Chemical Pathways of Peptide Degradation. VII. Solid State Chemical Instability of an Aspartyl Residue in a Model Hexapeptide[5]

Cecilia Oliyai,[1,3] Jitendra P. Patel,[2] Linda Carr,[2] and Ronald T. Borchardt[1,4]

Received June 16, 1993; accepted January 5, 1994

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 lyophilized 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

[1] Department of Pharmaceutical Chemistry, The University of Kansas, 3006 Malott Hall, Lawrence, Kansas 66045.
[2] Pharmaceutical Products Division, Abbott Laboratories, 1401 Sheridan Road, North Chicago, Illinois 60064.
[3] Current address: Chiron Corporation, Formulation & Pharmaceutical Development, 4560 Horton Street, Emeryville, California 94608.
[4] To whom correspondence should be addressed.
[5] Unless otherwise noted, all amino acids listed are L-enantiomers of the 20 common amino acids and are referred to by their three letter abbreviations. Asu is used as an abbreviation of the cyclic imide form of Asp.

0724-8741/94/0600-0901$07.00/0

Table I. Factorial Experimental Design for the Solid State Stability Study of Asp-hexapeptide

Trial Number	pH	Bulking Agent
1	3.5	mannitol
2	5.0	mannitol
3	6.5	mannitol
4	8.0	mannitol
5	3.5	lactose
6	5.0	lactose
7	6.5	lactose
8	8.0	lactose

At a given trial, three moisture levels (M1, M2, M3) were evaluated at 40°, 50°, and 60°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 μm resin, 4.6 × 250 mm). Lyophilization of all samples was carried out using an 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 μm 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 1.3×10^{-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 μL, moisture level 2; 1.6 μL, Moisture level 3) of distilled water was measured using a 10 μL 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 restoppered, capped with aluminum seals and stored at temperatures ranging from 40° to 60°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 "MINSQ" (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 ($p < 0.05$).

RESULTS AND DISCUSSION

Characteristics of the Asp-hexapeptide Formulations

All mannitol/peptide formulations lyophilized well, producing stable, homogenous cakes which retained their integrity under all conditions throughout the study. The lactose/peptide formulations generated acceptable, albeit less sturdy, lyophilized cakes. All lyophilized formulations reconstituted rapidly with water.

Based on the powder X-ray diffraction studies, the lyophilized cakes of mannitol/peptide formulations showed crystalline X-ray patterns that are characteristic of lyophilized mannitol (Fig. 1a) while those of lactose/peptide exhibited amorphous structure (Fig. 1b). Additionally, no physical transformations of the solids took place in either formulation with the progress of time and increasing hydration level (data not shown).

It was observed that the crystalline formulations did not retain and/or take up as much water as the freeze-dried amorphous formulations as evidenced by the experimentally measured moisture contents. Residual moisture contents for mannitol/peptide formulations were estimated to be 0.3% ± 0.08%, 0.4% ± 0.05%, and 0.6% ± 0.05% water for low, medium, and high moisture loading levels, respectively. The lactose/peptide formulations contained 1.9% ± 0.7%, 2.7% ± 0.2%, and 5.5% water at low, medium, and high moisture loading levels, respectively.

