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Solution Behavior of a Novel Biopharmaceutical Drug Candidate: A Gonadotropin-Toxin Conjugate

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ABSTRACT There is little known about the solution structure and stability of peptide-protein conjugates, which comprise a new class of potential biopharmaceutical agents. This study describes the solution behavior of gonadotropins-releasing hormone (GnRH) chemically conjugated to pokeweed antiviral protein (PAP). The conjugate adopts a well-defined conformation across a pH range of 4 to 8. Even after heating to 80°C, the conjugate retains a significant amount of secondary and tertiary structure. Heating for 1 h at 60°C does lead to chemical damage, as determined by cation exchange chromatography. Using an experimental design approach, the optimal pH and salt concentration for limiting chemical damage was determined.

KEYWORDS Solution structure and stability, peptide and protein conjugate

INTRODUCTION

The concept of using GnRH-toxin constructs as therapeutic agents has been reported in the literature (Oonk et al., 1998, Schlick et al., 2000). However, little, if anything, is known about the solution structure or stability of such conjugates. In this study, the GnRH peptide has been chemically linked to the toxin rather than by forming a fusion protein, as has been described by Schlick et al. (2000). In this case, the toxin of choice is pokeweed antiviral protein (PAP), a ribosome-inactivating protein from *Phytolacca americana*. The stoichiometry of the conjugate is approximately 1:1, although some small amount of 2:1 adduct is formed. The solution behavior of this conjugate, GnRH-PAP, is described herein.

While the structure of the GnRH-PAP conjugate is unknown, there have been reports on the conformation of PAP itself. For example, the crystal structure of PAP has been reported (Ago et al., 1994; Li et al., 1998). Other information on its activity, expression and primary sequence can be found as well (Blomgren et al., 1981; Rodes et al., 1981; Lodge et al., 1993). However, its behavior in solution has not been reported. Little is known about the solution behavior of protein conjugates, especially with respect to formulation and stabilization. Using approaches common for the stabilization of unconjugated proteins, the stability behavior of a novel peptide-protein conjugate was examined.

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A stability-indicating assay was developed, which provided information on both chemical and physical degradation. In addition, the secondary and tertiary structure of the conjugate was monitored with respect to elevated temperature. Optimization of the solution conditions (e.g., pH, ionic strength, and buffer composition) produced adequate stabilization of this peptide-protein-conjugate in aqueous solution, at least during accelerated storage at elevated temperatures.

MATERIALS AND METHODS

Materials

GnRH-PAP was provided by Gonex Pets Inc. (Fort Collins, CO) as a lyophilized powder. It is produced by chemical cross-linking of a GnRH analog with pokeweed antiviral protein (PAP). Authentic samples of PAP were provided by Gonex Pets as well.

Tertiary Structure by Near UV Circular Dichroism Spectroscopy

Near UV circular dichroism (CD) were obtained on an AVIV model 62 DS spectropolarimeter (Lakewood, NJ). Measurements of the samples were taken in a 1-cm pathlength quartz cell and placed in a thermostated cell holder. Concentrations were determined by UV/VIS spectrophotometry at 279 nm using an extinction coefficient of 1.18 (mL/mg*cm) for GnRH-PAP and 0.997 (mL/mg*cm) at absorbance maximum for PAP alone. Data were collected at 0.5-nm intervals using a 1.0-nm bandwidth, with an averaging time of 5 sec at each point. The appropriate buffer blank was collected and subtracted from each spectrum. All spectra were corrected and converted into mean residue ellipticity by the equation of [millidegrees*(mean residue weight/concentration (mg/mL)*pathlength (mm)].

Secondary Structure by Far UV Circular Dichroism Spectroscopy

Far UV CD spectra were collected on an Aviv model 62 DS spectropolarimeter (Lakewood, NJ) for the samples. Each sample was loaded into a 1-mm pathlength quartz cell and placed in a thermostated cell holder. Data were collected at 0.5-nm intervals using a 1.5-nm bandwidth, with an averaging time of 5 sec at each point. The appropriate buffer blank was collected and subtracted from each spectrum. All spectra were corrected and converted into mean residue ellipticity.

Thermal Melting Curves Monitored by Circular Dichroism Spectroscopy

Temperature melt curves were done by use of circular dichroism on an Aviv model 62-DS spectropolarimeter (Lakewood, NJ). Concentration determination was done on a Beckman DU-64 spectrophotometer using an extinction coefficient of 1.18 (mL/mg*cm) for GnRH-PAP and 0.997 (mL/mg*cm) at absorbance maximum for PAP alone. Each sample was loaded into a 1-mm pathlength quartz cell placed in a thermostated cell holder. Far and near UV CD wavelength data were collected prior to temperature treatment, at the highest temperature, and after cooled to the beginning temperature. Data were also collected during the temperature treatment by monitoring 222 nm and 293 nm, a band characteristic of alpha helix and of a well-defined tryptophan environment, respectively. All spectra were corrected and converted into mean residue ellipticity by the equation of [millidegrees* (mean residue weight/concentration (mg/mL)*pathlength (mm)].

