Chemical Pathways of Peptide Degradation. VII. Solid State Chemical Instability of an Aspartyl Residue in a Model Hexapeptide

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The chemical stability of an Asp-hexapeptide (Val-Tyr-Pro-Asp-Gly-Ala) in lyophilized formulations was evaluated as a function of multiple formulation variables—specifically pH of the bulk solution, temperature, moisture content, and type of bulking agent (amorphous vs. crystalline). The disappearance of the starting Asp-hexapeptide in the solid state conformed to pseudo-first-order reversible kinetics. This type of degradation profile was accounted for by the product distribution. The factorial experimental design of this study allowed statistical analysis of the effects of individual formulation variable (main effects) as well as those of two-factor interactions on the degradation of the Asp-hexapeptide. Analysis of Variance (ANOVA) calculations of the main effects indicated that while the influence of pH of the starting solution was not statistically significant, residual moisture level, temperature, and, especially, type of bulking agent had a significant impact on the solid state chemical reactivity of the hexapeptide. Furthermore, depending on which type of excipient was used in the lyophlized formulations, residual moisture level and temperature could be important stability variables. These types of factorial experiments have proven to be useful in the rapid identification of significant formulation variables in a given system and, consequently, in optimization of formulations.

KEY WORDS: lyophilization; lyophilized formulations; factorial experimental design; Asp-hexapeptide; solid state chemical stability; formulation development.

INTRODUCTION

A comprehensive understanding of the chemical reactivity of polypeptides at the molecular level is essential for their development as pharmaceutical agents. The stability of proteins is more complex and diverse than small molecules because in addition to the primary sequence, polypeptides possess higher order structures (i.e., secondary, tertiary, and quaternary) (1). Although extensive research efforts have been dedicated to studying the factors influencing the chemical stability of polypeptides in aqueous medium (2–10), our understanding of solid state stability of polypeptides is still limited and evolving.

Protein products are commonly administered intravenously because of their characteristically poor and erratic oral bioavailability (11), and often are lyophilized to achieve adequate shelf-life stability (12). However, development of freeze-dried formulations presents a new set of stability variables such as the effect of the type of excipients used, as well as those of residual moisture content and choice of optimum lyophilization cycle on the stability of the drug product. Assessment of these variables is imperative for designing the most stable dosage form with minimal lot-to-lot shelf-life variability.

We have recently described the solution kinetics and mechanism of the degradation of an Asp-hexapeptide (Val-Tyr-Pro-Asp-Gly-Ala) (13). The routes of degradation were found to be pH-dependent. Under highly acidic conditions, the Asp-hexapeptide predominantly underwent specific acid-catalyzed hydrolysis at the Asp-Gly amide bond to generate a tetrapeptide and a dipeptide. Simultaneously, the starting peptide, to a lesser extent, cyclized to form a cyclic imide (Asu-hexapeptide) whose breakdown was base-catalyzed. Consequently, the latter became more unstable with increasing pH, giving rise to formation of the isoAsp-hexapeptide and the regeneration of the parent Asp-hexapeptide at near neutral and alkaline pH values.

The present study evaluates the stability of the Asp-hexapeptide in lyophilized formulations as a function of pH, temperature, moisture level, and type of bulking agent using a factorial experiment design. This design made it feasible to examine the main and two-factor effects of the aforementioned variables on the stability of the hexapeptide in the lyophilized state. The complete study design is summarized in Table I.

MATERIALS AND METHODS

Materials

The Asp-hexapeptide (L-Val-L-Tyr-L-Pro-L-Asp-L-Gly-L-Ala) was synthesized by Dr. Madhup Dhaon (Abbott Laboratories, North Chicago, IL). L-Val-L-Tyr-L-Pro-L-Asu-L-Gly-L-Ala (Asu-hexapeptide) was isolated as a major side product during the HPLC purification of the Asp-hexapeptide. L-Val-L-Tyr-L-Pro-L-Asp (tetrapeptide) was synthesized by the Biochemical Research Services Laboratory (University of Kansas, Lawrence, KS). Lactose monohydrate (NF), mannitol (USP), anhydrous dibasic sodium phosphate (USP), and anhydrous citric acid (USP) were used as supplied by Abbott Laboratories (North Chicago, IL). Trifluoroacetic acid (TFA, HPLC grade) was purchased from Pierce Chemicals (Rockford, IL). HPLC grade acetonitrile was supplied by Fisher Chemical (Fair Lawn, NJ). The water used in all studies was from a Millipore MILLI-Q™ Water System.

Apparatus

High-performance liquid chromatography was done
Table 1. Factorial Experimental Design for the Solid State Stability Study of Asp-hexapeptide

<table>
<thead>
<tr>
<th>Trial Number</th>
<th>pH</th>
<th>Bulking Agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.5</td>
<td>mannitol</td>
</tr>
<tr>
<td>2</td>
<td>5.0</td>
<td>mannitol</td>
</tr>
<tr>
<td>3</td>
<td>6.5</td>
<td>mannitol</td>
</tr>
<tr>
<td>4</td>
<td>8.0</td>
<td>mannitol</td>
</tr>
<tr>
<td>5</td>
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<td>lactose</td>
</tr>
<tr>
<td>6</td>
<td>5.0</td>
<td>lactose</td>
</tr>
<tr>
<td>7</td>
<td>6.5</td>
<td>lactose</td>
</tr>
<tr>
<td>8</td>
<td>8.0</td>
<td>lactose</td>
</tr>
</tbody>
</table>

