Chemical Stability of Peptides in Polymers. 1. Effect of Water on Peptide Deamidation in Poly(vinyl alcohol) and Poly(vinyl pyrrolidone) Matrixes

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Abstract □ This paper examines the effect of water content, water activity, and glass transition temperature (T_g) on the deamidation of an asparagine-containing hexapeptide (VYPNGA; Asn-hexapeptide) in lyophilized poly(vinyl alcohol) (PVA) and poly(vinyl pyrrolidone) (PVP) at 50 °C. The rate of Asn-hexapeptide deamidation increases with increasing water content or water activity and, hence, decreasing T_g. The rate of deamidation is more sensitive to changes in these parameters in PVA than in PVP. Deamidation is clearly evident in the glassy state in both formulations. In the glassy state, the peptide is more stable in PVA than in PVP formulations but is less stable in the rubbery state. No single variable (water content, water activity, or T_g) could account for the variation in deamidation rates in PVA and PVP formulations. Deamidation rates were correlated with the degree of plasticization by water (distance of T_g from the dry intrinsic glass transition temperature); coincident curves for the two polymers were obtained with this correlation. Deamidation in PVA and PVP was closely correlated with the extent of water-induced plasticization experienced by the formulation relative to its glass transition at 50 °C, suggesting that the physical state of formulations could be used to predict chemical stability.

Introduction

Many proteins are formulated with polymers to protect against degradation during storage and/or processing or to provide a matrix for controlled release. Moisture content, polymer composition, and temperature can affect the stability of solid protein formulations by influencing the rates of chemical degradation reactions, such as asparagine deamidation.[1−4] Hydration of these solid formulations can easily occur during processing, storage, or after in vivo implantation. However, although the importance of polymer selection and moisture content is recognized, a complete mechanistic understanding of their effects on the chemical stability of proteins has not been developed. This manuscript addresses this mechanistic issue by examining the deamidation of a model hexapeptide (Val-Tyr-Pro-Asn-Gly-Ala, Asn-hexapeptide) in lyophilized poly(vinyl alcohol) (PVA) and poly(vinylpyrrolidone) (PVP) matrixes at various hydration levels at a constant temperature of 50 °C.

Water can affect the chemical stability of solid protein formulations in at least three ways: (1) as a solvent, (2) as a reactant in a reaction such as hydrolysis, and/or (3) as a plasticizer.[1,5] Water as a plasticizer of amorphous solids induces a physical transition from a brittle, dynamically constrained glassy state to a more mobile, less viscous, rubbery state at some temperature T_g, the glass transition temperature.[1] Because many reactions require sufficient mobility of the reactants to proceed, an increase in mobility with water content could promote chemical reactivity.[1,6,7] The decrease in T_g with increasing water content is often cited as an important factor in protein degradation in solids.[1,2,4,8] The effect of water as a plasticizer may be qualitatively described by changes in T_g produced by a given amount of water. The effect of water as a solvent or reactant should be related to its concentration (water content) or chemical potential (water activity). We report here the effects of water on the stability of the Asn-hexapeptide in solid formulations as a function of water content, water activity, and T_g.

Poly(Vinyl Alcohol) (PVA)	Poly(Vinyl Pyrrolidone) (PVP)
2 Molecular Weights	2 Molecular Weights
PVA 103 - 20,000	K12 - 4,000
PVA 125 - 125,000	K17 - 10,000

Scheme 1—Structures of poly(vinyl alcohol) (PVA) and poly(vinyl pyrrolidone) (PVP).

Deamidation at asparagine residues is one of the most prevalent chemical instabilities in proteins and peptides. The Asn-hexapeptide was selected as a model compound because its deamidation kinetics and mechanisms have been well characterized in solution.[9,10] This degree of mechanistic understanding makes the Asn-hexapeptide ideally suited for a study of the effect of water on reaction kinetics and mechanisms in solid polymer matrixes. PVP and PVA were selected as model polymers because they are commonly used pharmaceutical excipients with a simple chemical structure. Both polymers are linear, amorphous, polar, and hydrophilic (Scheme 1). Each consists of a vinyl backbone with pendant functional group (*N*-pyrrolidone for PVP and hydroxyl for PVA) at a 1,3-separation.

This paper examines the effect of water on Asn-hexapeptide deamidation in PVA and PVP at 50 °C. The relationships between water content, water activity, and T_g in these two polymer formulations will first be determined to characterize the effect of water on these polymer matrixes. Then the relationship between deamidation rates and residual moisture will be explored by correlating deamidation rates with formulation water content, water activity,

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and T_g. The impact of polymer composition on chemical stability is addressed by comparing these correlations in PVA and in PVP.

Experimental Section

Materials—L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala (Asn-hexapeptide) was synthesized by Dr. Madhup Dhaon (Abbott Laboratories, North Chicago, IL). The buffers and salts used in this study, together with organic solvents used in the HPLC mobile phase, were purchased from Mallinckrodt Chemical, Inc. (Paris, Kentucky). The two types of PVA, under the trade names of Airvol 103 and 125 [average MW 20 000 and 125 000], were obtained from Air Products and Chemicals, Inc. (Lehigh Valley, PA). The two types of PVP, under the trade names of Kollidon K12 and K17 [MW 4000 and 10 000], were obtained from BASF Corporation (Parsippany, NJ). Trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL). Deionized and distilled water was used throughout.

Preparation of PVA and PVP Formulations—Prior to use in formulations, the polymers were dissolved in water and dialyzed. Spectra/Pro molecular weight cutoff (MWCO) cellulose membranes from Spectrum Medical Industries (Houston, TX) were used to remove low molecular weight impurities. Membranes of MWCO 1000 and 3500 were used for PVP K12 and PVP K17, respectively. PVA 103 and PVA 125 were dialyzed using 10 000 and 50 000 MWCO membranes, respectively. After dialysis, the polymers were lyophilized in a VirTis Unitop 600SL Freeze-Dryer (Gardner, NY).