Tertiary Structure by Second Derivative Ultraviolet Spectroscopy

UV data were collected in a 1-cm pathlength quartz cell on a Hewlett Packard 8452A diode array spectrophotometer. The data were analyzed using Grams 386 software by use of the truncate, spline, and derivative functions to manipulate the data. The second derivative data were imported into a spreadsheet for data analysis. The a, b, and c values were calculated from this second derivative curve using the peak-to-valley distances from tryptophan and phenylalanine peaks. The conformational information is determined by calculating the peak-to-trough values near 284 nm (a), near 292 nm (b), and near 258 nm (c), which reflect the microenvironments of tyrosine, tryptophan, and phenylalanine, respectively. The relative exposure of the side chains to the solvent in the native protein can be expressed from the ratio of "a" and "b" (a/b ratio)

or the ratio of "a" and "c" (a/c ratio). Both parameters appear to reflect similar changes in the conformational integrity of the protein in solution.

Tertiary Structure by Fluorescence Spectroscopy

Fluorescence spectroscopy data were collected on the GnRH-PAP on an Aviv spectrofluorometer model ATF105. Emission spectra were collected from 450 to 305 nm with an excitation at 295 nm with an emission step of 1 nm, emission bandwidth of 8 nm, an excitation bandwidth at 4 nm, preset the photo multiplier tube high voltage (PMT HV) at 500, and quantum counter high voltage (QC HV) of 227.78.

Size Exclusion Chromatography (SEC)

Size exclusion chromatography was performed on a TSK Gel Super SW 2000 Tosohaas column, using either 200 mM sodium phosphate pH 7 or 20 mM sodium phosphate/100 mM sodium chloride pH 6 as the mobile phases. The GnRH-PAP elutes at approximately 11.3 min at a flow rate of 0.3 mL/min.

Cation Exchange Chromatography (CEX)

Cation exchange chromatography was performed on a TSK-GEL SP-NPR Tosohaas column, using 20 mM sodium phosphate buffer pH 6 as the mobile phase at a flow rate of 0.3 mL/min. Under these conditions GnRH-PAP elutes at approximately 0.85 min.

Freeze-Thaw Studies

GnRH-PAP was dissolved in 20 mM potassium phosphate buffer (pH 6). Triplicate samples were subjected to five cycles of freeze-thaw. Each freezing cycle was performed in a –20°C freezer and each thaw cycle was performed in a temperature-controlled water bath set at 25°C for 1 min for a total of five freeze-thaw cycles. The overall sample volume was 0.5 mL. After each freeze-thaw cycle, visual inspection was performed to detect any visible precipitates.

Solubility Assessment

Estimation of the maximum solubility of GnRH-PAP was performed using a protocol similar to one by Middaugh et al. (1979). A stock solution concentration of PEG 8000 and a stock solution of GnRH-PAP (pH 6) were prepared. A variety of polyethylene glycol (PEG) solutions were made to various concentrations (0%–35% PEG 8000) and diluting the protein to an approximate final concentration of 1 mg/mL in each of the PEG solutions. The concentration in solution was determined by UV spectroscopy, using an extinction coefficient of 1.18 (mL/mg*cm). The logarithm of soluble protein concentration was plotted versus the PEG concentrations. Extrapolation to zero PEG concentration provides an estimation of the intrinsic maximum solubility of GnRH-PAP.

RESULTS

Secondary Structure of GnRH-PAP

The far UV CD spectrum of GNRH-PAP in phosphate buffer (pH 7) displays negative bands at 222 and 208 nm and a positive band at approximately 190 nm (Fig. 1). Analysis of the CD spectrum indicates a secondary structure composition of approximately 35% of α-helix and 18% β-sheet. This secondary structure is consistent with the crystal structure of PAP (Ago et al., 1994; Li et al., 1998), as well as what one would predict from an analysis of the primary structure of PAP (Schlick et al., 2000). Therefore, it appears that conjugation of GnRH to PAP does not alter the secondary structure of the protein (Fig. 1).

