of the unit domains (*5*, *6*). Recently, Ejima *et al*. (*7*), analysed the effect of pH on the conformation, stability and aggregation of humanized mAbs (hIgG4-A, -B) using CD, DSC and sedimentation velocity. Therefore, DSC analyses of IgG or its fragments may be useful to investigate the mechanism of unfolding and aggregation of IgG.

In this study, we analysed the heat-denatured aggregation of humanized whole antibody and its fragments in the presence of several buffer species, in order to know the optimal condition where humanized IgG was stable and the mechanism of heat-denatured aggregation of humanized IgG.

MATERIALS AND METHODS

Materials and Reagents—The immunoglobulin (IgG), a humanized mAb specific for the human interleukin-6 receptor, was donated by Chugai pharmaceuticals (Tokyo, Japan). TSK-gel G4000SWXL column (7.8 × 300 mm) was purchased from Tosoh (Tokyo, Japan), and the HiTrap Protein A HP column was obtained from Amersham Biosciences (Piscataway, USA). All other chemicals were of analytical grade for biochemical use.

Preparation of the Samples of Whole IgG and the IgG Fragments—Sample solutions of whole IgG at a concentration of 10 mg/ml (65 μM), differing in pH and containing different buffer species were evaluated in this study. We used the buffer species phosphate, citrate, imidazole, 2-(N-Morpholino) ethane sulfonate (MES) and 3-(N-Morpholino) propane sulfonate (MOPS) at pH 6.5 and phosphate, citrate, acetate and MES at pH 5.5. The buffer concentration in all the solutions was 15 mM. The solutions were prepared from an IgG stock solution that contained 50 mg/ml of IgG in 15 mM sodium phosphate buffer, pH 6.5, by dialysis and dilution.

Fab and Fc fragments of IgG were obtained by papain digestion at 37°C, and separated on a HiTrap Protein A HP column. The Fab fragments were further purified using a Resource S column by cation exchange separation. Sample solutions of the Fab and Fc fragments (1 mg/ml) dissolved in various buffer solutions were prepared by dialysis and dilution. In this case, we used the buffer species, phosphate, citrate and MES at pH 6.5, and, phosphate, citrate and MES at pH 5.5.

Assessment of stability of the IgG sample solution—In order to evaluate the stability of the whole IgG and the IgG fragments in solution, the sample solutions were subjected to heat treatment at 60°C for 4 weeks. In the case of the whole IgG solutions, additional heat treatment at 80°C for 2 h was also performed.

The percentages of the residual monomer IgG and of the soluble aggregates formed were evaluated by size-exclusion chromatography (SEC–HPLC). The residual percentages of IgG fragments were also measured by the same method. Untreated and heat-treated sample solutions were diluted with water to 1 mg/ml and applied to a HPLC system (Waters) with a TSK-gel G4000SWXL column and eluted with 50 mM phosphate buffer, pH 7.0, containing 0.3 M NaCl.

The purity of the whole IgG was evaluated by SDS-PAGE (*8*). Untreated and heat-treated sample solutions, either with or without 2-mercaptoethanol, were diluted with 10% SDS-containing buffer to obtain a final protein concentration of 100 μg/ml. Samples containing 2-mercaptoethanol were boiled for 5 min for reducing the IgG, and then applied to a 12% polyacrylamide gel under reducing conditions or 8% polyacrylamide gel under non-reducing conditions and electrophoresed at 200 mA for about 40 min. Protein bands were stained with Coomassie brilliant blue.

Furthermore, 8-Anilino-1-naphthalene sulfonate (ANS) binding was measured to evaluate the surface hydrophobicity of the IgG fragments. ANS solution (0.4 mM) was added to each diluted heat-treated or untreated sample solution to obtain a final molar ratio of ANS to each IgG fragment of 10. The fluorescence spectra of the ANS solutions between 450 and 550 nm after excitation at 365 nm were measured at room temperature using a F-2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

Differential Scanning Calorimetry—The DSC measurements were carried out using a VP-DSC calorimeter (MicroCal). Each sample solution of whole IgG, Fab or Fc fragment was diluted to 0.5 mg/ml with the same buffer solution without IgG. DSC scans were

performed at a rate of 0.5°C/min in the temperature range of 40—105°C. Data were analysed using the Origin software (MicroCal).

Aggregation of the Heat-denatured Intermediates—Rayleigh scattering of the heat-denatured intermediates was monitored to evaluate their aggregation propensity. The heat-denatured intermediate stock solution with an IgG concentration of 10 mg/ml dissolved in 5 mM MES buffer, pH 5.5, was prepared by heat treatment of the IgG solution, by gradually raising the temperature from 40°C to 90°C at the rate of 0.17°C/min. Then, denatured IgG stock solution was added to the buffer solutions without IgG to adjust the IgG concentration to 500 μg/ml. The buffer species used in this experiment were phosphate (pH 5.5 and 6.5), citrate (pH 5.5), acetate (pH 5.5) and MES (pH 5.5 and 6.5). After the addition of denatured IgG, the Rayleigh scattering intensity of each buffer solution was monitored at 340 nm by an RF-5300 fluorescence spectrophotometer (Shimadzu, Kyoto, Japan) at room temperature.

