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Research Article

The Stabilization of a Human IgM Monoclonal Antibody with Poly(vinylpyrrolidone)

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An IgM anti-group B *Streptococcus* monoclonal antibody (4B9) was found to undergo irreversible heat-induced aggregation at 50°C. A variety of excipients was tested for their ability to inhibit antibody aggregation. The amount of 4B9 aggregation, which was determined by analysis on a size-exclusion HPLC, was significantly reduced in the presence of low concentrations [between 0.1 and 1.0% (w/v)] of poly(vinylpyrrolidone) (PVP) molecules ranging in molecular weight from 10 to 40 kDa. When the PVP concentration was greater than 1.0%, antibody aggregation was enhanced, and with the highest molecular weight PVP, antibody precipitation occurred. HPLC was used to show that more PVP was associated with the 4B9 at 50°C than at 25°C. Differential scanning calorimetry revealed that PVP concentrations greater than 2.0% decreased the antibody thermal transition temperature. Enzyme-linked immunosorbent assays were used to assess the effects of PVP on the antigen binding capacity of 4B9 and on 4B9 quantitation. At 4°C, PVP solutions of up to 5.0% had no effect on either 4B9 quantitation or antigen binding. At 50°C, however, less 4B9 was detected in the 5.0% PVP solution. The heat stabilization of the 4B9 antibody by low concentrations of PVP can be explained by a weak binding of PVP to the native protein. The PVP may sterically interfere with protein–protein interactions, thus reducing aggregation. Higher concentrations of PVP lead to protein aggregation and precipitation, probably by a volume-exclusion mechanism. Low concentrations of less than 1.0% PVP can be used to stabilize proteins against heat-induced aggregation, but care should be exercised, since even slightly higher concentrations of PVP can also lead to protein destabilization.

KEY WORDS: protein stability; protein aggregation; monoclonal antibodies; volume exclusion; poly(vinylpyrrolidone).

INTRODUCTION

The stabilization of protein pharmaceuticals is of critical importance to the development of successful therapeutic products. Protein molecules are intrinsically unstable in the simple aqueous solutions that are often employed in their manufacturing processes and can undergo a variety of physical and/or chemical reactions which result in loss of potency (1,2). Thermally induced denaturation can be a significant problem because proteins are often exposed to higher than ambient temperatures during different stages of their production and purification. High temperatures increase the flexibility and collision frequency of proteins in solution which can result in aggregation and/or precipitation. Aggregated proteins may exhibit decreased bioactivity, altered half-life, and enhanced immunogenicity (3,4). A goal of this study was to investigate formulations capable of stabilizing an IgM monoclonal antibody against aggregation at elevated temperatures.

Numerous compounds have been reported to stabilize proteins in solution. Several excellent reviews have been written on this subject (1,2,5,6). Particular interest has focused on the mechanisms by which the additives or cosolvents either enhance or decrease protein solubility. Solvent additives can affect protein stability by direct interaction with the protein (binding to the protein), by indirect action through effects on the solvent (i.e., increasing solvent surface tension or increasing solution viscosity), or by a combination of both of these mechanisms (7,8). Preferential hydration has been proposed to account for the interaction between proteins and cosolvents, and results from the cosolvent being excluded from the protein's surface (9). A small preferential hydration can prevent protein aggregation and lead to increased protein stability, while a large preferential hydration can lead to phase separation and protein precipitation (10).

Many additives have been shown to enhance the solubility of proteins including sugars (8,11), polyols such as

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glycerol (12), and salts (13). Water-soluble synthetic polymers have also been used for protein stabilizing agents. Poly(ethylene glycol) (PEG)[5] is one such polymer that has been studied extensively as both a protein stabilizer (10,14,15) and a protein precipitant (16–18). The polymer, at high concentrations (>10%, w/v) has been shown to cause a preferential hydration of proteins in their native state and can lead to protein precipitation. At low concentrations (<1%, w/v), however, PEG binds to proteins upon denaturation and can stabilize them against aggregation (19). Surprisingly few studies have been reported which examine the mechanisms of interaction between other water-soluble synthetic polymers and proteins in as much detail as the PEG/protein system.

This manuscript describes the stabilization of an IgM class human monoclonal antibody (4B9) against heat-induced aggregation by the addition of the water-soluble polymer poly(vinylpyrrolidone) (PVP) as a cosolvent. The protein is of clinical interest because of its ability to provide therapeutic protection in animals infected with group B *Streptococci* (GBS) (20). Due to its low toxicity, PVP has been used extensively as an excipient in oral dosage forms (21). PVP has been used parenterally as a blood plasma volume expander (21) and has been shown to stabilize several proteins in aqueous solution including hyaluronidase (22) and bovine ribonuclease during lyophilization (23). PVP also acts as a protein precipitant (24). Little work, however, has been done to determine the mechanism of protein stabilization or destabilization by PVP.

In this study, different concentrations and molecular weights of PVP were evaluated for their ability to stabilize 4B9 against heat-induced aggregation at 50°C. Size-exclusion high-performance liquid chromatography (HPLC), differential scanning calorimetry (DSC), and two enzyme-linked immunosorbent assays (ELISA) were used to study the nature of the interactions between the PVP and antibody.

MATERIALS AND METHODS

The recombinant 4B9 monoclonal antibody was produced by Bristol-Myers Squibb, Pharmaceutical Research Institute, Seattle, WA. The PVPs, K15 (MW 10,000) (PVP10), K25 (MW 24,000) (PVP24), and K30 (MW 40,000) (PVP40), were purchased in solid form from Fluka Chemika. The dextran (MW 40,000), PEG (MW 5000–7000), maltose, glycine, sucrose, and glycerol were all purchased from Fluka. The human albumin (fraction V) was purchased from Sigma Chemical Co.