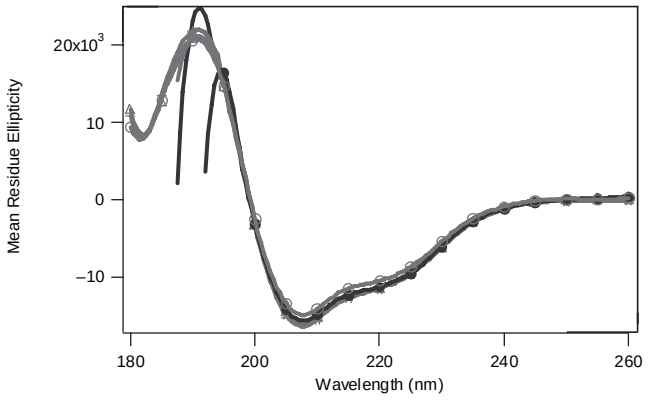

FIGURE 1 **Far UV CD of PAP-GnRH at pH 4, 5, 6, 7, 8 With pH 4.0, Solid Line, pH 5.0 # Sign, pH 6.0 +, pH 7.0 △, pH 7.0 and 100 mM NaCl •, and pH 8.0 O.**

The effect of pH on the secondary structure was investigated using far UV CD spectroscopy as well. Figure 1 shows that the secondary structure of GnRH-PAP does not change across the pH range of 4 to 8 (using 20 mM potassium phosphate for pH 6, 7, and 8 and using 20 mM acetate for pH 4 and 5), indicating that the conjugate is conformationally stable across a range of solution conditions. Furthermore, addition of 100 mM sodium chloride does not seem to perturb the secondary structure of GnRH-PAP either (data not shown).

Tertiary Structure of GnRH-PAP

Near UV CD spectroscopy (wavelength range of 340–250 nm) provides information about the tertiary structure of the protein. In this wavelength region, the side chains of aromatic amino acids (tyrosine, tryptophan, and phenylalanine) give rise to CD signals, depending on their local environment. Discrete signals in the CD spectra indicate that the chromophores are in a well-defined environment. In other words, sharp bands of substantial intensity indicate the presence of well-defined tertiary structure.

The near UV CD spectra of GnRH-PAP displays well-resolved vibronic fine structure between 295 and 260 nm (Fig. 2). The strong negative band around 292 nm and the sharp positive band near 285 nm arise from tryptophan residues. The positive bands at approximately 280 nm are due to tyrosine residues. The weak negative band near 260 nm is probably due to phenylalanine residues. The tertiary structure of the protein is not disrupted, as the pH is adjusted from of pH 4 to pH 8 (Fig. 2). Therefore, both the native secondary and tertiary structure of GnRH-PAP seems to remain intact across a fairly broad pH range. Addition of 100 mM sodium chloride does not affect the near UV CD spectrum (data not shown), similar to the results with the far UV CD spectrum.

Another method used to monitor the tertiary structure of proteins is fluorescence spectroscopy. The intrinsic fluorescence of a protein will change if the solvent accessibility of the aromatic residues is altered. Fluorescence spectra were collected for both PAP and GnRH-PAP. Excitation at 295 nm was used to excite Trp residues selectively. The observed emission (Fig. 3) displays a maximum at 345 nm for both the PAP and the GnRH-PAP. Proteins with fully solvent exposed Trp residues exhibit fluorescence emission maxima near 355 nm. Therefore, at least one Trp residue is partially solvent protected in GnGH-PAP, consistent with the crystal structure of PAP itself (Ago et al., 1994; Li et al., 1998). Moreover, the overall structure of PAP and the conjugate are similar, indicating that conjugation does greatly perturb the three-dimensional structure of the protein.

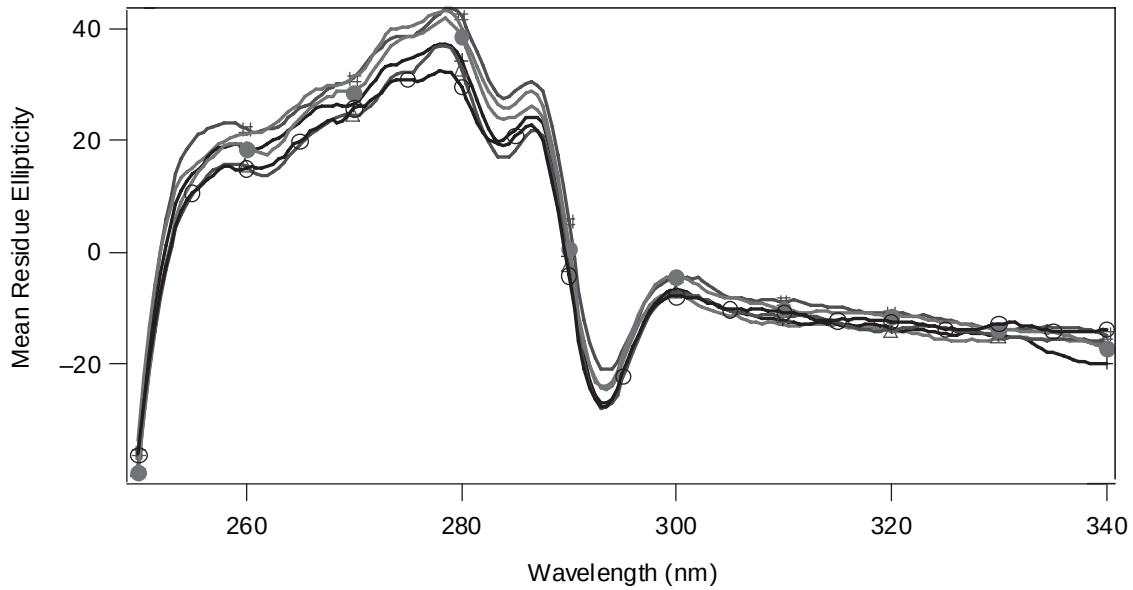

FIGURE 2 Near UV CD of PAP-GnRH in the pH Range of 4.0–8.0 With pH 4.0, Solid Line, pH 5.0 # Sign, pH 6.0 +, pH 7.0 △, pH 7.0 and 100 mM NaCl •, and pH 8.0 O.