Effect of the Ionic Strength of the Solution on the Aggregation of Whole IgG—The IgG solutions with various buffer species with differing ionic strengths, with ratios of 0.001, 0.01 and 0.1, were prepared by the dialysis to each buffer. We used phosphate (pH 5.5 and 6.5), citrate (pH 5.5), acetate (pH 5.5) and MES (pH 5.5 and 6.5) as the buffer species. The IgG concentration of each solution was 10 mg/ml. Then, the percentage of the residual monomer IgG after heat treatment at 60°C for 4 weeks was evaluated by SEC-HPLC, as described earlier.

RESULTS

Aggregation of the Whole IgG After Heat Treatment—The propensity to aggregation of the humanized IgG after heat treatment at 80°C for 2 h and 60°C for 4 weeks was evaluated in solutions containing each of the six buffer species at a constant molar concentration, by SEC–HPLC and SDS-PAGE. The typical SEC–HPLC chromatographs of the IgG solutions before and after heat treatment showed aggregation of most of the IgG monomers by the heat treatment (Fig. 1). The percentages of the residual monomer and the aggregates of IgG after heat treatment in solutions containing each of the buffer species are shown in Table 1. After the heat treatment at 80°C for 2 h, percentage of residual monomer differed markedly among buffer species. In particular, MES buffer on pH 5.5 showed quite high residual monomer of 75%. In addition, MES, MOPS and imidazole buffers at pH 6.5 showed relatively high residual monomer of the range from 50% to 60%. Citrate and phosphate buffers on both pH showed low percentage of residual monomer. On the contrary, after the heat treatment at 60°C for 4 weeks, marked percentage difference of residual monomer was not found among buffer species except for MES

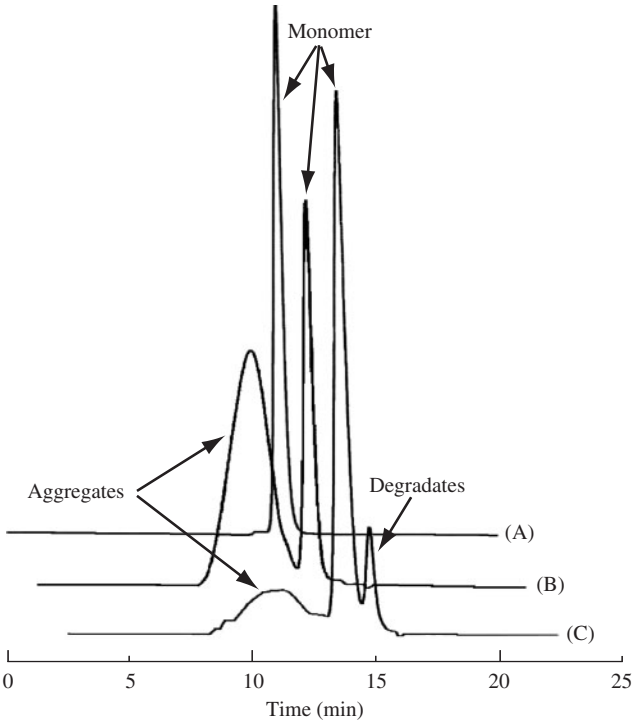

Fig. 1. **Size exclusion chromatograms of humanized IgG in 15 mM phosphate buffer, pH 5.5 before and after heat treatment.** (A) Before heat treatment, (B) After 80°C storage for 2 h, (C) After 60°C storage for 4 weeks.

Table 1. **Percentages of the residual monomer and the soluble aggregates in humanized IgG solution containing different buffer species after heat treatment.**

		80°C/2 h		60°C/4 weeks	
Buffer species		Monomer (%)[a]	Aggregates (%)[a]	Monomer (%)[a]	Aggregates (%)[a]
Phosphate	pH 6.5	28.3	68.1	49.4	8.3[b]
Citrate	pH 6.5	22.5	63.4	54.1	14.9[b]
Imidazole	pH 6.5	60.6	31.7	60.0	25.5
MES	pH 6.5	53.2	44.4	59.7	31.0
MOPS	pH 6.5	51.9	39.0	58.7	31.5
Phosphate	pH 5.5	49.1	48.9	47.1	43.2
Citrate	pH 5.5	21.4	75.3	21.4	65.0
Acetate	pH 5.5	58.7	36.8	58.7	32.8
MES	pH 5.5	75.4	9.9	72.1	16.0

[a]Percentages of the monomer and the aggregate content was the ratio of the peak area after heat treatment to the monomer peak area before heat treatment. [b]Insoluble aggregates were formed after heat treatment at 60°C for 4 weeks.

buffer at pH 5.5 which showed high residual monomer of 72%. However, total percentage of residual monomer and aggregate fell much below 100% in phosphate and citrate buffers at pH 6.5, because insoluble aggregate was formed in IgG solutions with such a condition. In other buffer species, such insoluble aggregate was not detected.

In order to confirm the presence or absence of covalent bonds in the aggregates, the IgG solutions was subjected to non-reduced and reduced SDS-PAGE after heat treatment at 80°C for 2 h and at 60°C for 4 weeks. The electrophoregrams of IgG solutions in phosphate buffer at pH 5.5 and MES buffer at pH 5.5 after heat treatment at 80°C for 2 h are shown in Fig. 2 as typical results of buffers with different stability. Some bands, corresponding to the aggregates, were detected in the higher molecular weight range than monomers by SDS-PAGE under non-reducing conditions. However, the proportion of the aggregates as calculated from the band intensity was lower than that calculated by SEC–HPLC. On the other hand, the band distribution in the SDS-PAGE under reducing conditions was not changed by heat treatment. Next, the electrophoregrams of IgG solutions in phosphate buffer at pH 5.5 and MES buffer at pH 5.5 after heat treatment at 60°C for 4 weeks are shown in Fig. 3 and the results were similar to those obtained following heat treatment at 80°C for 2 h. In this figure, not only the significant band corresponding to the aggregates seen in the electrophoregrams of SDS-PAGE under non-reducing conditions, but also the band in a higher molecular weight range than that corresponding to the H chain seen in those of SDS-PAGE under reducing conditions was observed. Therefore, most of the increase of the aggregates observed after heat treatment at 80°C storage represented the non-covalent species, whereas some covalent aggregates formed by inter-chain disulfide bonding. On the other hand, the