The polymers were then mixed with 0.01 M potassium phosphate buffer (pH 6.8) to yield 5% w/w polymer solutions. The PVP dissolved readily in buffer. The PVA mixtures were heated at 120 °C for 25 min to dissolve the polymers and then allowed to cool to 25 °C. The Asn-hexapeptide, first dissolved in a small amount of buffer, was added to the polymer solutions to produce a peptide concentration of 1.0−1.5 mg/mL (1.5−2.5 mM), as verified by HPLC. The pH of the resulting solution was measured with a Corning pH/ion Analyzer 350 with a Corning Semi-micro Combination Electrode (Corning, NY) and, if needed, was adjusted to pH 6.8 with NaOH.

The polymer and peptide solutions were placed into individual syringes fitted with 21-gauge needles. The solutions were then dispensed in a dropwise manner into liquid nitrogen to form individual frozen pellets ~2 mm in diameter. The frozen pellets were placed in a FTS Dura-Stop MicroProcessor Freeze-Dryer (FTS Systems, Inc., Stone Ridge, NY) with the shelves precooled to −40 °C. The chamber vacuum was set at 150 mTorr. The shelf temperature was ramped in 10 °C intervals every 3−4 h up to −5 °C. Afterward, shelf temperature was increased in 10 °C intervals every 6−8 h. The final drying was conducted at 25 °C for 17 h. After lyophilization, the resulting white spherical pellets were transferred into containers in a PlasLabs Dry Box (Lansing, MI), which was purged with dry nitrogen, and placed in a desiccator containing $CaSO_4$. The peptide loads for PVP and PVA were 0.017 ± 0.0006 and 0.024 ± 0.001 g/g solid, respectively, based on the maximum amount released. To determine peptide load, both PVA and PVP formulations were immersed in water. The PVP formulations dissolved completely, whereas the PVA pellets released >95% of their theoretical load after 4−8 h. The final composition of the dry formulation (in w/w) was ~2% peptide, 2% buffer, and 96% polymer, with a residual moisture content of <1%.

Characterization of Water Sorption Behavior—Sorption isotherms relating formulation water content to relative humidity (RH, water activity) at 50 °C were generated using a Controlled Atmosphere Microbalance (CAM), which monitored the sample mass at a specified relative humidity. The CAM was built at Pharmacia & Upjohn, Inc., as previously described.[11] The CAM consisted of a Cahn microbalance within an enclosed glass chamber, a nitrogen gas saturator assembly, water baths, and digital interfaces. Data were collected on a 486 PC computer and stored in a Microsoft Excel spreadsheet. Each CAM run was conducted on 5−10 mg of sample over a range of 0 to 85% RH at 5% RH step increases. When equilibrium was achieved, the system would step the chamber humidity to the next level. The criterion for equilibrium was defined as no more than a 0.5 μg change in sample mass over a 20-min interval. Water sorption was rapid, with 95% of the equilibrium mass reached within 20 min. The CAM took, on average, 2−3 h to achieve equilibrium at 5% RH intervals. Water desorption of the PVP and PVA formulations showed little or no hysteresis (data not shown), evidence that equilibrium water content was reached.

Two points on each isotherm were verified independently by thermogravimetric analysis (TGA). Samples from each formulation were stored at two RHs. The water contents were then determined with a DuPont 2050 TGA with a 2200 Data Analysis System from TA Instruments (Newcastle, DE). The water content of each sample was defined as the weight loss during heating from 25 to 120 °C at a heating rate of 10 °C/min.

The water sorption data obtained by the CAM were fitted to the Guggenheim−Anderson−deBoer (GAB) equation (eq 1), which describes the sorption of water by heterogeneous sorbents or solids.[1]

$$W = \frac{W_m C_g K_{GAB}(\rho/\rho_o)}{[1 - K_{GAB}(\rho/\rho_o)][1 - K_{GAB}(\rho/\rho_o) + C_g K_{GAB}(\rho/\rho_o)]} \quad (1)$$

In eq 1, W is the mass (mg) of water vapor adsorbed per mg of dry solid at (ρ/ρ_o), (ρ/ρ_o) is the relative vapor pressure, W_m is generally regarded as the amount of water vapor necessary to saturate the heterogeneous active sorption sites, and C_g and K_{GAB} are dimensionless constants that are related to the thermodynamic parameters for sorption of strongly and weakly interacting water.[12−14] The GAB equation (eq 1) was used because it provides the ability to relate vapor pressure and water content of the systems under investigation. Alternatively, material science-based descriptions for water sorption isotherms of amorphous solids may be more thermodynamically and physically relevant for these systems.[15] However, these alternative models are not necessary to empirically describe the relationship between vapor pressure and water content. All curve fitting of data was carried out with SigmaPlot Version 3.0 (Jandel Corporation, San Rafael, CA).

The relative water vapor pressure (ρ/ρ_o) or humidity was taken to be equal to the activity of water in the systems studied. These systems were assumed to be in equilibrium because the formulations were stored in a closed system (glass chamber) at constant temperature (50 °C) and pressure during the study.

Characterization of Formulation Glass Transition Temperature—A Thermal Analyst Instruments 2920 DSC outfitted with a TA 2200 Data Analysis System (Newcastle, DE) was used to determine T_g values. The DSC was calibrated with indium. A modulated temperature ramping program was used in which the sample temperature was increased at 2 °C/min with a modulation of ±1 °C/min. The DSC was cooled with a liquid nitrogen cooling apparatus. After storage at 50 °C under various RHs for 4 days, ~3−5 mg of each sample was sealed in aluminum DSC pans obtained from TA Instruments (Newcastle, DE). The T_g was defined as the midpoint of the glass transition. The T_g measurements for each formulation at each RH were made in triplicate.