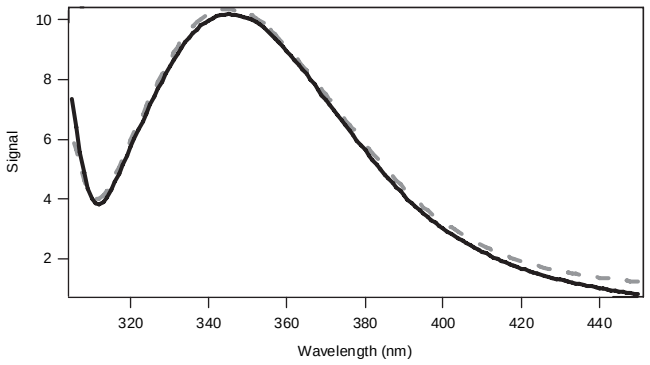

FIGURE 3 **Fluoresence of PAP and PAP-GnRH at pH 6.0. PAP is the Dashed Line and PAP-GnRH is the Solid Line.**

Thermal Unfolding of GnRH-PAP

To obtain an assessment of the overall conformational stability of GnRH-PAP, thermal unfolding studies were conducted. By monitoring a spectral signal that correlates to a specific structural characteristic of the protein, one can obtain an unfolding curve. Under conditions where the unfolding is reversible, it is possible to determine the free energy of unfolding.

Following the far UV CD spectrum, only modest decreases in intensity were observed up to 60°C for GhRH-PAP at pH 6. Full wavelength scans were obtained, beginning at 5°C, then at 80°C (the highest temperature examined), and after cooling back at 5°C (Fig. 4). Even at 80°C, GnRH-PAP still retains a significant amount of secondary structure, suggesting that is quite stable. Cooling the sample back to 5°C leads partial recovery of some of the secondary structure that was lost upon heating, indicating that the unfolding is at least partially reversible.

Thermal unfolding was also monitored using the near UV CD spectrum, focusing on the negative band

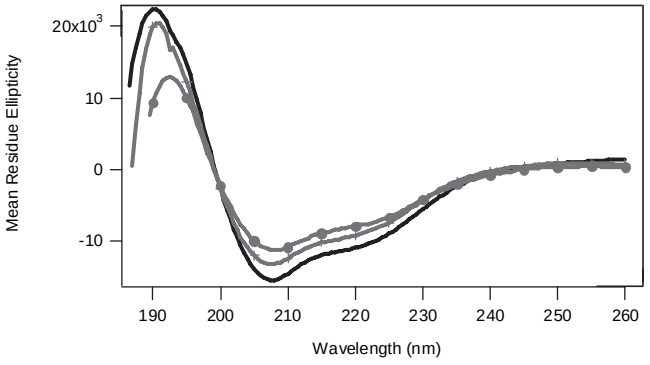

FIGURE 4 **Far UV CD Scans at 5⌋C, 80⌋C and #2 is Cooled Back to 5⌋C. PAP-GnRH at 5⌋C is the Solid Line, at 80⌋C •, and #2 at 5⌋C +.**

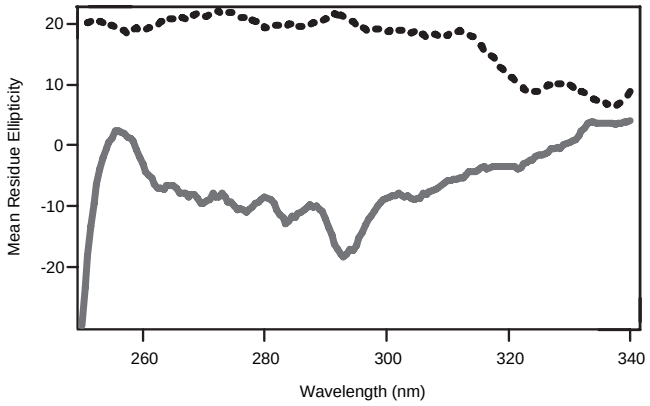

FIGURE 5 **Near UV CD Scans at pH 5.0 and 25°C After Heating to 80°C and Cooling Back to 25°C. PAP Alone, Dashed Line; PAP-GnRH, Solid Line.**

at 293 nm. Thermal unfolding was conducted from 25°C to 80°C at a heating rate of 1°C/min. Upon cooling back to 25°C, some tertiary structure in GnRH-PAP appears to remain, while it is not the case for PAP (Fig. 5). Apparently, the conjugation process confers some degree of stabilization of the tertiary structure of GnRH-PAP.