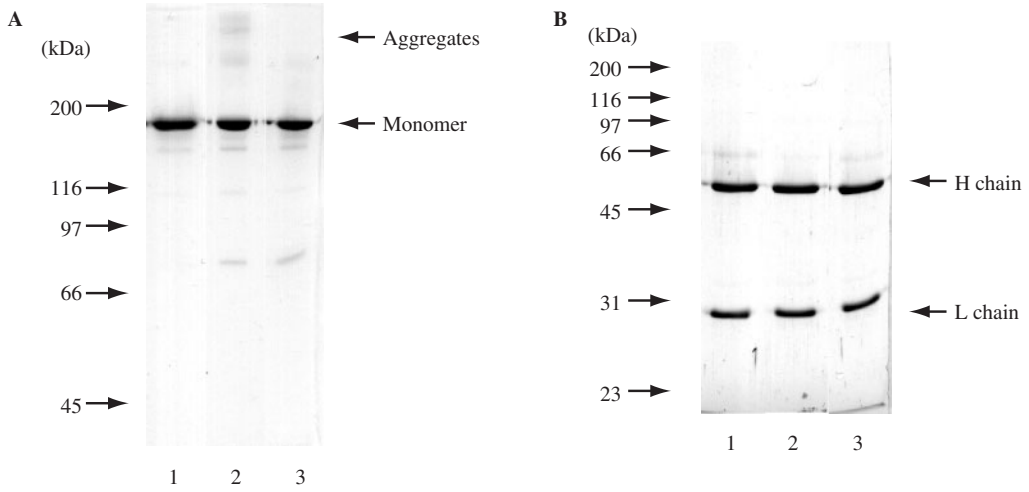

Fig. 2. **Non-reduced (A) and reduced (B) SDS-PAGE analysis of humanized IgG before and after 80°C storage for 2 h.** Lane 1: Before storage in 15 mM phosphate buffer, pH 5.5; Lane 2: After 80°C storage in 15 mM phosphate buffer, pH5.5; Lane 3: After 80°C storage in 15 mM MES buffer, pH 5.5.

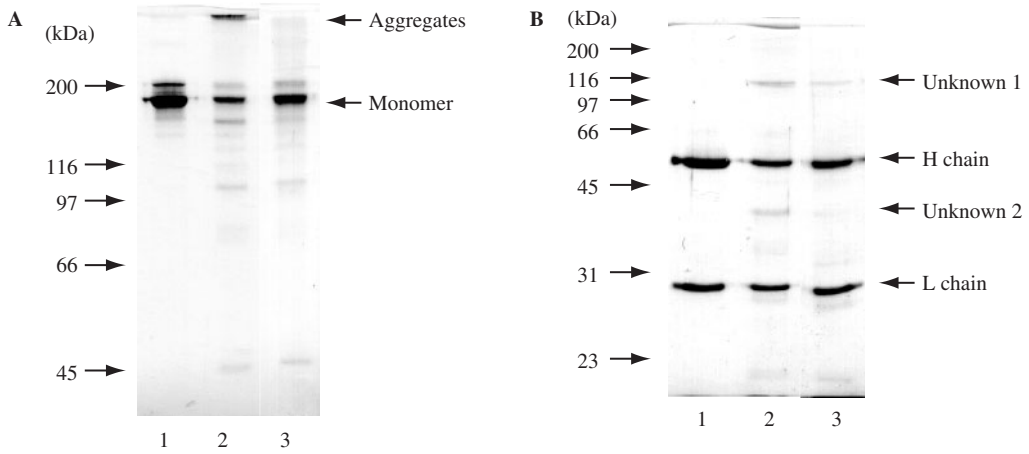

Fig. 3. **Non-reduced (A) and reduced (B) SDS-PAGE analysis of humanized IgG before and after 60°C storage for 4 weeks.** Lane 1: Before storage in 15 mM phosphate buffer, pH 5.5; Lane 2: After 60°C storage in 15 mM phosphate buffer, pH 5.5; Lane 3: After 60°C storage in 15 mM MES buffer, pH 5.5.

aggregates formed after heat treatment at 60°C were shown to be cross-linked by various covalent bonds, including inter-chain disulfide bonds. In addition, the bands corresponding to the aggregates in phosphate and citrate buffers showed relatively high intensity, irrespective of the heat treatment condition.

Evaluation of the Unfolding Temperature by DSC—Direct scanning calorimetry of the antibody solutions was performed to evaluate the unfolding temperature of the IgG in solutions containing each of the five buffer species. The DSC thermograms and the unfolding temperatures determined are shown in Fig. 4 and Table 2, respectively. These results suggest that heat denaturation of this IgG occurred in three steps, that is, unfolding of the Fc domain corresponding to the first two endothermic peaks, and that of the Fab domain corresponding to the last endothermic peak. However, the unfolding temperature of each buffer species with any of the unfolding steps did not correlate with the difference of aggregation propensity. In contrast, because of the appearance of an exothermic signal during the third unfolding step, the corresponding unfolding temperature could not be measured in the phosphate and citrate buffer solutions. This phenomenon was thought to be attributable to convection in the DSC cell due to protein aggregation, therefore it was suggested that the IgG aggregation occurred during the third unfolding step. In fact, sample solution of these buffer solutions recovered from DSC cell was clouded.