[5] *Abbreviations used:* A, aggregated state; Ag, antigen; D, denatured state; DSC, differential scanning calorimetry; ELISA, enzyme-linked immunosorbent assay; 4B9, IgM anti-group B *Streptococcus* human monoclonal antibody; GBS, group B *Streptococci;* HPLC, high-performance liquid chromatography; MW, molecular weight; N, native state; PBS, 10 m*M* sodium phosphate, 150 m*M* NaCl, pH 7.4; PEG, poly(ethylene glycol); PVP, poly(vinylpyrrolidone); PVP10, poly(vinylpyrrolidone), molecular weight 10,000; PVP24, poly(vinylpyrrolidone), molecular weight 24,000; PVP40, poly(vinylpyrrolidone), molecular weight 40,000; w/v, weight/volume.

Size-Exclusion HPLC for Protein Analysis

Protein samples were analyzed by size-exclusion HPLC at a wavelength of 280 nm on a Hewlett Packard 1090M Chemstation equipped with a diode array detector. The column (7.5 mm × 60 cm) was a TSK 4000 SW made by Toso Haas. Sample volumes of 1000 μl were injected onto the column at an antibody concentration of 1 mg/mL. The mobile phase, which consisted of a 10 m*M* phosphate buffer containing 100 m*M* NaCl at pH 7.4, was run at a flow rate of 0.5 mL/min. The column was calibrated with five gel filtration standards including vitamin B-12 (mw 1350), equine myoglobin (17,000), chicken ovalbumin (44,000), bovine γ-globulin (158,000), and thyroglobulin (670,000) (Bio-Rad Laboratories, No. 151-1901).

Heat Stability Studies with Different Excipients

A variety of excipients was tested for their ability to reduce heat-induced aggregation of the 4B9. Previous studies in our laboratory determined that the pH optimum for the antibody was 7.4. The following additives were therefore individually evaluated in 10 m*M* phosphate with 150 m*M* NaCl, pH 7.4 (PBS): 5.0% (w/v) sucrose, 5.0% glycerol, 5.0% maltose, 2.0% (w/v) dextran, 100 m*M* glycine, 2.0% (w/v) PEG, and 0.1, 0.5, 1.0, 2.0, 5.0, or 10.0% (w/v) solutions of PVP10, PVP24, or PVP40. The samples were prepared by first dissolving the additives in phosphate-buffered saline (PBS) and then adding the mixture to 4B9 solutions. The final 4B9 concentration was 1 mg/mL. The antibody solutions were analyzed for aggregation by size-exclusion HPLC before and after heating at 50°C for various times. ELISA was used to determine if the excipients had any adverse effect on the 4B9 quantitation or antigen binding activity. To determine if the aggregation was reversible, the 4B9 samples were incubated at 50°C for 72 hr and subsequently stored at either 4 or 37°C for 1 day. All samples were then analyzed by HPLC. The aggregated antibodies were subjected to both centrifugation or filtration through a 0.2-μm filter and subsequently analyzed by HPLC to determine if the aggregates could be removed from solution. The effect of PVP10 on preformed aggregates was studied by aggregating a sample of the antibody at 50°C for 96 hr, adding PVP10 to the aggregated solution, incubating at 37 or 4°C, and then assaying by HPLC.

PVP Binding Experiments Using Size-Exclusion HPLC

The 4B9 (1 mg/mL) was mixed with different concentrations of PVP10 and either heated to 50°C for 24 hr or kept at ambient temperature. PVP10 solutions containing no 4B9 were also prepared. The PVP peaks were detected at a wavelength of 214 nm by HPLC using the same chromatography conditions described above. The peak areas of PVP10 solutions in PBS were determined and compared to the peak areas of the same concentrations of PVP10 containing 1 mg/mL of 4B9.

DSC

The DSC curves were recorded on a Seiko Model SSC/5200 instrument calibrated with an indium standard. 4B9 solutions (70-μl samples ranging in concentration from 3 to 10

mg/mL) in crimped silver containers were heated from 25 to 100°C at a rate of 1°C/min. Faster scan rates of 5 and 10°C/min were initially used, but the slower scan rate of 1°C/min produced the most well-defined thermal transition peaks. Samples contained varying concentrations and molecular weights of PVP. Reference cells contained the buffer and additives without the antibody. A 4B9 sample in 6% urea was evaluated as a positive control for a protein denaturant. Samples containing a protein concentration of 1 mg/mL were also evaluated, but the thermal transitions obtained at this concentration were very small and often difficult to differentiate from background. For this reason only the samples containing 3 and 10 mg/mL of 4B9 were used.

Molecular Weight Determination of 4B9

A light-scattering technique in conjunction with size-exclusion chromatography was used to determine the absolute molecular weight of the 4B9 molecule. The IgM was chromatographed on two size-exclusion columns (Shodex Protein KW 803 and KW 804) in series. This procedure separated out any contaminating aggregates that could skew the average molecular weight calculations. A DAWN F multiangle light-scattering photometer (Wyatt Technology Corp.) equipped with a helium–neon laser was situated postcolumn, followed by a refractive index detector. The instrument, which is capable of performing both low-angle and high-angle measurements, collected data in the low-angle mode. Data collected from both the laser and the detector were processed with ASTRA and EASI software (Wyatt Technology Corp.). The molecular weight was calculated using the differential refractive index increment (dn/dc) method (25). For a fixed sample concentration and dn/dc (0.18 for this study), the greater the light scatter intensity, the greater the molecular weight of the sample. Molecular weights were determined for the main 4B9 peak and aggregate peaks.