Freeze-Thaw Lability of GnRH-PAP

GnRH-PAP was dissolved in 20 mM potassium phosphate buffer (pH 6). Triplicate samples were subjected to five cycles of freeze-thaw. Each freeze-thaw cycle (total of five cycles) was performed according to the method described in the materials and methods section. After each freeze-thaw cycle, visual inspection did not reveal any visible precipitates. After the five freeze-thaw cycles, far UV CD spectra were collected (Fig. 6). All of the replicate samples display CD spectra thatare identical to a pH 6 GnRH-PAP control sample. Therefore, it does not appear that GnRH-PAP is freeze-thaw labile at all, at least under these conditions.

Solubility Assessment of GnRH-PAP

Estimation of the maximum solubility of GnRH-PAP was performed using the protocol as described in the materials and methods section using a stock solution of 60% (w/v) PEG 8000 and a stock solution of GnRH-PAP at pH 6.0 and 3.17 mg/mL. PEG solutions were made to 0%, 10%, 20%, 25%, 30%, and 35%, and diluting the protein to an approximate final concentration of 1 mg/mL in each of the PEG solutions.

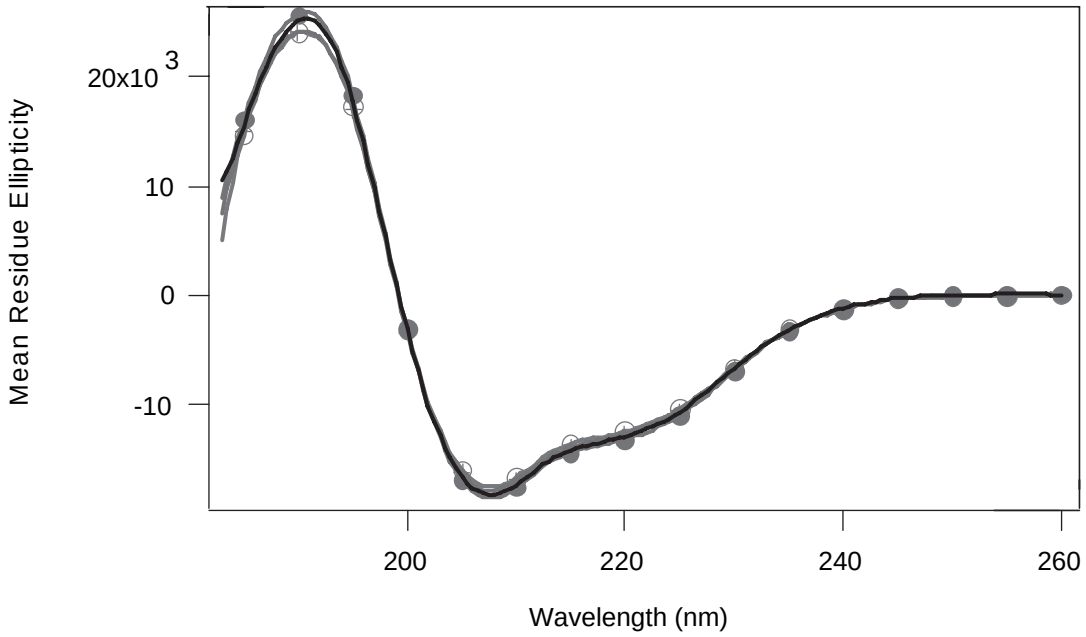

FIGURE 6 **Freeze-Thaw Stability of PAP-GnRH at pH 6.0. The Freeze-Thaw Cycle Consisted of 5 Cycles of Freezing at −20°C and Thawing in a 25°C Water Bath. PAP-GnRH Control, Solid Line; Freeze-Thaw Replicate 1; O; Freeze-Thaw Replicate 2, •; and Freeze-Thaw Replicate 3, +.**

The concentration in solution was determined by UV spectroscopy, using an extinction coefficient of 1.18 (mL/mg*cm). The logarithm of soluble protein concentration was plotted versus the PEG concentrations. A linear fit was performed on the data points (Fig. 7). Extrapolation to zero PEG concentration provides an estimation of the intrinsic maximum solubility of GnRH-PAP. The estimate for the protein solubility at pH 6 was 41 mg/mL.

To determine if any structural changes occur in the presence of high concentrations of PEG, second derivative UV absorbance data were collected and analyzed. By measuring the peak-to-peak amplitude distances of specific regions of the spectra, as described by Ragone et al. (1984), values for a and b were obtained. The ratio of the a and b values gives a parameter called the *r*-value. This r-value provides information regarding the microenvironments of tyrosine residues in a given protein. A change in the *r*-value indicates structural rearrangement has occurred. The *r*-values were obtained for spectra in 0% PEG and 25% PEG were 0.73 and 0.76, respectively, suggesting that the structure of GnRH-PAP in concentrated PEG solutions is nearly native-like.