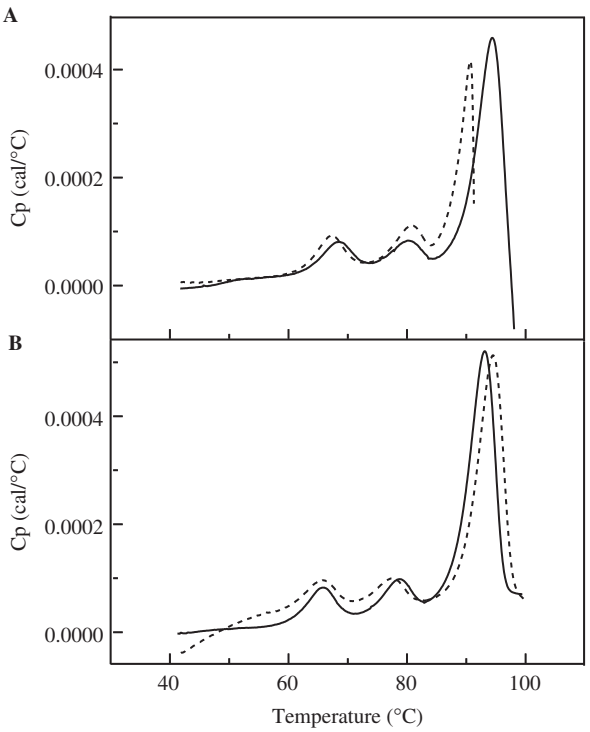

Fig. 4. **DSC thermograms of humanized IgG in different buffer species.** (A) Group H buffers; 15 mM phosphate buffer, pH 5.5 (solid lines) and 15 mM citrate buffer, pH 5.5 (dotted lines). (B) Group L buffers; 15 mM MES buffer, pH 5.5 (solid lines) and 15 mM acetate buffer, pH 5.5 (dotted lines). The protein concentration was 0.5 mg/ml.

Aggregation of the Heat-denatured Intermediate—The light scattering intensity of each buffer species after addition of the non-aggregated heat-denatured intermediate was measured to evaluate the aggregation propensity of the intermediate. The time-courses of the changes in the Rayleigh light scattering intensity at 340 nm in the phosphate, citrate, acetate and MES buffer solutions are shown in Fig. 5. A light scattering intensity after the addition of heat-denatured intermediate was not altered in MES and acetate buffers. On the contrary, this light scattering intensity was increased in phosphate and citrate buffers and the rate of increase was in the order of citrate pH 6.5 > phosphate pH 6.5 = citrate pH 5.5.

Effect of the Ionic Strength of the Solution on the Aggregation of Whole IgG—In order to evaluate the effect of the ionic strength of the solution on the IgG aggregation, IgG solutions with a similar ionic strength of the buffer species were stored at 60°C for 4 weeks. The percentages of the aggregates after heat treatment in the phosphate, citrate, acetate and MES buffer solutions are shown in Fig. 6. The percentages of the aggregates increased with increasing ionic strength for all the buffer species. Correspondingly, the percentage of residual monomer after heat treatment decreased with increasing percentage of the aggregates. On the other hand, the percentage of the aggregates did not differ among the different buffer species (phosphate, acetate and MES) having an equal ionic strength. Interestingly, the percentage of residual monomer was relatively high

Table 2. **Unfolding temperatures of whole and each fragments of humanized IgG in different buffer species.**

Buffer species		Tm[a] (°C)					
		Whole IgG			Fc		Fab
		First step	Second step	Third step	First step	Second step	
Phosphate	pH 6.5	NT[b]	NT[b]	NT[b]	70.3	–[d]	92.9
MES	pH 6.5	70.7	79.7	94.3	69.8	81.7	94.3
Imidazole	pH 6.5	67.3	76.3	93.9	NT	NT	NT
Phosphate	pH 5.5	68.6	80.2	94.3[c]	NT	NT	NT
Citrate	pH 5.5	67.7	81.3	91.5[c]	67.0	–[d]	93.1
Acetate	pH 5.5	65.6	77.5	94.4	68.8	81.1	94.3
MES	pH 5.5	66.5	79.2	93.6	66.5	80.8	93.6

[a]Tm was defined as the peak top of IgG or its fragments during thermal unfolding. [b]NT, Not tested. [c]Whole IgG was aggregated during the third denaturation step. [d]Fc fragment was aggregated during the second denaturation step.