ELISA

Two ELISAs were used to evaluate the different 4B9 formulations: an antigen (Ag) binding assay and a human IgM quantitation assay. The Ag binding assay measures the ability of the 4B9 to bind to the GBS group polysaccharide, while the IgM quantitation assay determines the amount of 4B9 bound by an anti-human immunoglobulin. Both assays were run at 25°C. Samples were serially diluted starting at 20 ng/mL and applied to 96-well microtiter plates which were coated, for the quantitation assay, with goat anti-human immunoglobulins G, A, and M (Cappel) and, for the Ag binding assay, with purified GBS capsular polysaccharide (in-house preparation). The bound 4B9 was detected with affinity-purified goat anti-human IgM, horseradish peroxidase conjugate (Tago, Burlingame, CA). The reaction was developed with a hydrogen peroxide solution containing the chromogen 3,3′,5,5′-tetramethylbenzidine (26). Absorbances were determined at 450 nm with a 630 reference filter on a microtiter plate reader. In experiments using purified antibody as the standard, the results were highly sensitive and reproducible, giving linear binding curves ranging in antibody concentration from 1 to 20 ng/mL. ELISA was used to evaluate 4B9 solutions with or without different concentrations of PVP10. The solutions were stored at either 25°C or incubated at 50°C for different times.

Protein Precipitation by High Concentrations of PVP

A study was done to determine the ability of PVP to precipitate 4B9 or human albumin from PBS. All solutions had an initial protein concentration of approximately 5 mg/mL. Different amounts of PVP10 and PVP40 were added to the protein solutions at concentrations ranging from 6 to 18%. The samples were assessed by visual analysis immediately after preparation. Solutions were then incubated at 60°C for 1 hr and reevaluated.

RESULTS

Heat-Induced Aggregation of 4B9

A typical size-exclusion HPLC chromatogram of a 1 mg/mL 4B9 sample which was frozen at −70°C in PBS after purification, thawed, and stored at 4°C is shown in Fig. 1A. The majority of the protein eluted between 28 and 29 min as a single peak. There were two smaller peaks that eluted between 20 and 24 min that can be attributed to 4B9 aggregation. The combined area of both peaks was used to calculate the total amount of aggregation in a given sample. Aggregates made up between 5 and 10% of the total protein present in an unheated sample. Stability studies indicated that the percentage aggregate in a given lot of 4B9 stored at 4°C remained unchanged over a period of 6 months.

After heating the antibody at 50°C for 96 hr, the amount of aggregate increased significantly as shown in Fig. 1B. At least two distinct populations of aggregated proteins eluted at 20 and 23 min. The combined percentage area of these peaks was 35% of the total protein. The size of the main peak decreased in this sample when compared to the control, indicating that the aggregate peaks formed from the monomeric 4B9 molecules. A lower molecular weight protein peak, resulting from 4B9 degradation, was observed at about 42 min. The same HPLC profile was seen after the sample was filtered through a 0.2-μm filter or after centrifugation, indicating that the aggregates were soluble. A kinetic aggregation study was carried out by incubating 4B9 at 50°C in PBS for up to 120 hr, followed by size-exclusion HPLC analysis. The results show that there was a linear increase in the amount of 4B9 aggregation over time at 50°C (Fig. 1C).

Molecular Weight Determination by Light Scattering

The molecular weight of the unaggregated 4B9 molecule was determined to be approximately 920,000 daltons based on light-scattering analysis. The two aggregate peaks which eluted between 20 and 24 min were also analyzed and found to have molecular weights of approximately 3,090,000 and 1,840,000 daltons. These are most likely trimers and dimers of the native IgM molecule. The HPLC and light-scattering studies established that the 4B9 antibody was subject to heat-induced aggregation. A next study was designed to determine the effects of various additives on protein aggregation.