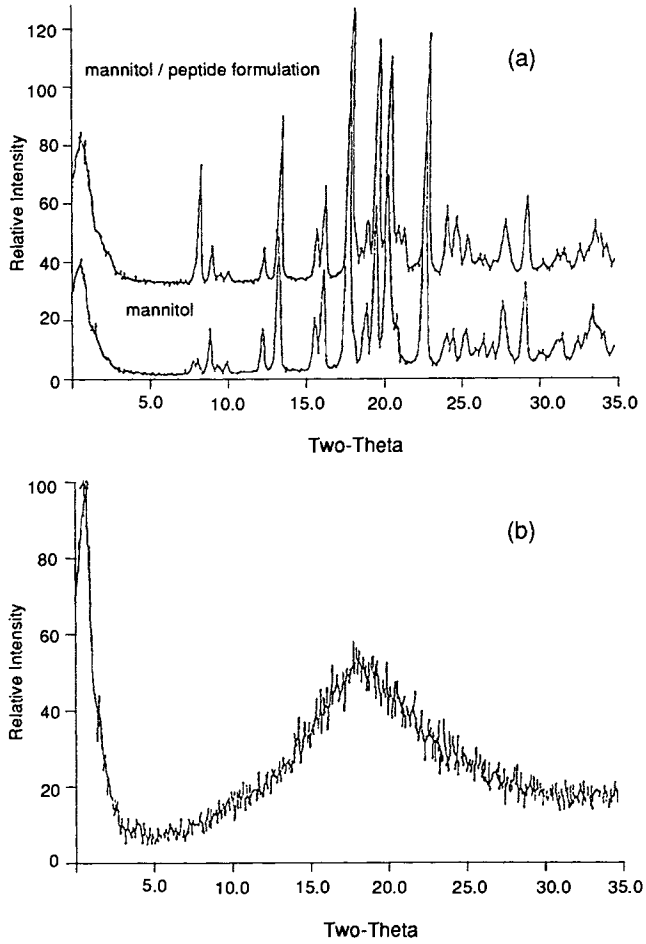
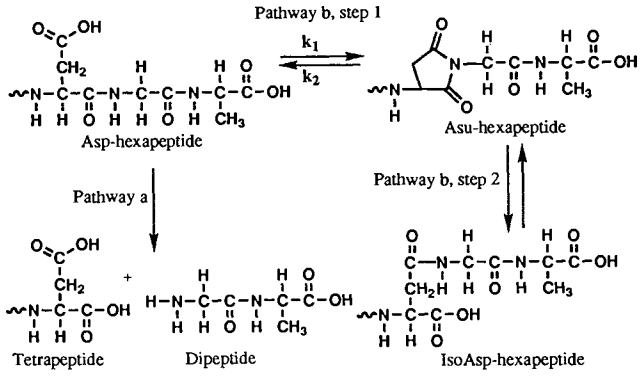


Fig. 1: Powder X-ray diffraction of mannitol/peptide formulation versus lyophilized mannitol 4% w/v solution (panel a) and lactose/peptide formulation at 3 months (panel b).

Degradation Pathways in the Lyophilized State

The degradation pathways were found to be dependent on the pH of the bulk solutions and moisture content of the freeze-dried formulations. In general, the kinetics of the disappearance of Asp-hexapeptide followed pseudo-first-order reversible kinetic behavior under all experimental conditions (Scheme I, Fig. 2). This type of kinetic profile was justified by the product distribution observed after 6 months of degradation at 60°C. Under acidic conditions (pH 3.5 and 5.0), the lyophilized Asp-hexapeptide predominantly decomposed to generate the Asu-hexapeptide, irrespective of the type of excipient present in the formulation (Scheme I, Figs. 3a and 3b). The hydrolysis of Asp-Gly amide bond constituted a much less significant pathway under these conditions (Scheme I, Figs. 3a and 3b). At pH 6.5 and 8.0, the parent hexapeptide exclusively isomerized via formation of the Asu-hexapeptide to produce the isoAsp-hexapeptide (Figs. 3a and 3b). The extent of hydrolysis of the Asu-hexapeptide intermediate at pH 8.0 exceeded that at pH 6.5, rendering the isoAsp-hexapeptide the major product of degradation in the basic environment.

Upon examination, the product distribution in solid state was significantly different from that in solution, probably because most of the water, an important participant in hydrolytic reactions, was removed by lyophilization. Evidently, the hydrolysis of the Asu-hexapeptide intermediate and that of the Asp-Gly peptide bond (formation of tetrapeptide) were suppressed in water-deficient environments. Thus, at lower pH values (pH 3.5, 5.0, and 6.5), the major product observed was the Asu-hexapeptide, and only trace amounts of tetrapeptide and isoAsp-hexapeptide were detected at pH 3.5 and 6.5, respectively (Scheme I, Figs. 3a



	Pathway a	Pathway b Step 1	Pathway b Step 2
pH 3.5	minor	major	undetected
pH 5.0	undetected	major	undetected
pH 6.5	undetected	major	minor
pH 8.0	undetected	minor	major

Scheme I. Pathways of degradation of the Asp-hexapeptide in solid state.