The T_g data were fitted to the Gordon−Taylor equation (eq 2) for two miscible components:

$$T_g = \frac{w_1 T_{g_1} + K_{GT} w_2 T_{g_2}}{w_1 + K_{GT} w_2} \quad (2)$$

where T_g is the glass transition temperature of the mixture, w_1 and w_2 are the weight fractions of the individual components, T_{g1} and T_{g2} are the intrinsic T_gs of each component, and K_{GT} is a constant that can be considered to be a ratio of the free volumes of the two components.[16,17] The T_g for water was set at the estimated value of 135 K.[17]

Stability Study—For the stability study, the solid formulations were transferred to 2-mL glass lyophilization vials, with each vial containing ~5 mg of pellets. The vials were placed in several closed controlled RH chambers, which were maintained at various specified RHs (11−75% RH) using saturated salt solutions.[18] The solid formulations were allowed to equilibrate in chambers at 20 °C for 12 h. Afterward, samples from various RHs chambers were removed and analyzed for Asn-hexapeptide and its degradation products. No degradation products were observed after this initial equilibration period. The samples were then transferred to chambers with similar RHs in a 50 °C room for the stability study. Saturated solutions of LiCl, $MgCl_2$, $Mg(NO_3)_2$, KI, and NaCl were

used to maintain RHs of 11, 30, 45, 64, and 75%, respectively, at 50 °C. $CaSO_4$ was used as the desiccant for the 0% RH condition. Samples were prepared in triplicate. Peptide composition was assayed by HPLC after various storage times to determine degradation kinetics and product distribution. The duration of the study was 6 months.

Peptide Analysis—Analyses of Asn-hexapeptide and its degradation products were performed by reversed-phase HPLC according to an established method.[10] The system consisted of a Varian VISTA 5500 liquid chromatography system with a Varian UV200 detector and a Varian Series 600 Data System (Walnut Creek, CA). Peptide separation was performed with an Alltech Econosphere C18, 5 μm, 250 × 4.6 mm analytical column (Deerfield, IL) in conjunction with a Applied Biosystems Brownlee Spheri-5, C-18, 5 μm, 30 × 4.6 mm guard column (San Jose, CA). A Perkin-Elmer ISS-100 Autoinjector (Norwalk, CT) was used to deliver 50-μL sample injections. An isocratic elution method was used at a flow rate of 0.8 mL/min. The mobile phase consisted of 6% (v/v) acetonitrile and 0.1% (v/v) TFA in 40 mM ammonium acetate at a pH of 4.4, which was adjusted using 1 N HCl. An ultraviolet (UV) detection wavelength of 218 nm was used to detect Asn-hexapeptide and its degradation products, which were identified via co-injection with known standards.

Kinetic Measurements—After a specified time interval, triplicate samples of each formulation at each RH were removed for peptide analysis. A 0.5-mL aliquot of deionized water was added to each vial. The PVP formulations dissolved readily in water. However, PVA was not soluble in water without heating. Therefore, the PVA pellets were immersed in water at 4 °C for 6 h and were shaken periodically. Less than 0.5% peptide decomposition occurred during this extraction ($t_{1/2}$ = 1150 h). More than 90% of the peptide was released in the first 2 h, with >95% released after 6 h. The pH of the resulting solution was ~6.8. The content of Asn-hexapeptide in the resultant solution was assayed by the HPLC method already described.

The observed rate constant (k_{obs}) for the disappearance of Asn-hexapeptide was determined from the slope of the plot of ln(% peptide remaining) versus time. This calculation was based on the pseudo-first-order degradation kinetics previously described for this peptide in solution[10] and solid[8] states according to the following relationship:

$$\ln\frac{A}{A_o} = -k_{obs}t \qquad (3)$$

where A is the amount of peptide at time t, and A_o is the initial peptide concentration. Analysis of kinetic data was handled with Excel Version 5.0 (Microsoft Corporation, Redmond, WA).

Results

Physical Characterization of Formulations—*Physical Appearance*—PVP and PVA formulations stored under desiccated or dry conditions were white, smooth, spherical pellets. The texture for the dry PVP formulation (water content < 0.004 g/g wet solid) was powdery, whereas dry PVA (water content < 0.003 g/g wet solid) had a fibrous, Styrofoam-like texture. As the RH increased, both PVP and PVA pellets decreased in diameter. At lower RHs (11% RH), this decrease in pellet size was the most noticeable change in PVP and PVA. At intermediate RHs (30−45% RH), the PVP pellets were dome shaped and sticky and had a yellowish, melted appearance, whereas the PVA pellets took on a wrinkled appearance and were less foamy in texture. At high RH (65−75% RH), the PVP pellets had become an opaque, viscous fluid, which coated the bottom of the vial. The PVA pellets had collapsed into a hard sphere with a wrinkled surface.

Water Sorption Isotherms—Researchers have reported that residual water in solid formulations can compromise drug stability.[1,19] Therefore, characterizing the hydration behavior of these polymers is important. The water sorption isotherms, which relate formulation water content to relative water vapor pressure or humidity, are shown in Figure 1 for PVA and PVP at 50 °C. The water content of both polymers increases with increasing RH. PVP and PVA formulations have different water sorption behaviors, with PVP being more hygroscopic than PVA. Thus, water activity is greater in PVA than in PVP at a given water content.

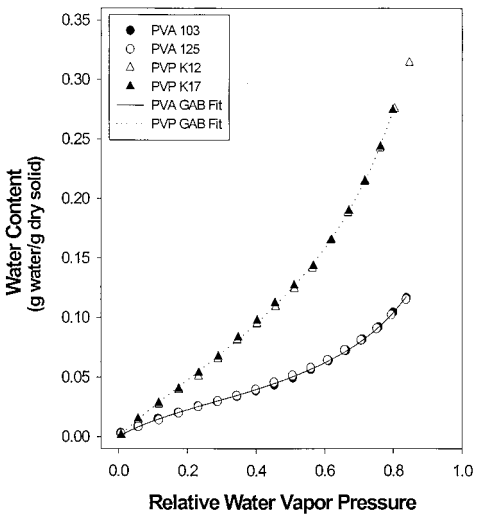


Figure 1—Water sorption isotherms for PVA and PVP formulations at 50 °C. The curved lines are nonlinear least-squares fits of the sorption data to the GAB equation (eq 1).