At a given trial, three moisture levels (M1, M2, M3) were evaluated at 40\(^\circ\), 50\(^\circ\), and 60\(^\circ\)C.

with a system consisting of a Shimadzu LC-6A pump, a SCL-6B system controller, a SPD-6A variable wavelength UV detector, a Perkin Elmer ISS-100 autosampler, and a C-R4A Chromatopac integrator. The pH readings were recorded using a POPE Model 1501 pH/ion meter. The HPLC analysis of the Asp-hexapeptide and its products was performed on an Alltech ODS Hypersil C\(_18\) column (5 \(\mu\)m resin, 4.6 \times 250 mm). Lyophilization of all samples was carried out using Edwards lyophilizer. Residual moisture was determined by coulometric Karl Fischer assay (Photovolt Aquatest 8 Moisture Analyzer). Powder X-ray patterns were obtained using a Nicolet 12 diffractometer with monochromatized copper radiation (50 kilovolts).

Lyophilization Studies

All buffer solutions were prepared by mixing solutions of 0.01 M dibasic sodium phosphate and 0.01 M citric acid at room temperature to obtain the desired pH. The resulting buffer solutions of pH 3.5, 5.0, 6.5, and 8.0 were filtered through a 0.2 \(\mu\)m nylon filter (Pall Trinity Micro Corp., Cortland, NY) into autoclaved flasks. Sufficient quantities of peptide and excipient were dissolved in 500 ml of pH-adjusted buffer solutions to yield initial peptide and bulking agent concentrations of 1.3 \times 10^{-4} M and 4\% w/v, respectively. Aliquots of 2 mL were filled into 10 mL USP Type I flint vials using Fill-O-Matic filler. The filling machine was calibrated and in process weight checks were done to assure filling accuracy. These vials were then labeled, loaded onto trays, and lyophilized. Lyophilization stoppers (West Co., Phoenixville, PA) were placed on top of vials prior to loading into the lyophilizer. The following lyophilization cycle was used for this study: hard freezing−shelf (−35°C), hold product (−25°C) for 1 hour; primary drying−shelf (0°C), hold product (−2°C) for 2 hours, vacuum (250 microns); secondary drying−shelf (6°C), hold product (4°C) for 1 hour, vacuum (250 microns); third stage drying−shelf (15°C), hold product (12°C) for 1 hour, vacuum (250 microns) for 1 hour; fourth stage drying−shelf (40°C), hold product (30°C) for 10 hours, vacuum (100 microns); shelf temperature for unloading (20°C). At the completion of the cycle, nitrogen was introduced into the freeze drying chamber, and the shelves were collapsed to seal the vials with lyophilization stoppers. The stoppers used in this study had been washed, siliconized, and dried using heat and reduced pressure.

Moisture Loading

All lyophilized samples, except for the control (moisture level 1) samples, were loaded with moisture. The required volumes (0.8 \(\mu\)L, moisture level 2; 1.6 \(\mu\)L, moisture level 3) of distilled water were measured using a 10 \(\mu\)L Hamilton syringe into plastic WISP (Waters Associates) inserts. The inserts with liquid water, open at one end, were placed inside the vials on top of the lyophilized cakes. This placement of inserts into the vials was done in an atmosphere of dry nitrogen created by using an inverted funnel. The vials were then restopped, capped with aluminum seals and stored at temperatures ranging from 40\(^\circ\) to 60\(^\circ\)C.

Determination of Crystallinity of Samples

The crystallinity of the lyophilized samples was determined by powder X-ray diffraction. The relative intensity was determined by comparing the resulting integrated peak areas with those of the standard (bulk powder).

Moisture Content Determination

Residual moisture content was determined by Karl Fischer assay. With the exception of the lactose/peptide formulation at high moisture loading level, the reported moisture percentages for all formulations were expressed as average values of duplicate sample vials.

HPLC Protocol

The vials were removed at designated time intervals and placed in a freezer (−15°C or lower) prior to HPLC analysis. At the time of analysis, the vials were then reconstituted with 2 mL of distilled water and the resulting solutions were injected directly onto the HPLC column. Because of the size of the study, multiple repetitions of experiments (e.g., duplicates, triplicates) were not feasible. Single determinations were made for all kinetic data points except the initial time (t = 0) points for which triplicates were determined.

The HPLC separation was carried out at ambient temperature, using an isocratic system consisting of 12\% (v/v) acetonitrile and 0.1\% (v/v) TFA in water at a flow rate of 1.0 mL/min and detection at 214 nm.

Data Fitting and Statistical Analysis

The disappearance of the Asp-hexapeptide was fitted to the pseudo-first-order reversible kinetic model from which the rate constants \(k_1\) (forward rate constant) and \(k_2\) (reverse rate constant) were generated. This non-linear curve fitting was accomplished with the Least Squares algorithm in the program "MINNSQ" (MicroMath) for IBM compatible personal computers. Analysis of Variance (ANOVA) calculations were performed separately on the rate constants \(k_1\) and \(k_2\) at all temperatures using Statgraphics (STSC, Rockville, MD) software application. However, only trends that are applicable to the forward rate constant \(k_1\) are discussed in this paper since those that pertain to the reverse rate constant proved to be equal and opposite to the forward rate constants. The significance of trends presented here fall within the 95\% confidence interval (p < 0.05).