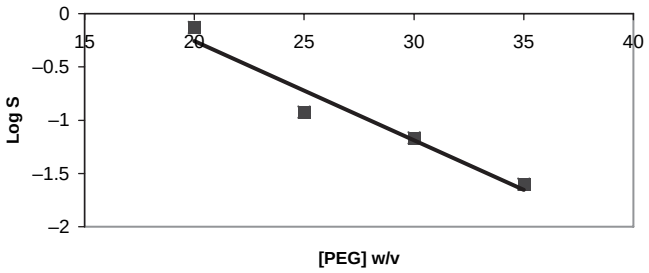

FIGURE 7 **Solubility of PAP-GnRH at pH 6.0.**

Concentration Effects on GnRH-PAP

GnRH-PAP was moderately concentrated in potassium phosphate buffer (pH 6) to a concentration of 3.3 mg/mL to determine if an approximate 3-fold increase in concentration (from that previously studied) would change the solution conformation. The near UV CD spectrum does not appear to be changed upon increasing the concentration (data not shown). Likewise, the far UV CD spectrum of GnRH-PAP does not seem to be altered with an increase in concentration. Therefore, the native structure of GnRH-PAP at 3.3 mg/mL appears to be identical to that at 1 mg/mL.

Chemical Stability of GnRH-PAP

A strong cation exchange (CEX) HPLC method was used to evaluate chemical stability. Typically, changes in the CEX chromatogram are indicative of deamidation, as

this reaction results in a change in the overall charge on the protein, although other degradation may be detected as well. Given the high pI value of GnRH-PAP (theoretical pI: 9.24), the pH of the mobile phase was lower than found for many published CEX methods.

A single peak was observed for GnRH-PAP, as it was for PAP (data not shown). Incubation of GnRH-PAP at 60ºC was conducted at three different pH values (2.5, 6, and 11). At pH 6, no degradation peaks are observed after heating for 3 h (Fig. 8A). However, at pH 2, a distinct