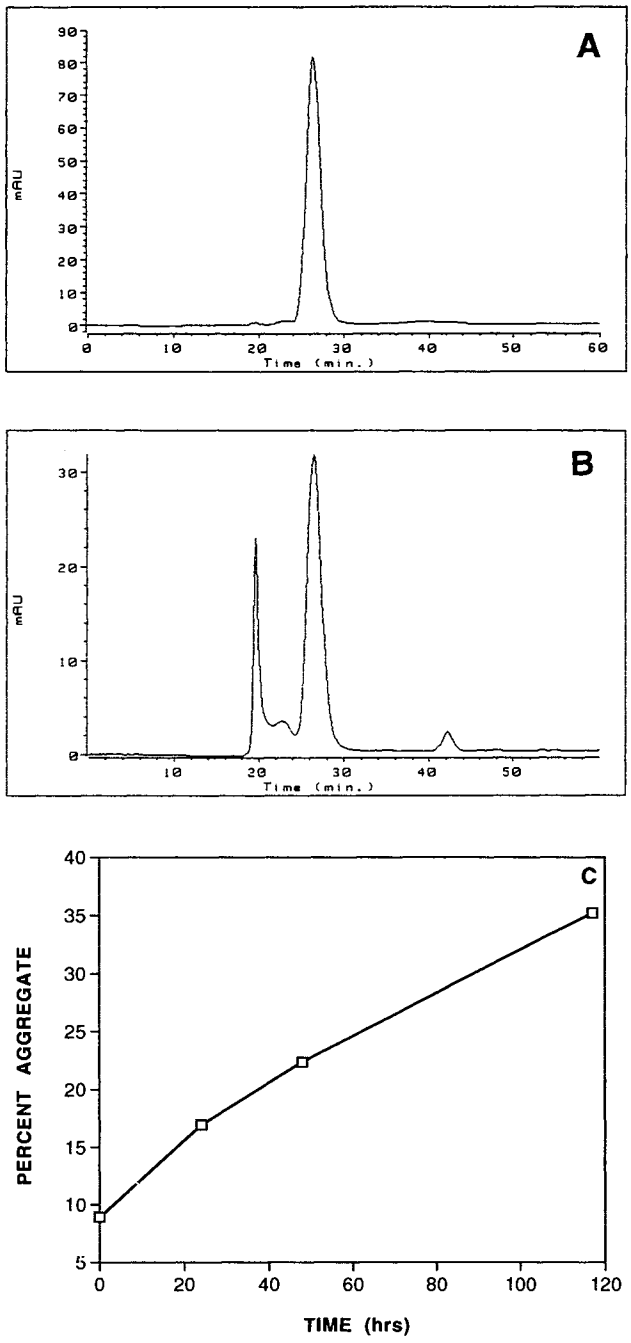


Fig. 1. Size-exclusion HPLC chromatograms of an IgM anti-group B *Streptococcus* monoclonal antibody (4B9) in (A) 1.0 mg/mL in 10 m*M* phosphate buffer, pH 7.4, at 4°C and (B) after heating in the same buffer at 50°C for 96 hr. (C) The percentage aggregation of 4B9 heated in PBS at 50°C as a function of time. The percentage aggregate was determined by size-exclusion HPLC.

Heat Stability Studies with Different Excipients

The percentage aggregation of 4B9 after heating at 50°C for 96 hr was compared in the presence or absence of different additives (Fig. 2). Two different lots of 4B9, produced from two different hybridoma cell lines, were used in this experiment. These lots had different amounts of aggregation in the starting material that had been stored at −70°C. The 2.0% PVP40 was the most effective additive for the prevention of heat-induced protein aggregation in both lots of 4B9.

Heat Stability Studies Varying PVP Molecular Weight and Concentration

The reduction in 4B9 aggregation by PVP motivated closer examination. The influence of PVP concentration and molecular weight was evaluated regarding 4B9 aggregation. Representative HPLC chromatograms of 4B9 samples incubated at 50°C for 96 hr with different concentrations of PVP10 are shown in Fig. 3. The 1.0% PVP sample (Fig. 3A) had less aggregation than a heated 4B9 sample containing no PVP (Fig. 1B). Increasing the PVP10 concentration to 2.0% (Fig. 3B) resulted in a slight increase in the amount of 4B9 aggregation relative to 1.0% PVP10, but it was still less than the control sample heated with no PVP. The sample containing 5.0% PVP10 had a large amount of 4B9 aggregation. No shifts in the elution time of the main 4B9 peak or changes in the degradation product (42 min) were observed with any of these samples. As the PVP concentration was increased, the PVP peak area (48 min) also increased.

Different molecular weights and concentrations of PVP were compared for their ability to reduce aggregation of 4B9 which was heated at 50°C for 96 hr (Fig. 4A). All of the PVP samples inhibited 4B9 aggregation when used at concentrations of 2.0% or less compared to the control 4B9 heated with no PVP. Solutions containing 5.0% PVP10 exhibited an increase in 4B9 aggregation compared to the controls. The 5.0% PVP24 caused an even greater increase in 4B9 aggregation (50%). The antibody precipitated from a heated solution containing 5.0% PVP40.

The total 4B9 main peak areas eluting between 28 and 29 min (which are proportional to amount of unaggregated 4B9 antibody in solution) of the same samples described in Fig. 4A are shown in Fig. 4B. At PVP concentrations of up to 1.0%, an increase in the 4B9 main peak area was observed in samples containing all three molecular weight PVPs com-

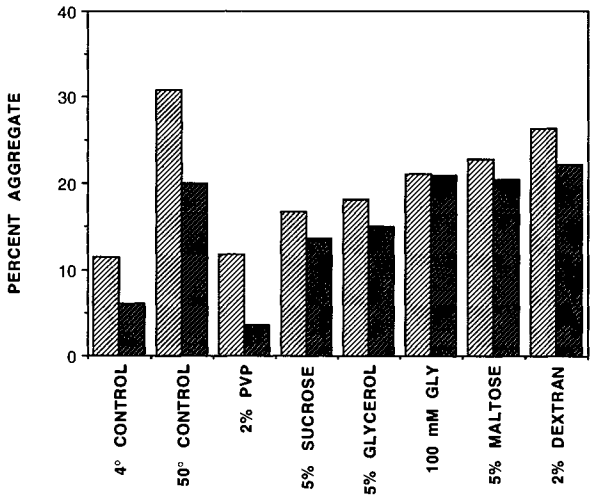


Fig. 2. Percentage aggregation as determined by size-exclusion HPLC of two lots of 4B9 antibody (represented by the different pattern bars) that were heated at 50°C for 96 hr in the presence of different additives. Samples were analyzed at a wavelength of 280 nm.