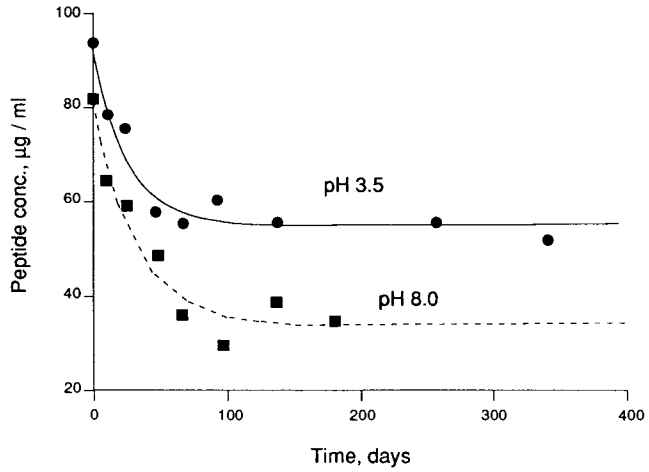


Fig. 2: Typical curve-fitted plots of the disappearance of Asp-hexapeptide at low moisture level (60°C) in lactose.

and 3b). It was observed that the stability of the Asu-hexapeptide in lyophilized formulations was dependent on not only the moisture content [e.g., 13 µg/mL (low moisture level), 6.3 µg/mL (medium moisture level), 3.8 µg/mL (high moisture level) of Asu-hexapeptide remained at pH 8.0 and 60°C after six months] but also the pH of the bulk solution (Fig. 4). Thus, at pH 8.0, the base catalysis component of the hydrolysis of Asu-hexapeptide intermediate offset the nearly inoperative water catalysis component. Consequently, the rate of decomposition of the Asu-hexapeptide was sufficiently significant to afford the isoAsp-hexapeptide as a major degradation product at pH 8.0 (Scheme I, Figs. 3a and 3b).

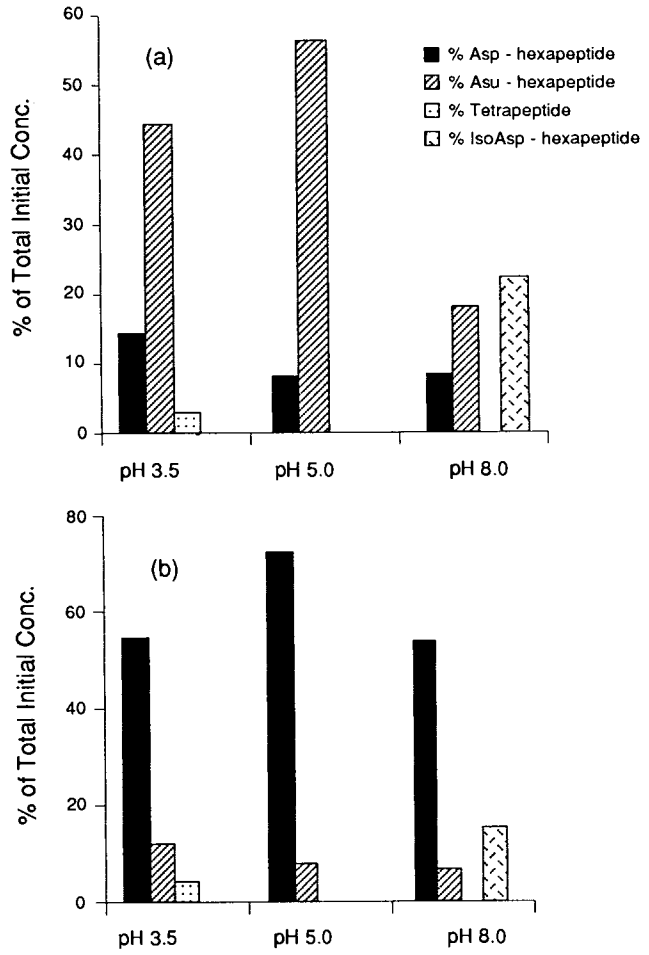


Fig. 3: Peptide distribution at 60°C and low moisture level after 6 months for mannitol/peptide (panel a) and lactose/peptide formulations (panel b).

Effects of Individual Formulation Variables

Nature of Excipient

It has been reported that the crystallinity of excipients in the final formulation depends on the lyophilization cycle (14) and potential interactions between the bulking agent and other components in the formulation may accelerate solid state chemical decomposition of drugs (15,16). Residual water in lyophilized products which can interact with the excipient to facilitate physical transformation of solids (i.e., excipients, drug) has been shown to compromise the overall stability of the active drug (17).