Table 1—Parameter Values and Standard Errors Derived from Fitting the Water Sorption Data at 50 °C to the GAB Equation (eq 1) for PVA and PVP Formulations

formulation	W_m (g H_2O/g dry solid)	C_g	K_{GAB}
PVA 103	0.0350 ± 0.0010	5.54 ± 0.42	0.861 ± 0.008
PVA 125	0.0378 ± 0.0007	5.00 ± 0.20	0.835 ± 0.006
PVP K12	0.111 ± 0.003	2.61 ± 0.11	0.825 ± 0.007
PVP K17	0.101 ± 0.003	3.13 ± 0.17	0.849 ± 0.009

The curved lines in Figure 1 represent the nonlinear least-squares fit of the water sorption data to the GAB equation (eq 1). The fitted parameter values of the GAB equation are shown in Table 1. Polymer molecular weight did not appear to significantly affect water sorption behavior for either PVA or PVP at 50 °C. The W_m value for PVP was ~3 times greater than for PVA, which is consistent with the greater affinity of PVP for water. The GAB equation, together with these fitted values, was used to relate water content and water activity in the analysis of stability data.

Formulation T_g as a Function of Water Content—Water in amorphous polymers can act as a plasticizer, increasing both the segmental mobility of the polymer chains and the flexional and translational mobility of incoporated low molecular weight solutes.[20] This increased mobility of the peptide in water-plasticized PVA and PVP systems may contribute to reactivity. One way to monitor the plasticizing effect of water is to measure the T_g. Figure 2 shows the effect of water content on formulation T_g. As water content increased, the T_g of PVA and PVP formulations decreased, indicating that water acts to plasticize both polymers. The dry PVP T_g was 80° greater than that of PVA (Table 2). At similar water contents, the PVP T_g was greater than the PVA T_g. Formulations with T_gs above the experimental temperature (T_{exp}= 323 K or 50 °C) were considered to be glassy, whereas those with T_gs of <323 K were assumed to be in the rubbery state.

The solid (PVA 103) and dashed (PVP K17) curves in Figure 2 represent the nonlinear least-squares fits of the

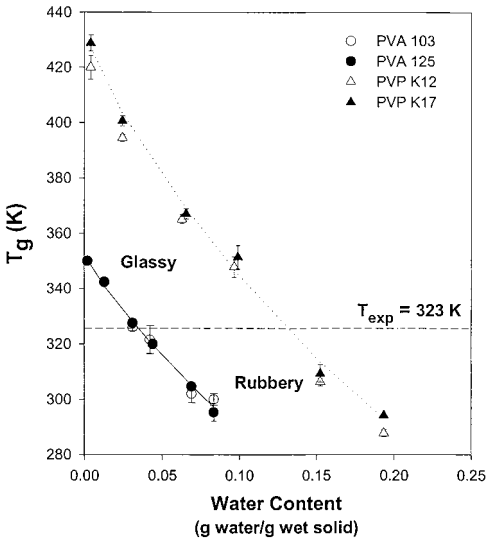


Figure 2—Glass transition temperature (T_g) as a function of water content. The curved lines are nonlinear least-squares fits of the sorption data to the Gordon–Taylor equation (eq 2). The labels "Glassy" and "Rubbery" indicate the physical state of the polymer matrix at the experimental temperature, T_{exp} (dashed line). Matrixes with $T_g > T_{exp}$ are glassy at T_{exp}; matrixes with $T_g < T_{exp}$ are rubbery at T_{exp}. Error bars represent standard deviations ($n = 3$).

Table 2—Glass Transition Data for PVA and PVP[a]

formulation	intrinsic T_g (K)[b]	fitted K_{GT} value	W_g (g H_2O/g wet solid)[c]	A_g[d]
PVA 103	346.3 ± 3.5	0.276 ± 0.04	0.0383	0.40
PVA 125	347.0 ± 1.1	0.266 ± 0.09	0.0384	0.40
PVP K12	421.1 ± 3.8	0.265 ± 0.08	0.127	0.58
PVP K17	429.4 ± 4.4	0.281 ± 0.09	0.135	0.60

[a] The K_{GT} values are from data fitted to the Gordon–Taylor equation (eq 2), and the T_g for water was assumed to be 135 K. [b] T_g value for "dry" formulations (water content < 0.004 g/g dry solid). [c] Amount of water required to reduce T_g to the experimental temperature (323 K). [d] Water activity corresponding to the W_g value.

data to the Gordon−Taylor equation (eq 2). The observed depression of T_g by an increase in water content was well described by the equation, suggesting that the mixing behavior of water with PVA and PVP was ideal.[17] The fitted K_{GT} values (Table 2), which can be considered to be the ratio of free volumes of water and polymer, were similar to the estimated K_{GT} values of 0.29 (PVA) and 0.25 (PVP). The K_{GT} values were estimated based on T_g and density values given for water, PVP, and PVA in the cited references.[17,21]

From the Gordon−Taylor equation (eq 2), the amount of water (W_g) required to induce the glass transition (i.e., to bring T_g into equality with T_{exp}) was calculated for PVA and PVP. The W_g values and the corresponding water activities at T_g (A_g) are listed in Table 2. Three times more water was required to induce the glass transition at 50 °C in PVP than in PVA, in part because of the greater dry T_g of PVP. Correspondingly, the water activity at the glass transition ($T_g = T_{exp}$) was higher in PVP than PVA. Initially, the same amount of water had a greater plasticizing effect on PVP. We will correlate formulation T_g with deamidation rates to evaluate the potential for a relationship between chemical instability and formulation viscosity.