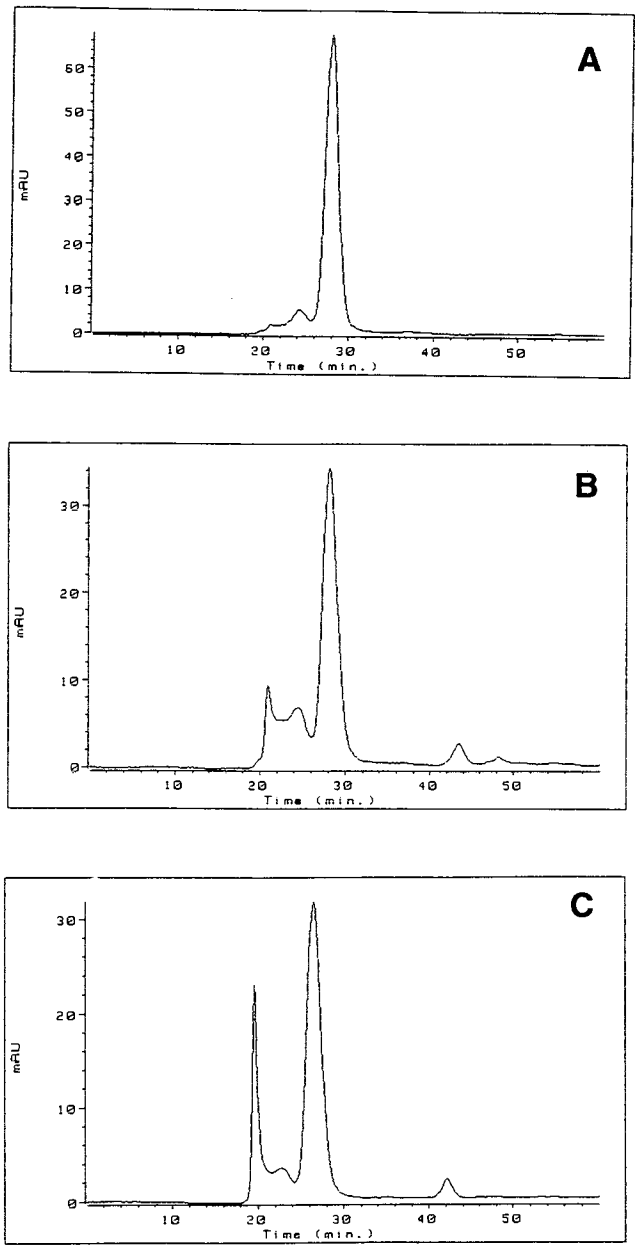


Fig. 3. Size-exclusion HPLC chromatograms of 4B9 at 1.0 mg/mL incubated at 50°C for 96 hr in 10 m*M* phosphate buffer, pH 7.4, at 4°C containing (A) 1.0%, (B) 2.0%, and (C) 5.0% PVP10. Samples were analyzed at a wavelength of 280 nm.

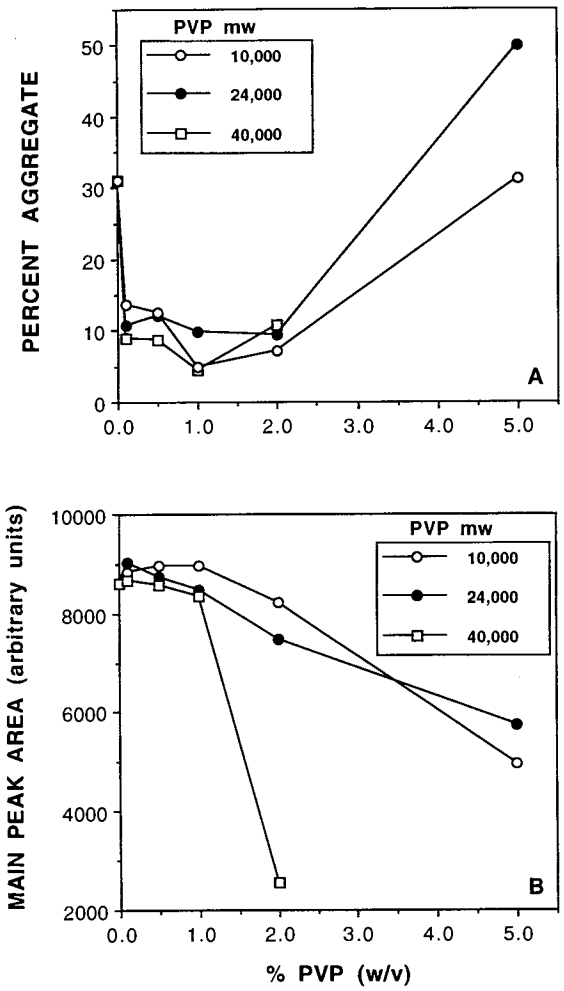


Fig. 4. (A) Percentage aggregation as determined by size-exclusion HPLC of 4B9 samples at 1 mg/mL that were heated at 50°C for 96 hr in the presence of different molecular weights and concentrations of PVP and (B) main HPLC peak area of the same samples.

pared to the heated control. This is consistent with the reduction in aggregation shown in Fig. 4A. Less 4B9 aggregation would result in more monomeric protein in solution. At a concentration of 2.0% the PVP10 and PVP25 samples were still effective in reducing aggregation compared to the controls, but had less unaggregated antibody than samples containing 1.0% PVP. The 2.0% PVP40 caused a significant reduction in the 4B9 concentration compared to the control. The 5.0% PVP10 and PVP25 samples caused a further decrease in antibody concentration to levels below that of the control sample. This result is consistent with the increased aggregation seen in these samples (Fig. 4A). Visual analysis of the samples showed that PVP40 at a concentration of 5.0% caused antibody precipitation. The same concentrations of PVP10 or PVP24 did not precipitate the 4B9. Antibody aggregation and/or precipitation, therefore, increased with increasing PVP molecular weight at PVP concentrations greater than 1.0%. Preformed 4B9 aggregates were not able to be dissociated when incubated with 1.0% PVP 10 solutions.