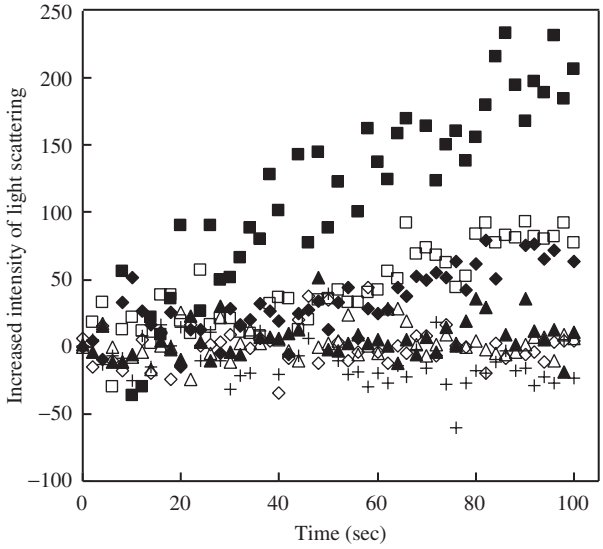

Fig. 5. **Time courses of the light scattering intensity at 340 nm after the heat-denatured intermediate addition in different buffer species:** 15 mM phosphate buffer, pH 6.5 (closed diamonds) and 5.5 (open diamonds), 15 mM citrate buffer, pH 6.5 (closed squares) and 5.5 (open squares), 15 mM MES buffer, pH 6.5 (closed triangles) and 5.5 (open triangles), and 15 mM acetate buffers, pH 5.5 (crosses).

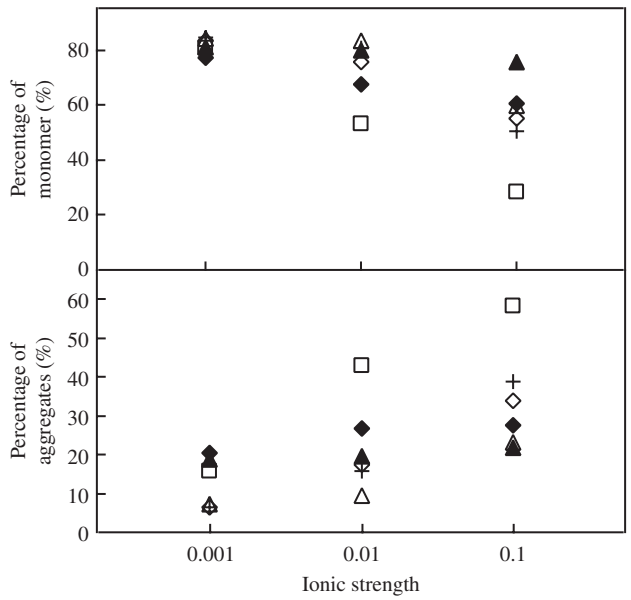

Fig. 6. **Effect of ionic strength on the aggregation of humanized IgG in different buffer species:** 15 mM phosphate buffer, pH 6.5 (closed diamonds) and 5.5 (open diamonds); 15 mM citrate buffer, pH 6.5 (closed squares) and 5.5 (open squares); 15 mM MES buffer, pH 6.5 (closed triangles) and 5.5 (open triangles); 15 mM acetate buffers, pH 5.5 (crosses).

even at a high ionic strength of the MES buffer, pH 6.5. Also, the percentage of aggregates formed in the citrate buffer was higher than that in the phosphate, acetate and MES buffers, even for an equal ionic strength.

Table 3. **Residual percentage of each fragment and increase of ANS fluorescence intensity[a] in the solutions of humanized IgG fragments containing different buffer species after heat treatment.**

Buffer species		Fab (60°C/4weeks)		Fc (60°C/7days)	
		Residual (%)	Increase of ANS fluorescence	Residual (%)	Increase of ANS fluorescence
Phosphate	pH 6.5	94.1	25	65.1	311
Citrate	pH 5.5	67.9	-20	55.6	1365
MES	pH 6.5	93.4	8	62.5	137
MES	pH 5.5	99.8	7	51.1	532
Acetate	pH 5.5	90.5	0	68.6	246

[a]ANS fluorescence intensity was monitored at 470 nm after excitation at 365 nm.

Thermal Stability of the Fab and Fc Domains—The effect of the buffer species on the thermal stability of the Fab and Fc fragments prepared by papain digestion of IgG was evaluated after heat treatment at 60°C. The percentage of the residual monomer and the ANS protein binding fluorescences of the Fab and Fc fragments in phosphate, citrate, acetate and MES buffer solutions after heat treatment at 60°C are shown in Table 3. The percentage of the Fab fragment monomer after storage at 60°C for 4 weeks was almost 100% in all the buffer species, except citrate. In addition, no ANS protein binding was detected in any of the buffer species because fluorescence intensity was not increased after ANS addition. On the other hand, the percentage of the residual Fc fragment monomer was decreased to 50–70% even after storage at 60°C for 1 week, without any significant differences among the buffer species. Furthermore, the ANS protein binding fluorescence was increased in the Fc fragment solutions, and the maximum increase was observed in the solution in citrate buffer, pH 5.5.

The DSC thermographs and the determined unfolding temperature of each IgG fragment in each of the buffer species are shown in Fig. 7 and Table 2, respectively. The Fab fragment solution showed an endothermic peak corresponding to the third unfolding step of whole IgG, and the unfolding temperature determined was 94°C, irrespective of the buffer species used. On the other hand, the Fc fragment solutions showed two endothermic peaks corresponding to first and second unfolding steps of whole IgG. While the first unfolding temperature of the Fc fragment was 67–70°C, irrespective of the buffer species used, the second unfolding temperature of the Fc fragment differed significantly among the buffer species, and a dramatic exothermic signal appeared in all except the MES buffer, pH 5.5, possibly due to the aggregation propensity of the partially unfolded molecule. The signal of the second unfolding step was, however, especially difficult to confirm in the phosphate and citrate buffer solutions.