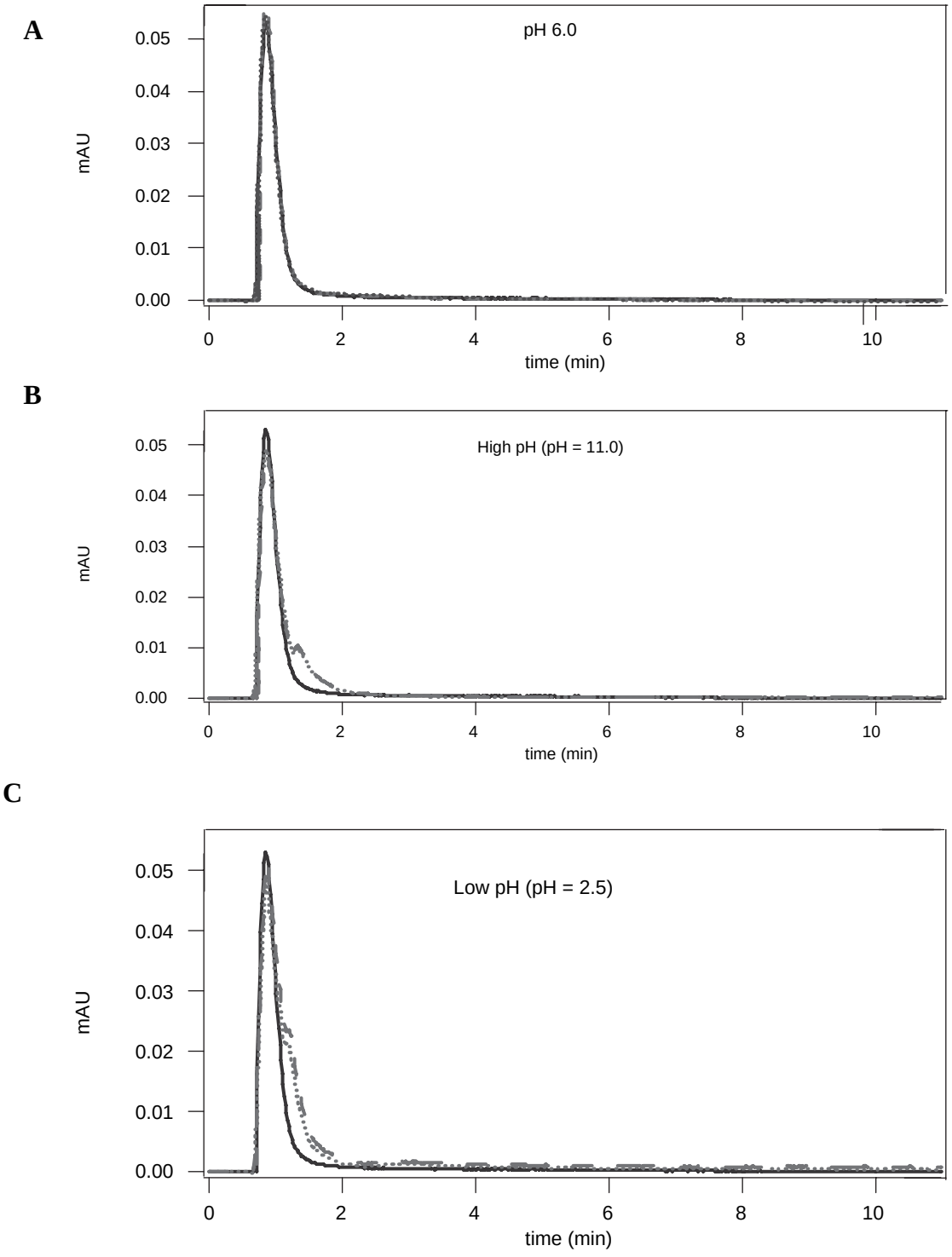

FIGURE 8 **Cation Exchange Chromatograms at Three Different pH values (2.5, 6, 11) Upon Incubation at 60°C for 0, 1, and 3 h. Control PAP-GnRH, Solid Line; 1 h, Dashed Line; and 3 h, Dotted Line.**

TABLE 1 Experimental Design Matrix: pH and Sodium Chloride

pH	NaCl
8	0
8	0
8	33
6	50
6	50
5.5	0
5.5	33
8	50
8	50
8	16.7
6.7	0
4	50
4	50
4	25
4	25
4	0

shoulder appears after 1 h (Fig. 8B). Heating for 2 h more produces little further change (Fig. 8A, B, and C).

At pH 11, a new peak appears upon heating as well, which is better resolved than in the low pH samples (Fig. 8B). Again, the peak can be observed after only 1 h, but, once again, little other change was seen after heating for 2 h more. These data suggest that there is a less stable subpopulation in these preparations that degrades quickly, leaving the more stable fraction(s) intact.

Using the CEX method, the effect of pH and salt on the chemical stability GnRH-PAP was evaluated. Using experimental design, solution conditions were selected that would allow for identification of the optimal pH and ionic strength with the fewest number of trials. The pH range was chosen to cover pH 4 to 8 and the sodium chloride concentration was varied from 0 to 50 mM (Table 1). Samples were prepared and incubated in a temperature controlled water bath at 60°C for 0 h, 2 h, 3 h, 4 h, and 5 h. The samples were removed and analyzed by CEX.

These results were analyzed using both multiple linear regression (MLR) and a quadratic partial least squares (PLS) model. As can be seen in the response surface plots (Fig. 9), the smallest amount of degradation, as detected by CEX, occurs at the lowest pH (pH 4). This is true independent of the mathematical model used. As for NaCl, it is predicted to have a much smaller effect than pH. In the MLR model, some added salt is beneficial (Fig. 9A). In the quadratic PLS model, the optimal amount of salt appears to be near 30 mM (Fig. 9B). In any case, low pH and a modest amount of salt produce the least amount of chemical degradation at elevated temperature.

It is possible that other excipients might be beneficial in slowing degradation. Using stabilizers that are commonly found in other protein formulations, samples were stored at 40°C and samples withdrawn after 4 weeks. The pH of these samples was adjusted to 7.4 to ensure that pH was not dominating the stability profile. All of the samples were analyzed at the same time by the CEX method.

Upon storage at elevated temperatures, the formulation containing 6% sucrose exhibited the greatest loss of native protein, with nearly 40% degrading over the time of storage (Table 2). The other two formulations (containing sucrose and glycine) only displayed ~30% loss. Similarly, the sucrose formulation also showed the largest change in conformation, with the *r*-value increasing from 0.68 to 0.76. The other two formulations had a change in the *r*-value of only 0.01. These data indicate that there is some stabilization gained by the addition of a combination of sucrose and glycine. The actual ratio of the two excipients does not appear to be important. Meanwhile, sucrose alone was less potent at stabilization of GnRH-PAP, at least for protecting against chemical degradation. Formulations at pH 4 showed no degradation over the 4-week storage time (data not shown). The reason for increased stabilization by sucrose/glycine relative to sucrose alone is not clear and requires further investigation.