Polymer molecular weight did not significantly affect the T_g values for either PVA or PVP. In general, T_g values are expected to increase with increasing polymer molecular weight up to a limiting or "persistent" T_g value, although both decreases in T_g with molecular weight and molecular-weight-independent T_g values have also been observed.[22]

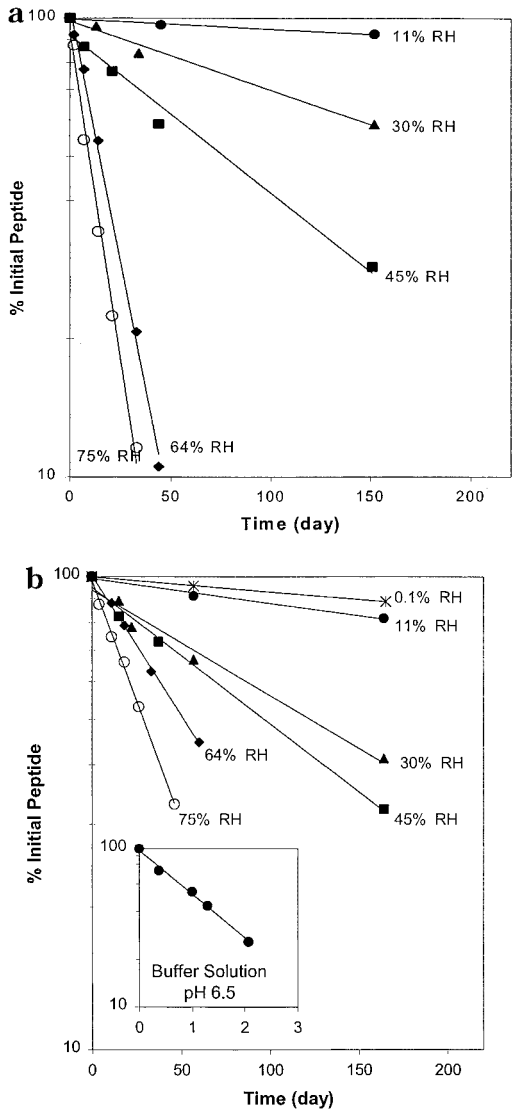


Figure 3—Asn-hexapeptide degradation profiles at various RHs at 50 °C for (a) PVA (Airvol 103) and (b) PVP (Kollidon K12) formulations ($n = 3$). The insert in Figure 3b shows the disappearance of the Asn-hexapeptide in polymer-free phosphate buffer, pH 6.8.

The lack of molecular weight dependence observed here may be due to the narrow range of molecular weights studied (particularly for PVP), to the removal of low molecular weight components during extensive dialysis prior to use, or to unusual intrinsic T_g behavior of the polymers. For our purposes, this result effectively removes polymer molecular weight from the list of independent variables that can be manipulated to control T_g.

Degradation Products—The major degradation products of Asn-hexapeptide deamidation were the isoAsp-hexapeptide (isoAsp), the Asp-hexapeptide (Asp), and the cyclic imide hexapeptide (Asu). Although isoAsp and Asp are commonly observed in solution-state deamidation at neutral pH, the cyclic imide is usually not observed because it is rapidly hydrolyzed to produce the isoAsp and Asp hexapeptides.[9,10] The dominance of isoAsp and Asu suggests that formation of the cyclic imide is the major route of Asn-hexapeptide deamidation in these polymer formulations.[9,10] Oliyai et al. have noted that Asn-hexapeptide deamidation in solid sugar formulations also occurs through a degradation pathway similar to that in solution.[8]

Deamidation Kinetics—Figures 3a and 3b show representative time-dependent disappearances of Asn-hexapeptide in PVA and PVP at different RHs. The disappearance

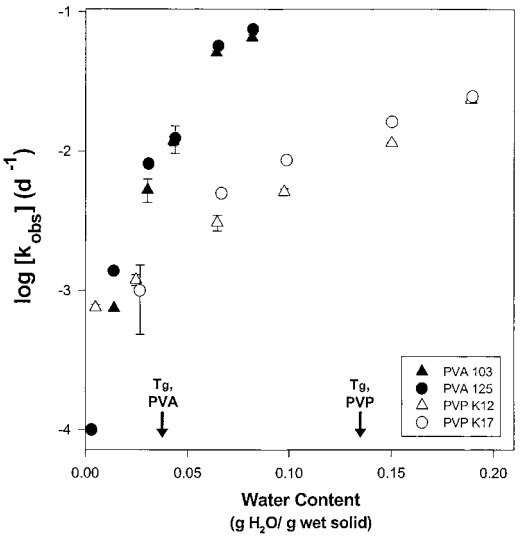


Figure 4—Effect of water content on the observed rate constant of Asn-hexapeptide deamidation (k_{obs}) in PVP and PVA at 50 °C. Error bars represent standard deviations ($n = 3$). The approximate water contents needed to lower the T_g of the PVA and PVP formulations to the experimental temperature are noted with arrows labeled "Tg, PVA" and "Tg, PVP", respectively.

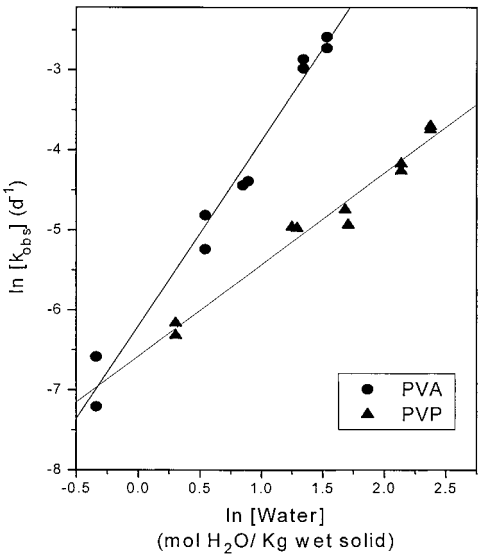


Figure 5—Plot showing the apparent dependence of deamidation rates on water content. Error bars represent standard deviations ($n = 3$).

of Asn-hexapeptide was observed to follow pseudo-first-order kinetics in peptide content at all RHs studied. Deamidation of this peptide in solution is known to exhibit pseudo-first-order kinetics.[9,10] The reaction order in peptide content appears to be similar in the solution and in these solid polymer formulations. As the RH increased, the rate of peptide degradation also increased. The observed rate constants (k_{obs}) were obtained from the slopes of the plots of ln [%Asn-hexapeptide remaining] versus time. Subsequent discussion of Asn-hexapeptide deamidation rates will employ the observed pseudo-first-order degradation rate constant.