DISCUSSION

Effect of Buffers on the Aggregation of IgG—Because of the restricted pH range in which high protein stability

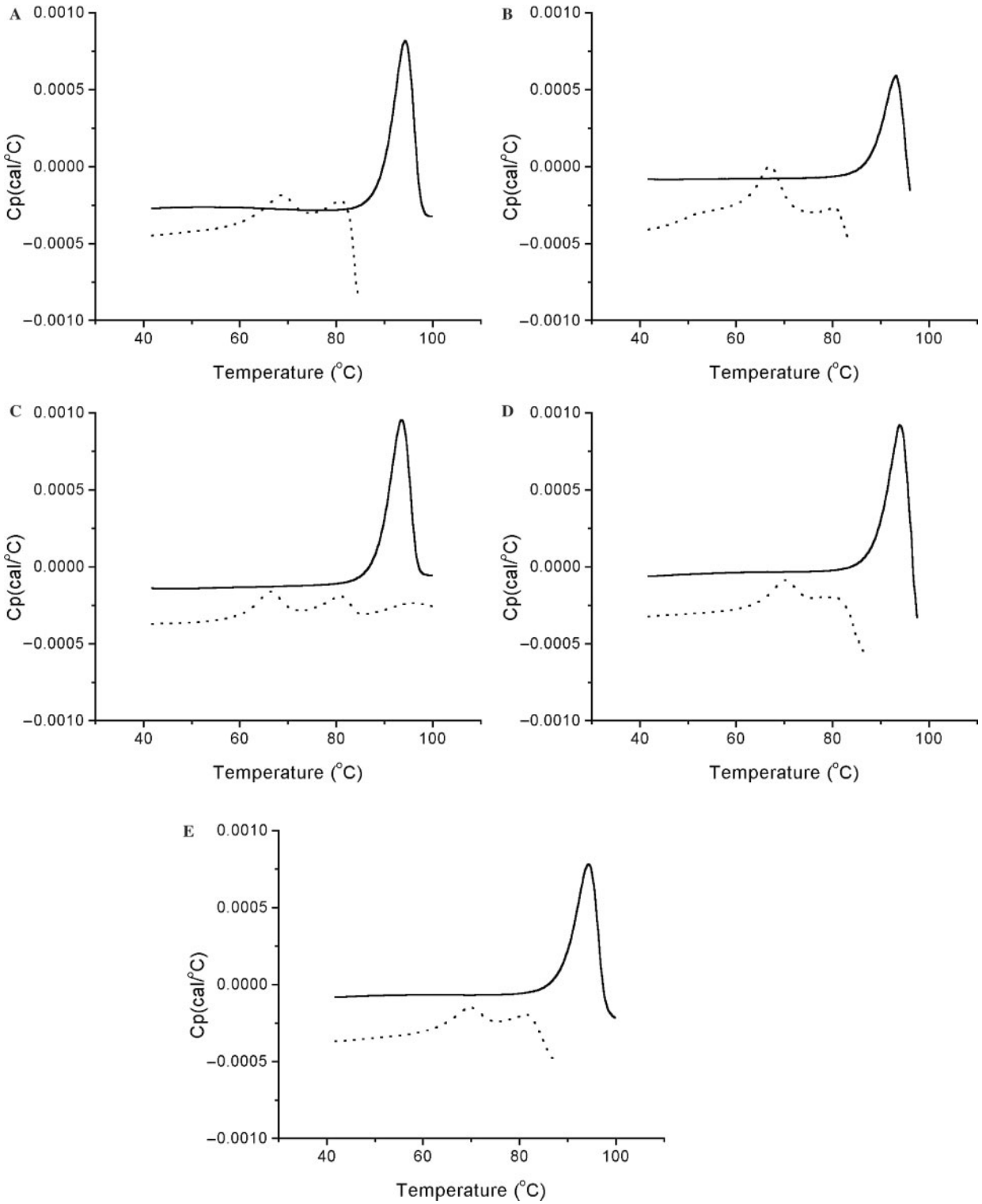

Fig. 7. **DSC thermogram of each humanized IgG fragments in different buffer species.** (A) 15 mM acetate buffer, pH 5.5; (B) 15 mM citrate buffer, pH 5.5; (C) 15 mM MES buffer, pH 5.5; (D) 15 mM MES buffer, pH 6.5; (E) 15 mM phosphate buffer, pH6.5. Solid line, Fab fragment; dotted line, Fc fragment. The protein concentration was 0.5 mg/ml.

can be maintained, the pH of protein solutions should be controlled precisely within the appropriate range for each molecule to maintain the protein stability and prevent protein aggregation. Therefore, buffer solutions are employed in many cases for long-term protein storage. To determine the most suitable buffer species for each protein solution, the effects of the buffer species on the protein stability should be evaluated. In this study, the

influence of six buffer species on the aggregation of humanized IgG was evaluated. As a result, low aggregation propensity in MES, MOPS and imidazole buffers and high aggregation propensity in phosphate and citrate buffers were found (Table 1). Therefore, for humanized IgG, it was suggested that the buffer containing morpholine, sulfonic acid and imidazole retarded the formation of heat-denatured intermediates. So, in order to know which functional groups retard the formation of heat-denatured intermediates, further study will be necessary using the other types of buffers. And also, the possibilities to extrapolate the effect of buffer species on the inhibition of protein aggregation for other proteins need to be investigated.