PVP Binding Experiments Using Size-Exclusion HPLC

Size-exclusion HPLC was used to determine if the PVP10 was binding to the 4B9 in solution. Detection of PVP10 at an absorbance of 214 nm was much more sensitive than detection at an absorbance of 280 nm. A standard curve comparing PVP10 peak area to PVP concentration was generated for PVP in PBS solutions at 25°C (Fig. 5). The curve was linear for PVP10 solutions ranging in concentration from 0.007 to 2.0%. Heating the PVP10 solutions in PBS at 50°C for 24 hr had no effect on the PVP concentration in solution (data not shown). A similar PVP curve was generated for PVP10 solutions containing 1.0 mg/mL 4B9 which were incubated at 25°C for 24 hr (Fig. 5). A slight reduction in PVP

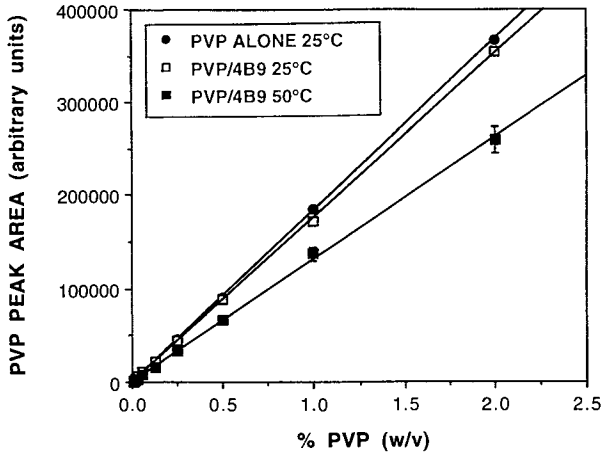


Fig. 5. Main PVP peak area determined by size-exclusion HPLC of PVP10 solutions alone in PBS at 25°C, PVP10 solutions with 1.0 mg/mL 4B9 stored at 25°C for 24 hr, and PVP10 solutions with 1.0 mg/mL 4B9 heated at 50°C for 24 hr. The PVP peaks were detected at an absorbance of 214 nm. Each data point represents the mean of three measurements ± standard deviation.

peak area was evident in samples containing PVP10 concentrations of 0.25% and higher. When the same samples were heated to 50°C for 24 hr, however, a significant reduction in PVP10 peak areas was observed for solutions containing PVP concentrations of 0.06% and higher, suggesting that some type of interaction was occurring between PVP and 4B9.

DSC

DSC has been used to determine how a cosolvent effects the thermal transition temperature (T_m) of a protein. Additives that induce an elevation in the T_m are generally protein stabilizers (8,12), while additives that decrease the T_m are protein destabilizers (10). Heating the 4B9 in PBS resulted in a thermal transition which could be seen as an endotherm at approximately 72°C (Fig. 6A). The addition of PVP10 and PVP40 to the samples at concentrations of 0.5 to 2.0% caused no significant change in the temperature at which the protein unfolded (Fig. 6B). When the amount of PVP was increased to 5.0%, however, a decrease in the T_m was observed. There appeared to be a trend in the reduction of the T_m with PVP molecular weight. The PVP40 was particularly effective, reducing the T_m from 72 to 66°C. A 10.0% PVP40 solution gave uninterpretable results due to precipitation of the sample. When the 4B9 was heated in the presence of 6.0% urea, the T_m also decreased to 66°C (data not shown).

ELISA

ELISA was used to determine if PVP10 had an effect on the GBS antigen binding activity of the 4B9 or its ability to be quantitated by binding to an anti-human immunoglobulin. The 4B9 samples stored at 25°C with PVP10 concentrations ranging from 0.1 to 5.0% were identical to controls in both 4B9 quantitation and GBS antigen binding. At 50°C, however, samples with 0 and 5.0% PVP underwent a 15 to 20% reduction in the detectable amount of 4B9 (Fig. 7). These samples had no visible precipitate indicating that the protein

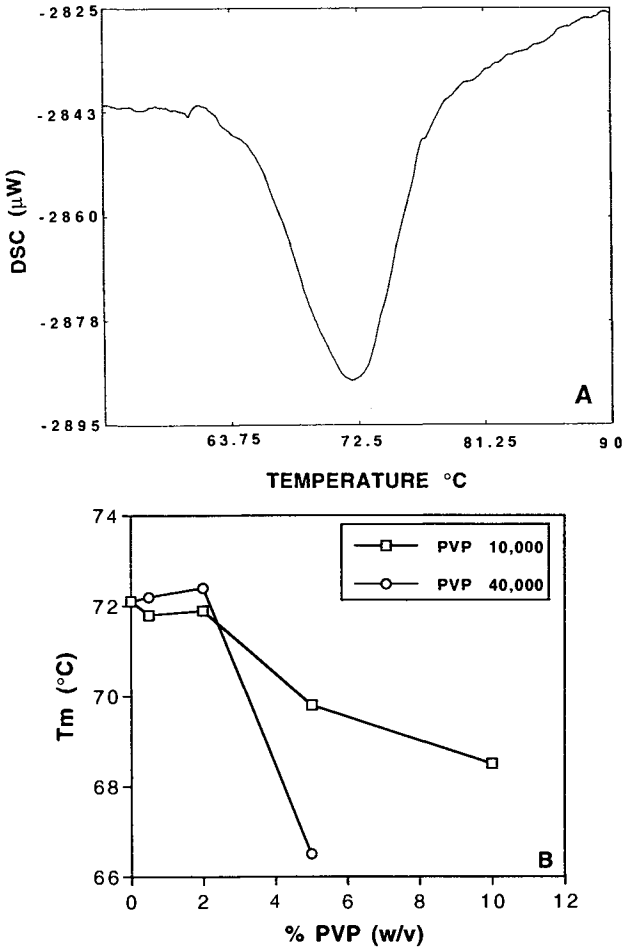


Fig. 6. (A) DSC thermogram for 4B9 in PBS heated from 25 to 100°C at a rate of 1°C/min. (B) Thermal transition temperatures (T_m) determined by DSC for 4B9 samples that were heated from 25 to 100°C at a rate of 1°C/min as a function of PVP concentration for solutions containing PVP10 or PVP40.

was either denatured or in the form of soluble aggregates. Samples containing from 0.1 to 1.0% PVP10 at 50°C still had up to 95% of the 4B9 quantitated by the ELISA (Fig. 7) and all of this antibody was effective in binding the GBS antigen. The results are consistent with the size-exclusion HPLC analysis of the 4B9 peak areas after heating to 50°C with different concentrations of PVP10 (Fig. 4B). In these HPLC studies, the 4B9 solutions containing between 0.1 and 1.0% PVP10 had the most detectable amount of monomeric protein present in solution, while samples heated with 0 or 5.0% PVP exhibited the smallest 4B9 peak areas and the greatest amount of soluble aggregate formation (Fig. 5A).