Upon exposure of the formulations to moisture during loading, the percent of moisture content increased, confirming that additional moisture was taken up into the lyophilized cakes. The lactose-containing cakes, being amorphous, had greater affinity for moisture than those containing mannitol. This was evident from the higher moisture content of lactose containing formulations. Yet, the peptide was significantly more stable in the lactose matrix compared to mannitol under all conditions (Fig. 5a). Pikal and co-workers (18) similarly observed greater stability for lyophilized human growth hormone against aggregation and chemical decomposition in *partially amorphous* formulation than in either totally amorphous or crystalline excipient systems. These authors concluded that this was because a partially amorphous system

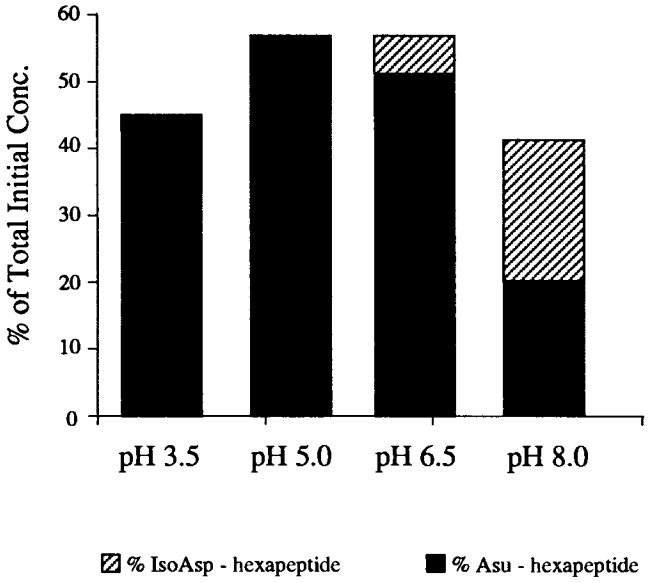


Fig. 4: Influence of pH on the stability of Asu-hexapeptide in mannitol/peptide formulations at 60°C and low moisture level after 6 months.

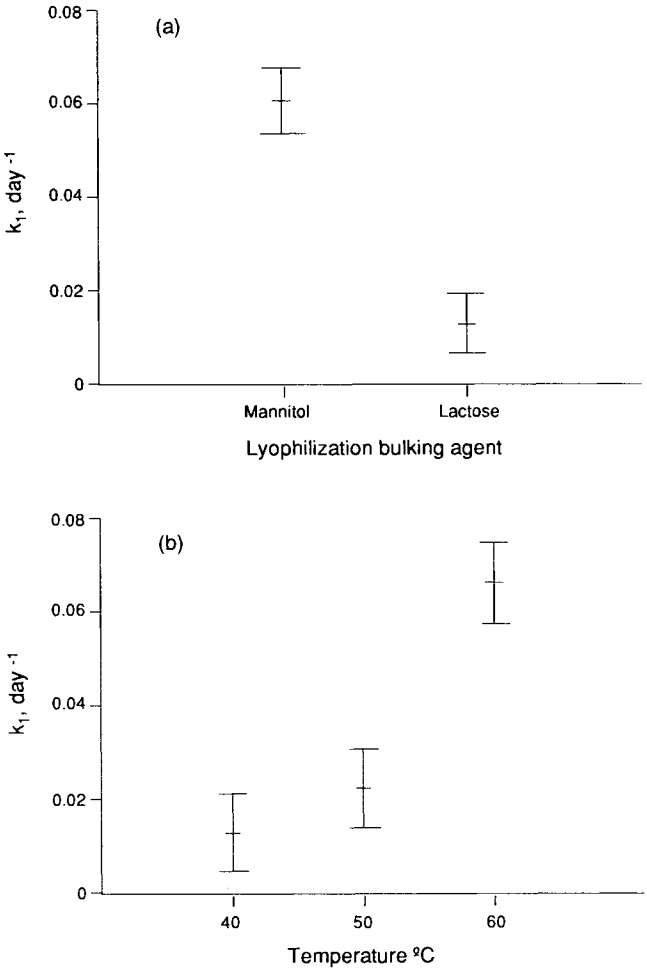


Fig. 5: Main effects of bulking agent (panel a), and temperature (panel b) on the mean rate constant of peptide degradation k_1 ($p \leq 0.05$).