Involvement of Heat-denatured Intermediate on Aggregation of IgG—Since no differences in the unfolding temperature were observed among several buffer solutions in this study, the DSC results could not be attributed to differences in the aggregation propensity of IgG (Table 2). However, the exothermic signal suggestive of aggregate formation during the 3rd unfolding step was detected only for phosphate and citrate buffer solutions which showed high aggregation propensity (Fig. 4). From these findings, it was suggested that the differences in the IgG aggregation propensity among buffer species were considered to be attributable to aggregation of the heat-denatured intermediate.

The aggregation mechanism of many proteins can be expressed as a two-step reaction consisting of denaturation and aggregation processes (*9*). One is the reversible process from native state to intermediate state, and the other is the irreversible process from the intermediate to aggregation state. This supposition implies that the aggregation propensity is closely related to the heat-denatured intermediates. The present results were consistent with the previous idea (*9*) because the light scattering intensity was increased in phosphate and citrate buffer solutions, where the heat-denatured intermediate was observed (Fig. 5). From the above results, IgG aggregation was affected by the stability of the heat-denatured intermediate rather than the unfolding temperature. Therefore, the suppressed aggregation of humanized IgG in these stable buffer solutions such as MES and acetate is considered to be attributable to the higher stability of the hydrophobic heat-denatured intermediate with a low aggregation propensity. In fact, the aggregation of ABX-IL8 antibody, as measured by DSC, was inhibited in histidine buffer containing an imidazole group (*10*). Since the finding for ABX-IL8 antibody was consistent with the present results, the information obtained from this study can generally be applied to many other humanized antibodies, especially subtype IgG1 antibodies.

Contribution of Ionic Strength of Buffer for the Aggregation of IgG—In this study, the effects of the buffer species on the aggregation of humanized IgG were compared under the condition of a constant molar concentration. However, the effects may originate from differences in the ionic strength, because the ionic charges were different among the various buffer species. In fact, evaluation of the influence of the ionic strength on the aggregation propensity after heat treatment showed that the percentage of aggregates in all the buffer species increased in proportion to the ionic strength (Fig. 6). In addition, the aggregation propensity was not different in the equal ionic strength, except for the case of citrate buffer. Generally, the hydrophobic interaction could represent the main intermolecular interaction under high ionic strength conditions after salt addition, because the electrostatic repulsion due to the negative charge on this molecule could be weakened. Therefore, the acceleration of protein aggregation under high ionic strength conditions observed in this study may imply that the hydrophobic interaction could be the dominant factor for aggregation. In other words, the higher aggregation propensity of humanized IgG in phosphate buffer as compared with that in the other buffer species containing equal buffer concentration could only be due to the higher ionic strength of phosphate buffer. On the other hand, the aggregation propensity in all the buffer species cannot be explained by only differences in the ionic strength. For example, MES buffer specifically inhibited the aggregation of humanized IgG at pH 6.5, but citrate buffer accelerated such aggregation under both the pH conditions examined, despite the equal ionic strength. From these results, it is suggested that some interaction of the buffer ions with the protein surface stabilized or destabilized the protein itself. This proposition is interesting from the viewpoint of resolving the effects of the buffer species on protein aggregation, and could be clarified from identification of the binding positions of the buffer ions on the protein surface. For example, comparative evaluation of the suppression of IL-1ra aggregation in high concentration solutions of three anion species showed the lowest effect of phosphate ions, perhaps attributable to the low affinity of this anion to its binding site on IL-1ra (*11*). Such knowledge is important to obtain an understanding at the molecular level of suppression mechanism of protein aggregation by buffer species, which has until now been discussed only on an empirical basis.

Aggregation of Whole IgG is Caused by that of Fc Fragment—The heat denaturation process of IgG can be divided into several denaturation steps of the Fc and Fab domains, and both of the denaturation processes occur independently. Vermeer *et al*. has discussed this thermodynamic fact in detail based on their series of research on isotype 2b IgG (*5*, *12*). In their study, the denaturation temperature of the Fab and Fc fragments of the above IgG prepared by papain digestion was comparable to that of whole IgG, and the heat denaturation of whole IgG can be expressed as the summation of that of the Fab and Fc domains. Moreover, the denaturation peak detected during DSC of the anti-p24 (HIV-1) mAb CB 4-1 was also assigned to the denaturation of the Fab and Fc domains, and the influence of pH on the denaturation temperature of each was confirmed (*13*). Taking into consideration these references, separation of the Fab and Fc fragments is often used to investigate the changes in the physicochemical properties of whole IgG and to discuss the denaturation mechanism of IgG. In this study, the influence of buffer species on the aggregation propensity and denaturation temperature of the Fab and

Fc fragments prepared by papain digestion was evaluated to clarify which domain might contribute to aggregation of the whole humanized IgG. The results revealed no aggregation of the Fab fragment after heat treatment at 60°C for 4 weeks in any of the buffer species, except citrate, but the aggregation propensity of the Fc fragment depended on the buffer species, as in the case of whole IgG (Table 3). On the other hand, the results of DSC revealed that the denaturation temperature of both fragments was equal to that of the whole IgG, but an exothermic peak was detected after the denaturation of the Fc fragment as in the case of the whole IgG in all the buffer species, except in the MES buffer at pH 5.5 (Table 2 and Fig. 7). From these results, since the aggregation propensity of the whole IgG and Fc fragment was similar, it was suggested that Fc was the important domain which governed the aggregation of whole IgG.