Correlation with Water Content—The effect of formulation water content on the deamidation rate of Asn-hexapeptide is shown in Figure 4. At higher water contents ($\cong$ 10% w/w, or 0.10 g H_2O/g wet solid), the rate in PVA is nearly an order of magnitude faster than in PVP. As the water content decreases from 10% to ~2% (w/w), the deamidation rates in the two polymers become nearly equal. At very low water content (<2% w/w), the rate in PVA appears to be approximately an order of magnitude slower than in PVP, but this observation should be regarded as tentative given the limited data in this region. Polymer molecular weight had no apparent effect on peptide reactivity in either polymer. The results are consistent with those of Oliyai et al., who observed an increase in the rate of cyclic imide formation with increasing moisture level in lyophilized sugar formulations.[8]

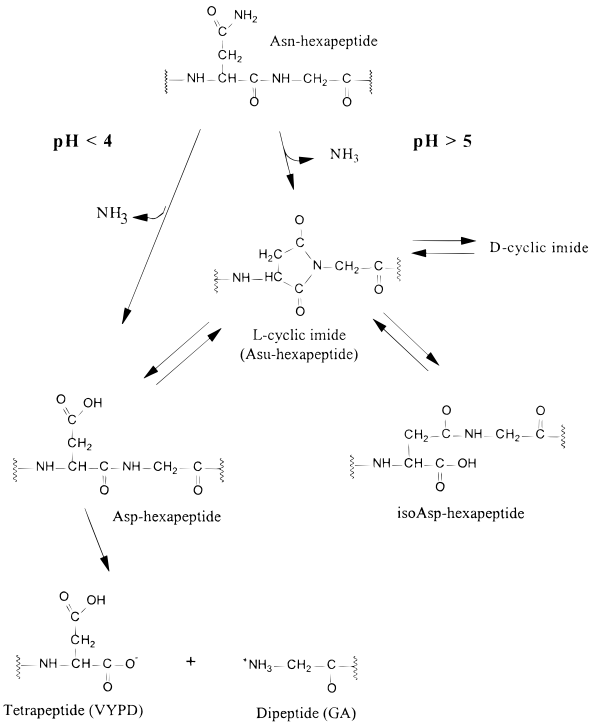
Peptide reactivity in PVA appeared to be more sensitive to increases in water content than in PVP at water contents >5%. An increase in water content of 0.083 g/g wet solid in the PVA formulation increased the observed deamidation rate constant by almost 3 orders of magnitude [$(1.0 \pm 0.05) \times 10^{-4}$ to $(7.5 \pm 0.4) \times 10^{-2}$ day^{-1}]. In contrast, the deamidation rate in PVP increased by only 1 order of magnitude over a wider range of water contents. Figure 5 shows that there is an apparent first-order dependence of the rate of Asn-hexapeptide deamidation on water content in PVP (slope = 1.15 ± 0.10), but an apparent second-order dependence on water content in PVA (slope = 2.31 ± 0.12), according to the following relationship:

$$k_{obs} = k\,[\text{water content}]^n \qquad (4)$$

where k is a constant and n is the order of the reaction with respect to water content in mol water/kg wet solid. The difference in reaction order between PVP (first order) and PVA (second order) is indicative of differences in reaction mechanism or environmental response, or both, in the two media. Further study will be required to establish the molecular origins of the difference because changes in water content can cause both mechanistic and environmental effects. For example, water may serve as a reactant or catalyst in the conversion of a reactant state to a transition state (a mechanistic effect) or may act as a plasticizer in affecting the ease of molecular motion in the medium (an environmental effect). Even a consideration of mechanistic effects alone is less straightforward than it might appear initially. If the reaction in polymeric media proceeds by the mechanism established in the solution state (Scheme 2), the initial cyclization step will not involve water stoichiometrically, whereas the hydrolysis step in ring opening of the cyclic imide will stoichiometrically require one molecule of water per molecule of cyclic imide. This conclusion might suggest that the higher order in water content observed in PVA is associated with rate-determining ring opening, whereas the lower order observed in PVP is indicative of rate-determining cyclization. However, catalytic involvement of unknown numbers of water molecules in either step of the solid-state reaction would render such a conclusion incorrect.

Correlation with Water Activity—The amount of water present is not always representative of the amount of water available for reaction; chemical potential or water activity may be a better indicator of its possible role as a reactant. In this study, the formulations were assumed to be in equilibrium within the closed RH chambers at constant temperature and pressure. Therefore, it is reasonable to assume that the relative water vapor pressure in the chambers is equal to the formulation water activity. Figure 1 shows that PVA and PVP have different water vapor sorption behavior and thus have different water activities when water content is similar.

Figure 6 correlates the rate of Asn-hexapeptide deamidation in PVA and PVP with water activity or RH. The rate of Asn-hexapeptide deamidation increases with increases in water activity (A_w). Reactivity may be better described by water activity (Figure 6) than by water content (Figure 4) in these systems because the differences between the PVA and PVP curves are reduced in Figure



Scheme 2—Solution state degradation pathways of Asn-hexapeptide (adapted from ref 10).

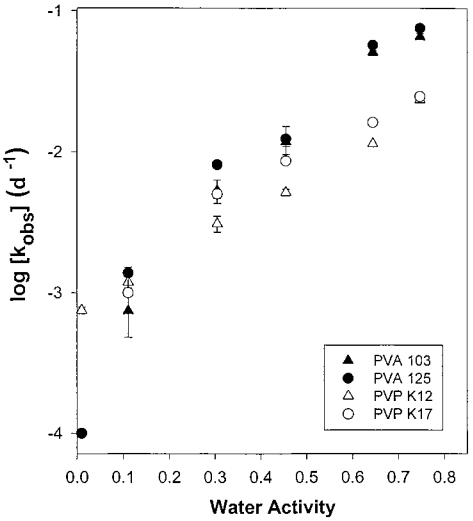


Figure 6—Effect of water activity on the observed rate constant of Asn-hexapeptide deamidation (k_{obs}) in PVP and PVA at 50 °C. Error bars represent standard deviations ($n = 3$).