Protein Precipitation Experiments

No visible protein precipitate was formed in any of the human albumin solutions containing PVP10 or PVP40. The 4B9 solutions containing PVP10 were clear at ambient temperature but became hazy after heating to 60°C. All of the 4B9 solutions containing PVP40 had precipitate present both before and after heating. Protein precipitation by PVP may depend on the molecular weight of both the protein and the PVP. The precipitate was unable to be dissolved upon dilu-

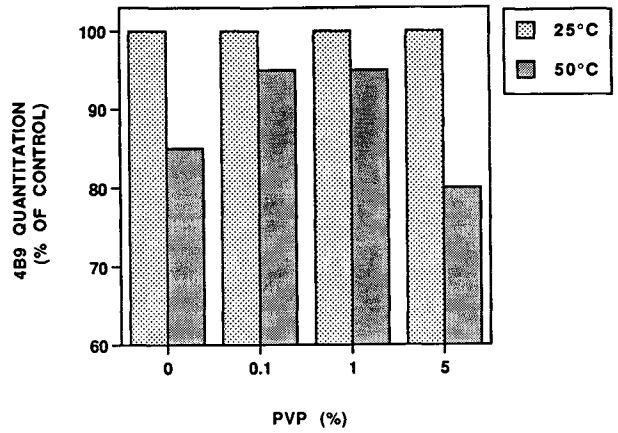


Fig. 7. ELISA 4B9 quantitation of 4B9 samples incubated at 25 or 50°C for 24 hr with different amounts of PVP10. The values are reported as percentage of the control sample held at 25°C with no PVP10. Values are reported as the mean of duplicate samples.

tion with buffer, indicating that protein precipitation by PVP is irreversible.

DISCUSSION

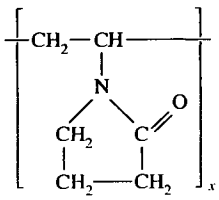
This report describes the stabilization of a human IgM monoclonal antibody against heat-induced aggregation using low concentrations ($\leq$1.0%) of PVP ranging in molecular weight from 10 to 40 kDa. Higher concentrations of PVP increased 4B9 aggregation and decreased the total amount of detectable antibody in solution. The protein destabilizing effect was proportional to PVP molecular weight at PVP concentrations of 2.0% or more. Solutions containing PVP40 concentrations of 5.0% or more caused precipitation of the antibody at 50°C, and 6.0% PVP40 solutions precipitated the 4B9 at room temperature. Thus, low concentrations of PVP stabilized the 4B9 against heat-induced aggregation, while high concentrations enhanced heat-induced aggregation and ultimately led to protein precipitation.

The simplest model that describes heat induced protein aggregation involves a two-step kinetic process:

$$N \leftrightarrow D \rightarrow A$$

where N is the native protein, D is the reversibly denatured protein, and A is the inactivated aggregate (27). Other more complex models have been proposed that involve a series of stable and misfolded intermediates, but for our discussion, the two-step model will suffice. In the first step, as the temperature rises, the conformational flexibility of the protein increases. The increased flexibility can result in a conformational change in protein secondary and tertiary structure. Reversible denaturation (D) involves the unfolding of a protein caused by an increase in temperature which can be reversed by subsequent lowering of the temperature. Irreversible denaturation disrupts the protein molecule to such an extent that it is unable to regain its native state. These changes are often the result of exposed hydrophobic amino acids which are normally buried in the interior of the protein. To minimize the free energy of the system, these amino acids may intra- or intermolecularly associate to reduce interactions with the aqueous solvent, ultimately leading to the formation of aggregates (A) and/or precipitate. The prevention of 4B9 aggregation by PVP must occur through interactions of PVP (either directly or through solution effects) with the native form (N) of the protein and/or the denatured state (D). These interactions shift the system's equilibrium to the native state (N). The protein destabilizing effect of higher PVP concentrations, on the other hand, shifts the equilibrium of the system to the denatured (D) and/or aggregated state (A).

The structure of PVP and its properties in simple aqueous solution may help explain the general interaction of PVP with proteins. The PVP monomer unit has an amphiphilic character. The highly polar amide group confers hydrophilic and polar-attracting properties, while the apolar methylene and methine groups in the backbone and the ring contribute to hydrophobic properties (28). The amide groups on PVP contribute to significant hydrogen bonding, and as a result, PVP has a high binding capacity for water. The molecule is soluble in aqueous solution in concentrations greater than 65% (28). Near-infrared spectroscopy measurements of PVP in aqueous solution indicate that



PVP has a structure making effect on the water as opposed to a structure breaking effect (28). The interaction between PVP and water is so strong that high PVP concentrations can prevent water from freezing, thus causing it to deviate from its normal behavior as a solvent (29). This unique behavior has led to the use of PVP as a cryoprotectant for a wide variety of cells (30) and as a lyoprotectant for proteins (23).