allowed molecular interaction with the protein and/or acted as a "sink" for residual water. However, the results from these studies clearly suggested that while an excipient system which is partially amorphous is *necessary* for stability enhancement, this condition alone is not *sufficient* for stability (18). Thus, it is not unreasonable that an amorphous lyo-protectant could stabilize a protein/peptide drug against chemical degradation better than a completely crystalline one. In this case, the stabilizing effect observed with lactose obviously is not due to any physical transformation whereby the amorphous peptide or excipient crystallizes, thus altering the stability of the peptide. Moreover, these results are not inconsistent with the view that the activity of water molecules in lactose/peptide formulations might be relatively lower as compared to that in mannitol/peptide systems due to greater interaction between water and the amorphous lactose. This association consequently decreased the number of *free* water molecules available for chemical reaction with the peptide.

pH of the Bulk Solution

Although the concept of pH has no meaning in the solid state, the pH of the unlyophilized bulk solution determines the extent of ionizations of the peptide and buffer species both in solution and in the solid state (18). In some cases, the rate constant of degradation in solid state can be comparable to or higher than that in solution at a given pH (19,20). Therefore, it is important that formulation scientists confirm whether the same pH dependence for the rate of degradation generated from solution stability studies applies in solid state.

While the bulk solution pH noticeably perturbed the product distribution, it had no statistically significant effect on the solid state chemical stability (mean rate constant k_1) of the Asp-hexapeptide within the pH range studied (data not shown). In solution, however, the pH of the medium governed not only the product distribution but also the rate of degradation of the Asp-hexapeptide (13). This peptide degraded approximately four times faster in solution at acidic pH values than at basic pH in solution. Under aqueous acidic conditions, specific acid-catalyzed hydrolysis of the Asp-Gly amide bond and cyclic imide formation constituted the major route of degradation in aqueous solution. In the solid state at pH 3.5, this predominant hydrolytic pathway was substantially suppressed due to lack of moisture. This accounted for a reduction in the overall rate of decomposition in solid state under acidic conditions.

In solution, at basic pH values, the Asp-hexapeptide exclusively undergoes pH-independent isomerization in which the slow kinetic step is the formation of cyclic imide (13). Unlike the acid-catalyzed formation of tetrapeptide, the rate of formation of cyclic imide was only slightly affected in the solid state as evidenced by the accumulation of cyclic imide product over a wide pH range except at pH 8.0 (Figs. 3a and 3b). Lack of moisture had little impact on this ring closure since it does not require water as a direct participant. Consequently, the rate of cyclic imide formation in the solid state would be somewhat comparable to that in solution. The slight difference in the rates may be attributed to lowered chain flexibility due to moisture deficiency.

Collectively, the depressed pH dependence of the overall rate of peptide decomposition observed in the solid state (data not shown) is a direct result of suppression of the rate of acid-catalyzed tetrapeptide formation (Asp-Gly amide bond hydrolysis) under acidic conditions and pH-independent isomerization reaction under basic conditions.

Moisture Level

Water has always played an important role in the chemical stability of drugs, whether they be macromolecules or small organic molecules. It is known that water facilitates spontaneous chemical degradation by increasing molecular mobility to encourage intermolecular encounters, chain flexibility to foster intramolecular rearrangements, and the number of water molecules available as direct participants in reactions.

It was observed that the rate of degradation of the Asp-hexapeptide increased as the vial moisture content increased (data not shown). In this case, increasing the moisture content augmented not only the number of free water molecules available for catalyzing the decompositions of the cyclic imide and cyclic anhydride intermediates, but probably also the molecular mobility and chain flexibility, which would in-

crease the propensity of the peptide to undergo cyclization in the formations of tetrapeptide and cyclic imide. However, the differences in rate constants were statistically significant only between low and high moisture levels (data not shown).

Temperature

In general, the stability of the Asp-hexapeptide varied inversely with temperature in a non-linear Arrhenius fashion. The difference in rate constants was minimal between 40°C and 50°C (Fig. 5b). On the other hand, the effect of temperature was statistically significant between 40°C and 60°C and between 50°C and 60°C (Fig. 5b). In other words, the rate constant increased substantially at 60°C, possibly indicating the existence of a critical temperature threshold.