Adequate Buffer Solution is Effective Aggregation Inhibitor—Many approaches for suppressing protein aggregation have been attempted to maintain the stability of protein solutions in storage. Addition of stabilizers, such as sugars and amino acids, is a widely used technique for suppressing protein aggregation. Sugars and sugar alcohols are preferentially excluded from the protein surface in the protein solution, and the protein is preferentially hydrated (*14*). As the free energy of the native protein is decreased by the preferential hydration, the hydrated protein shows an increase in the denaturation temperature. Moreover, basic amino acids, such as L-arginine suppress the aggregation and precipitation of the intermediates with high hydrophobicity during the protein refolding process by interacting with the hydrophobic protein surface (*15*). Arakawa *et al*. (*16*) reported that arginine suppressed aggregation of interleukin-6 (IL-6) and a mAb concentration-dependently during thermal unfolding. However, in such an approach, extensive additional amounts of the stabilizers are needed, which results in a marked increase in the osmotic pressure of protein solution. Such a high osmotic pressure shows little adequacy for pharmaceutical application, in particular, for subcutaneous injection because isotonicity of administrative solution is effective to reduce the pain on injection. Therefore, additives which can be added in small quantities to suppress protein aggregation are desirable. The results of this study revealed that the aggregation of humanized IgG can be inhibited without the addition of large amounts of stabilizers, by adding small concentrations, in the millimolar range, of suitably selected buffer species. Moreover, it was shown that the inhibition of humanized IgG aggregation was due to stabilization of the heat-denatured intermediates. Thus, humanized IgGs can be applied as a pharmaceutical possessing numerous different antigen-binding abilities only by inducing slight structural changes of the antigen-binding site. In conclusion, the method attempted for the suppression of protein aggregation in this study is expected to allow the application of humanized IgGs as pharmaceuticals.

The authors thank Dr Paul Langman (Chugai Pharmaceutical Co. Ltd.) for his useful advice in the language editing of this article.

REFERENCES

1. Chen, B., Arakawa, T., Hsu, E., Narhi, L.O., Tressel, T.J., and Chen, S.L. (1994) Strategies to suppress aggregation of recombinant keratinocyte growth factor during liquid formulation development. *J. Pharm. Sci.* **83**, 1657–1661
2. Roberts, C.J., Darrington, R.T., and Whitley, M.B. (2003) Irreversible aggregation of recombinant bovine granulocyte-colony stimulating factor (bG-CSF) and implications for predicting protein shelf life. *J. Pharm. Sci.* **92**, 1095–1111
3. Townsend, M.W. and DeLuca, P.P. (1990) Stability of ribonuclease A in solution and the freeze-dried state. *J. Pharm. Sci.* **79**, 1083–1086
4. Fatouros, A., Österberg, T., and Mikaelsson, M. (1997) Recombinant factor VIII SQ—influence of oxygen, metal ions, pH and ionic strength on its stability in aqueous solution. *Int. J. Pharm.* **155**, 121–131
5. Vermeer, A.W.P. and Norde, W. (2000) The thermal stability of immunoglobulin: unfolding and aggregation of a multi-domain protein. *Biophys. J.* **78**, 394–404
6. Tischenko, V.M., Zav'yalov, V.P., Medgyesi, G.A., Potekhin, S.A., and Privalov, P.L. (1982) A thermodynamic study of cooperative structures in rabbit immunoglobulin G. *Eur. J. Biochem.* **126**, 517–521
7. Ejima, D., Tsumoto, K., Fukada, H., Yumioka, R., Nagase, K., Arakawa, T., and Philo, J.S. (2007) Effects of acid exposure on the conformation, stability, and aggregation of monoclonal antibodies. *Proteins* **66**, 954–962
8. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **15**, 680–685
9. Fink, A.L. (1998) Protein aggregation: folding aggregates, inclusion bodies and amyloid. *Fold. Des.* **3**, R9–R23
10. Chen, B., Bautista, R., Yu, K., Zapata, G.A., Mulkerrin, M.G., and Chamow, S.M. (2003) Influence of histidine on the stability and physical properties of a fully human antibody in aqueous and solid forms. *Pharm. Res.* **20**, 1952–1960
11. Raibekas, A.A., Bures, E.J., Siska, C.C., Kohno, T., Latypov, R.F., and Kerwin, B.A. (2005) Anion binding and controlled aggregation of human interleukin-1 receptor antagonist. *Biochemistry* **44**, 9871–9879
12. Vermeer, A.W. and Norde, W. (2000) The unfolding/denaturation of immunogammaglobulin of isotype 2b and its Fab and Fc Fragments. *Biophys J.* **79**, 2150–2154
13. Welfle, K., Misselwitz, R., Hausdorf, G., Höhne, W., and Welfle, H. (1999) Conformation, pH-induced conformational changes, and thermal unfolding of anti-p24 (HIV-1) monoclonal antibody CB4-1 and its Fab and Fc fragments. *Biochim. Biophys. Acta* **1431**, 120–131
14. Arakawa, T. and Timasheff, S.N. (1982) Stabilization of protein structure by sugars. *Biochemistry* **21**, 6536–6544
15. Tsumoto, K., Umetsu, M., Kumagai, I., Ejima, D., Philo, J.S., and Arakawa, T. (2004) Role of arginine in protein refolding, solubilization, and purification. *Biotechnol. Prog.* **20**, 1301–1308
16. Arakawa, T., Kita, Y., Ejima, D., Tsumoto, K., and Fukada, H. (2006) Aggregation suppression of proteins by arginine during thermal unfolding. *Protein Pept. Lett.* **13**, 921–927