Based on these properties, several potential mechanisms can explain both the protein (4B9) stabilizing and the protein destabilizing effects of PVP. Under different conditions the PVP may be interacting with the 4B9 by (i) binding directly to the antibody through hydrogen bonding or hydrophobic interactions, (ii) changing the solvent surface tension and/or viscosity, or (iii) sterically excluding the antibody from solution by volume exclusion. The data suggest that the mechanism of interaction between PVP and 4B9 depends on the PVP concentration and temperature of the system.

At low PVP concentrations (below 0.25%) and temperatures of 25°C, there is no detectable association between the PVP10 and the 4B9 molecules (Fig. 5). As the system is heated to 50°C, we begin to see some interaction between PVP and 4B9. The PVP molecules are probably binding weakly to the 4B9 and this binding is enhanced at elevated temperatures as the protein begins to unfold. Since the binding is most likely a weak one, the observed decrease in PVP peak area observed when the sample is injected onto the column probably corresponds to only part of the bound PVP prior to injection. Upon injection, some of the weakly bound PVP will dissociate from the protein as it runs through the column.

The ELISA studies further support that PVP is binding

to the 4B9 at 50°C. At 25°C the 4B9 was unaffected in PVP10 solutions of up to 5.0%. However, at 50°C, PVP concentrations greater than 1.0% interfered with the ability of 4B9 to bind GBS antigen. At 25°C, therefore, either the PVP is weakly binding to the 4B9 or it is binding to sites on the 4B9 that do not interfere with the ELISAs. At 50°C, the PVP may be binding more strongly to exposed portions of the antibody. Therefore, as the temperature of the system is increased, the PVP is more likely to bind to the 4B9.

This conclusion is supported by the DSC results, which show that low concentrations (2.0% or less) of PVP10 or PVP40 had little effect on the T_m of the 4B9 molecule (Fig. 6B). Higher concentrations of PVP, however, caused a reduction in T_m of the 4B9. A similar reduction in the thermal transition of several proteins has been observed with PEG (10,14). The effect was attributed to a preferential interaction of the PEG with the denatured state of the protein. The authors hypothesized that the hydrophobic portion of the PEG bound to the hydrophobic side chains exposed upon protein unfolding, which led to a decrease in the T_m of the protein. Low concentrations of PEG have also been shown by fluorescence spectroscopy to bind to hydrophobic segments of carbonic anhydrase, thus preventing aggregation of the protein during refolding (19). The PVP appears to behave much like the PEG in its ability to decrease the T_m of a protein molecule. Both PVP and PEG have an amphiphilic character, are water soluble, and have hydrophobic moieties capable of binding to proteins. Spectral analysis indicates that hydrogen bonding is involved when PVP complexes with many molecules (21). The PVP in our system could be complexing to the 4B9 molecules at elevated temperatures through hydrogen or hydrophobic bonding.

Therefore, low concentrations of PVP may be stabilizing the 4B9 from heat-induced aggregation by binding weakly to the reversibly denatured forms of the protein (D), thereby inhibiting aggregation. A small amount of PVP interacting with the 4B9 at 50°C appears to be beneficial. As the PVP concentration becomes too high, however, the protein becomes more unstable. The DSC results provide evidence that too much PVP can reduce the T_m of the protein.

The HPLC data in Fig. 4B show that the 4B9 molecules are also being removed from heated solutions containing 2.0% PVP (particularly PVP40). Thus, both PVP and 4B9 are removed from 50°C solutions that contain high concentrations of PVP. No visible precipitate was observed in these samples. The PVP molecules may be forming a soluble complex with the denatured 4B9 molecules. The complex, which is too small to be seen visually, could be removed from solution by irreversible adsorption to the surfaces of vials and chromatography column media and tubing. The surface adsorption would result in an apparent decrease in concentration when analyzed by HPLC.

Some water-soluble polymers may induce a phase separation and result in protein precipitation when added to protein solutions at high enough concentrations. This type of protein precipitation has been observed with several water-soluble polymers including PVP (25) and has been studied extensively in protein solutions containing PEG (25,18). A steric or volume exclusion mechanism has been proposed to explain why proteins are precipitated out of solution by PEG (16). In general, higher molecular weight proteins are more prone to be excluded from a given polymer solution than lower molecular weight proteins. Increasing the protein concentration or the polymer molecular weight can also result in a more pronounced volume exclusion of the protein. In our studies, PVP more readily precipitated 4B9 (900 kD) from solution compared to BSA (45 kD). Solutions containing higher concentrations of 4B9 were more prone to aggregation and precipitation by PVP than more dilute 4B9 solutions. In addition, larger PVP molecules were more effective as protein precipitants than lower molecular weight PVPs. These trends are all consistent with the volume exclusion effect. This effect is not surprising in light of the extremely strong interaction between PVP and water (28). The irreversibility of the PVP induced protein precipitation (also observed by Polson *et al.* (24) may result from hydrophobic or hydrogen bonding of PVP to the protein, contributing to irreversible protein denaturation.

In summary, we have shown that the hydrophilic polymer PVP, at low concentrations, effectively stabilizes a human IgM monoclonal antibody against heat-induced aggregation. However, at higher PVP concentrations, protein aggregation and precipitation were apparent. In general, the PVP appears to stabilize the 4B9 by a weak binding of PVP to the 4B9. Destabilization and irreversible precipitation of the antibody occur at high PVP concentrations and elevated temperatures, most likely from a combination of mechanisms including protein denaturation through increased binding of PVP to the 4B9 and volume exclusion of the 4B9 from solution. Since PVP interacts with proteins by several mechanisms, care should be exercised when using PVP to stabilize other aqueous protein solutions, particularly at elevated temperatures.

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