6. Although the discrepancy in Asn-hexapeptide reactivity between PVA and PVP was less when differences in formulation A_w were taken into account, peptide reactivity at high (>0.6) or low (<0.1) water activity was still markedly different in PVA and PVP. Thus, water activity alone was not adequate to describe peptide reactivity in these different polymer solids, again implying that additional effects of water may be important in these systems.

Correlation with Formulation T_g—The finding that reactivity was not predicted by water content or activity alone suggests that the effect of water on Asn-hexapeptide deamidation in PVA and PVP was not entirely due to its role as a reactant. Another possible role of water is as a plasticizer to facilitate polymer chain mobility and decrease polymer viscosity, along with a decrease in T_g. The T_g was thus taken as a qualitative measure of matrix mobility. If deamidation in the solid state is controlled by reactant mobility, then the degradation rate should be affected by changes in T_g, assuming some degree of coupling between reactant and formulation mobility. It should be noted that because T_g measures a bulk property of the polymer matrix, it is not possible to distinguish the flexional and translational mobilities of the incorporated peptide using T_g.

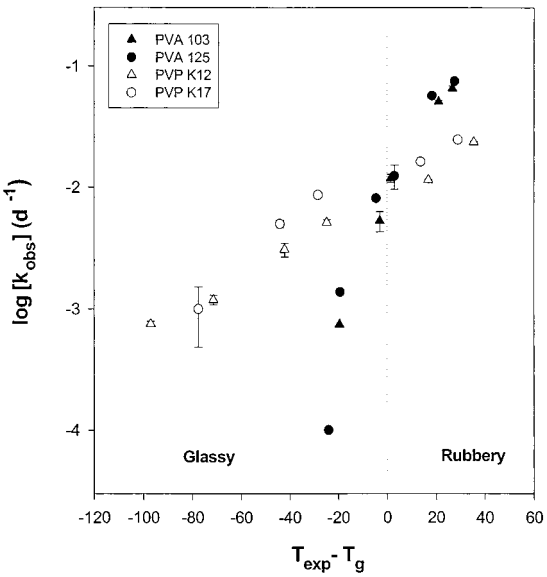


Figure 7—Effect of formulation T_g on the rate of Asn-hexapeptide deamidation in PVA and PVP at 50 °C. Temperatures are in °C. Error bars represent standard deviations ($n = 3$).

Figure 7 correlates the rate of Asn-hexapeptide deamidation to changes in formulation mobility, as expressed by ($T_{exp} - T_g$) based on the Vogel–Tamman–Fulcher and Williams–Landel–Ferry equations.[23–25] The T_{exp} parameter was chosen as the constant reference temperature with T_g as a variable. Negative values on the x axis denote formulations in the glassy state with $T_g > T_{exp}$, whereas positive values represent formulations in the rubbery state with $T_g < T_{exp}$. As the formulation T_g decreased (i.e., as $T_{exp} - T_g$ increased), the rates of deamidation increased in both PVA and PVP. In the glassy state, the rate of Asn-hexapeptide deamidation was greater in PVP than in PVA. However, as the formulations became more rubbery, peptide reactivity in the two polymers was reversed; that is, Asn-hexapeptide deamidation in the rubbery state was more rapid in PVA than in PVP. In going from a glassy to a rubbery state, the deamidation rate in PVA increased by 3 orders of magnitude with a 50 °C decrease in formulation T_g as compared with only 1 order of magnitude increase in PVP over the same range. At the glass transition, the rates of Asn-hexapeptide deamidation were similar in both polymers. The rate of Asn-hexapeptide deamidation seemed to be more sensitive to changes in the T_g of PVA than in PVP. If T_g alone described reactivity in these polymers, the curves for PVA and PVP would be expected to be coincident. That they differ suggests that formulation T_g alone was not adequate in describing Asn-hexapeptide reactivity in the two polymers.

Discussion

The rate of Asn-hexapeptide deamidation increased with increasing water content and water activity and decreasing formulation T_g in PVA and in PVP. However, degradation behavior in the two polymers differed so that chemical reactivity could not be predicted from water content, water activity, or formulation T_g alone. Thus, no single parameter seems to dictate the deamidation rate over the range of water content, water activity, and T_g studied.

Formulation mobility appears to affect the reactivity of the Asn-hexapeptide. All glassy formulations were more stable than rubbery formulations, regardless of water content. This result suggests that limited peptide mobility in the dynamically constrained glassy state may contribute to the greater stability of glassy formulations. This idea is consistent with the likely mechanism of Asn-hexapeptide degradation in these formulations. The degradation product data suggest that the mechanism of deamidation in these polymer solids is similar to that observed in solution. Thus, it is likely that deamidation in these systems proceeds via intramolecular cyclization to form a cyclic imide, a process that requires sufficient peptide mobility to adopt the necessary conformation for cyclization. Local segmental flexibility has been shown to influence the propensity for spontaneous cyclic imide formation from asparagine residues in calmodulin.[26] Yoshioka et al. observed that the γ-globulin aggregation rate was faster in lyophilized PVA than in dextran, although water content was lower in PVA than in dextran (0.098 versus 0.177 g/g of solid).[27] They attributed this lower stability to the lower critical temperature for mobility of PVA and proposed that the greater mobility in PVA accounted for the more rapid aggregation.

Although the less mobile glassy formulations were more stable, chemical reactivity was not negligible in these systems; as shown in Figure 7, deamidation occurred at a measurable rate in glassy matrixes of both PVA and PVP. Hancock et al. have suggested that glassy solids should be expected to experience significant molecular mobility at temperatures up to 50 °C below T_g,[28] which suggests that mobility-dependent reactions may still occur. Notably, the data in Figure 7 demonstrate that the rate of deamidation in glassy PVP matrixes is rapid enough to preclude adequate shelf stability. At an experimental temperature 100 °C below the T_g of the "dry" PVP formulation ($T_{exp} - T_g = -100$ °C, with T_g of the dry formulation = 150 °C), the Asn-hexapeptide was observed to have a half-life of only 2 years, suggesting that significant reactivity may be observed at temperatures far below T_g. Although the dry PVA formulation had a lower T_g (75 °C), the half-life for deamidation was much longer than that observed for the dry PVP formulations (20 versus 2 years). This result suggests that formulation mobility, as measured by T_g, is in itself insufficient to predict deamidation rates in these polymer systems.