SUMMARY

Conjugation of GnRH to PAP does not appear to cause any significant alterations in the solution conformation of the toxin. The conjugate has a well-defined secondary and tertiary structure and it is quite soluble at pH 6 (>40 mg/mL). Upon exposure to elevated temperature, GnRH-PAP displays only minor changes in conformation, but this loss is mostly irreversible. In addition, it appears to be chemically stable at pH values less than 6, but degradation occurs rapidly at neutral to basic pH. Overall, we have been able

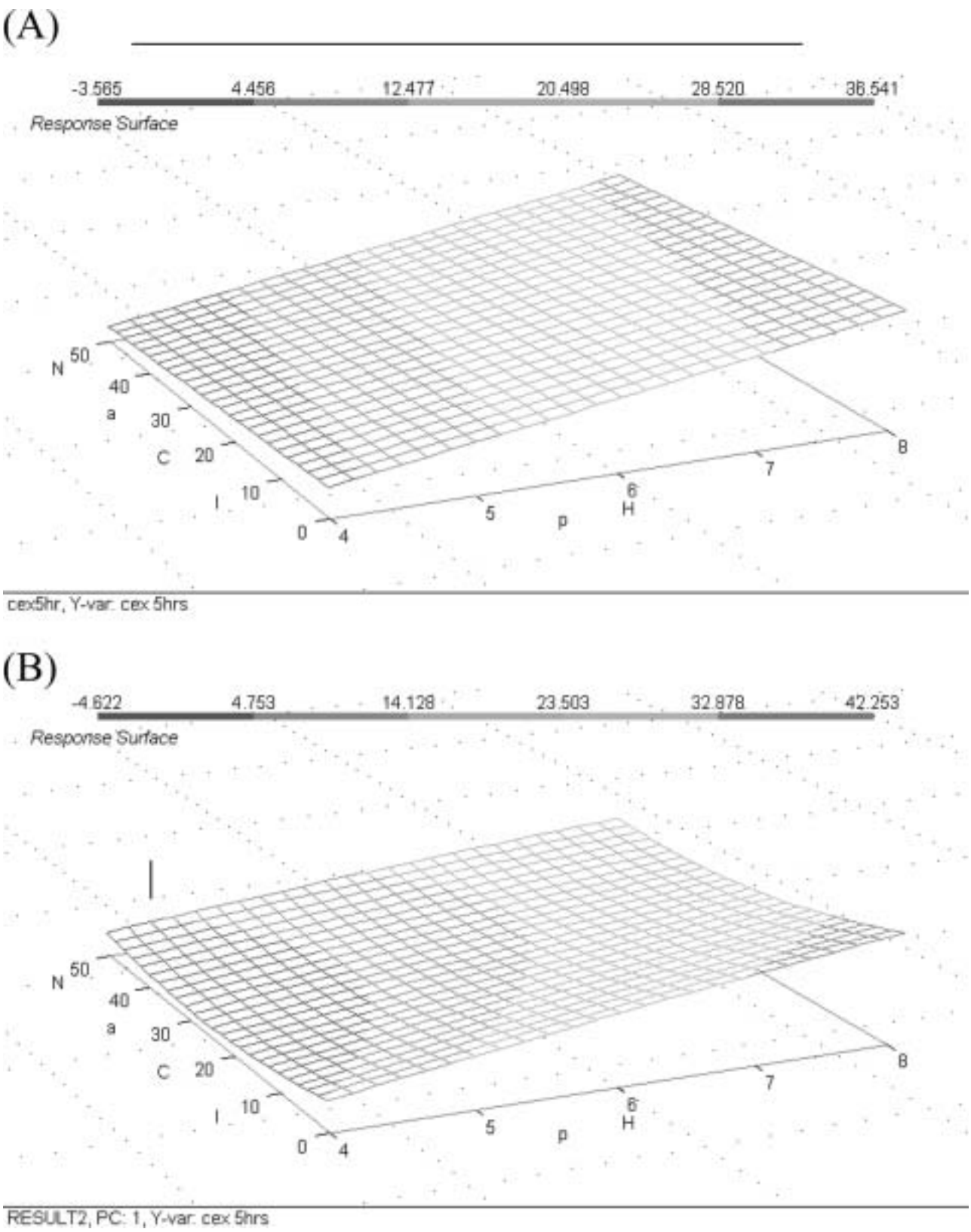

FIGURE 9 **Response Surface for Degradation of GnRH-PAP at 60°C for 5 h Using a MLR Model (A) or a Quadratic PLS Model (B).**

TABLE 2 **Stability of GnRH-PAP (pH 7.4, 20 mM Phosphate Buffer) After Storage at 40ºC for 4 Weeks**

Formulation	Percent remaining by CEX	r-values before and after storage
Formulation 1 6% sucrose	60.70	0.68/0.76
Formulation 2 1.5% sucrose/2% Gly	71.50	0.70/0.69
Formulation 3 1% sucrose/2.3% Gly	71.12	0.75/0.75

to approach the solution characterization and formulation of a peptide-protein conjugate in a manner similar to that of the characterization of an unconjugated protein by investigating the physical and chemical stability as well as structural characterization of this peptide-protein conjugate with regardto pH, ionic strength, concentration, and thermal and freeze-thaw stress. Additionally, we have made some valuable observations regarding the unique solution behavior and stability of this GnRH-PAP biopharmaceutical drug candidate.

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