An examination of Asn-hexapeptide reactivity and viscosity changes around the glass transition further supports this idea. The transition from a glassy to a rubbery state usually is characterized by a decrease in viscosity of >5 orders of magnitude.[6,24] Around this region ($T_g = T_{exp}$), the rates of deamidation increased by only 3 orders of magnitude in PVA and barely 2 orders of magnitude in PVP. The absence of an increase in rate comparable in magnitude to the decrease in viscosity at the glass transition may suggest incomplete coupling of deamidation rate to matrix mobility.

The inability of T_g alone to predict deamidation rates in PVA and PVP suggests that the level of mobility required for deamidation may be less than the bulk mobility represented by T_g. As already noted, the mechanism of deamidation in these solid polymer systems appears to be similar to that in solution, proceeding via a cyclic imide intermediate. Formation of the cyclic imide requires localized conformational flexibility of the peptide chain, allowing the attack of the backbone NH of the glycine residue on the side-chain amide function of the asparagine. Because T_g measures a bulk property of the system, it may not adequately reflect the localized molecular motions required for deamidation. Thus, although PVA and PVP formulations may have the same T_g, the degree of localized molecular mobility may differ. The characteristic length

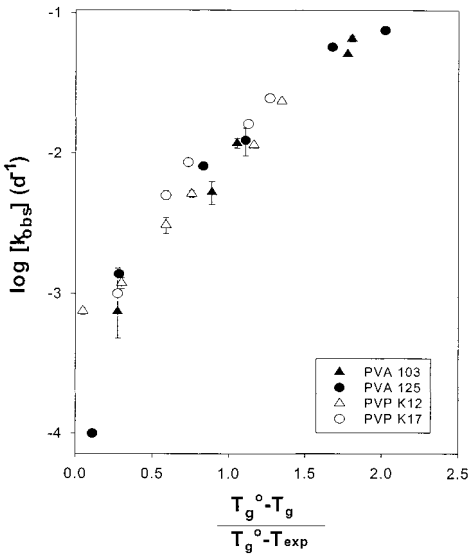


Figure 8—Correlation between deamidation rates and water plasticization in PVA and PVP at 50 °C.

scale for motions associated with the relaxation of the glassy modulus can have different temperature [or water] dependencies and can vary significantly for amorphous polymers.[29] Thus, the discrepancy in deamidation rates in PVA and PVP may be due to differences in polymer properties and structural responses to water, leading to differences in localized molecular mobility changes with increases in water content.

Although mobility affects deamidation, water may affect deamidation also as a solvent and proton-transfer agent. Among the three correlations made between deamidation rates and formulation parameters, water activity appeared to correlate best with Asn-hexapeptide instability in PVA and in PVP. The closer correlation between deamidation rates and water activity in PVA and PVP formulations suggests that some factor related to water activity may be influencing deamidation. In solution, solvent effects have been shown to affect deamidation due to changes in dielectric constant (polarity) and peptide pK_as. Brennan and Clarke have shown that deamidation rates decrease as solvent dielectric constant decreases.[30] Although water contents in these solid systems are low relative to solution, the "solvent" properties of water may still affect chemical reactivity, and changes in deamidation rate may be partially due to "polarity" changes upon hydration. The rate discrepancy in PVA and PVP further suggests that the "solvent" environment may affect deamidation because the PVA and PVP matrixes are serving as the main "solvent" for Asn-hexapeptide.

Water and polymer type both appeared to have an effect on deamidation, indicating that more than one parameter should be required to describe chemical reactivity in these systems. Figure 8 shows the correlation between deamidation rate and the degree of plasticization ($T_g^o - T_g$), normalized for the extent of plasticization required to induce the glass transition at 50 °C ($T_g^o - T_{exp}$). Data for both polymers are described by a single relationship. The expression was derived by making the approximation that the relationship between T_g and water content was linear over the range used in this study (see Figure 2) such that

$$T_g = T_g^o - \alpha W \qquad (5)$$

where α is a constant and W is the weight fraction of water. A similar relationship has been proposed for the more general case of plasticization by any additive.[31] Assuming

that α is constant for each polymer, eq 5 can be expected to hold when $T_g = T_{exp}$ and $W = W_g$ so that

$$\alpha = \frac{T_g^{\circ} - T_{exp}}{W_g} \tag{6}$$

where W_g is the water content needed to induce the glass transition at $T_{exp} = 50$ °C. Substituting this value for α (i.e., the slope) into eq 5 yields:

$$\frac{T_g^{\circ} - T_g}{T_g^{\circ} - T_{exp}} = \frac{W}{W_g} \tag{7}$$

This equation states that the degree of plasticization needed to induce a glass transition is related to water content, the experimental temperature, and the dry intrinsic T_g° of the polymer. A convergence of the deamidation rates in PVA and PVP was also observed when the rate constant (k_{obs}) was plotted against W/W_g. Although this parameter $[(T - T_g)/(T_g^{\circ} - T_{exp})]$ may be useful in correlating reaction rates in other systems, its utility in these studies (Figure 8) may be due to the near linear relationship between T_g and water content observed for PVA and PVP under these experimental conditions (Figure 2).

The results of this study suggest that several factors, including water content and mobility, may affect the chemical reactivity of Asn-hexapeptide in lyophilized polymer formulations. Isolating a single dominant mechanism driving deamidation is difficult because water content, water activity, and T_g are coupled. Future studies will attempt to vary T_g and water content independently with the use of a separate plasticizer to deconvolute their effects on deamidation in the solid state.

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