Effects of Two-Factor Interactions on the Stability of Asp-hexapeptide

Temperature/Bulking Agent

The statistically significant main effect of temperature on the mean degradation rate constant can be accounted for by examining the temperature and bulking agent two-factor interaction. While the stability of peptide formulations containing lactose was indifferent to the effect of temperature, the decomposition rate constants for mannitol formulations were sensitive to a ten-degree increase in temperature, particularly from 50°C to 60°C (Fig. 6a). When mannitol was used as a formulation excipient, increasing the temperature from 50°C to 60°C enhanced the average rate constant of peptide degradation fourfold (Fig. 6a). One plausible explanation for this temperature threshold observed in mannitol/peptide formulations would be the following. At 60°C in a lyophilized two-phase system, the experimental temperature might have approached or exceeded the glass transition temperature, T_g, of the amorphous hexapeptide in a predominantly crystalline mannitol matrix. An increase in temperature induces a transition from a glassy to a rubbery state in the amorphous peptide, resulting in an increase in the molecular mobility of both the peptide and water (21). This increased mobility, which occurs as the operating temperature is near or above T_g, has been shown to be sufficient to allow amorphous solids to readily undergo solid state chemical reactions (22–26). In the lactose/peptide formulations, the lyophilized hexapeptide is intimately mixed (a solid solution) in the lactose matrix after lyophilization to become a single amorphous (glassy) system. As such, the peptide in this matrix can be characterized with one "composite" glass transition temperature, which is probably higher than that of the peptide in the mannitol matrix. Therefore, the stability of the Asp-hexapeptide was not affected within the temperature range of 40° to 60°C in the presence of lactose. This hypothesis assumes that the lyophilized Asp-hexapeptide in the mannitol and lactose matrices is in an amorphous state. The ratio of the peptide to the excipient in the formulation was 1:500. Thus, it was not possible to test the validity of this assumption by using techniques such as X-ray diffraction and thermal methods (DSC, calorimetry) because of their inherent sensitivity limits.

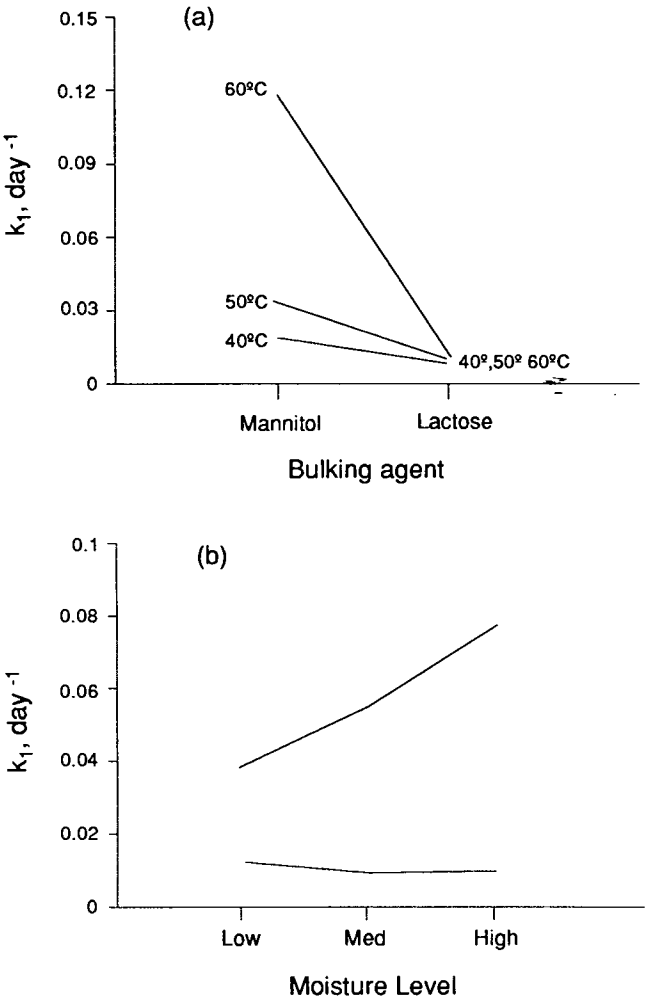


Fig. 6: Effects of two-factor interactions between temperature/bulking agent (panel a) and moisture level/bulking agent (panel b) on the mean rate constant of peptide degradation k_1.

Moisture Level/Bulking Agent

In this study, the mannitol/peptide formulation stability was significantly affected by temperature as well as residual moisture level (Fig. 6b). In contrast, the lactose/peptide formulations remained unaffected by the moisture level as well as the temperature (Fig. 6b). The stability of the Asp-hexapeptide decreased significantly with increasing moisture level in the mannitol matrix. As discussed in the previous section, the Asp-hexapeptide is assumed to be amorphous in both the predominantly crystalline mannitol and amorphous lactose matrices. Since amorphous solids have greater affinity for moisture than crystalline solids, the *effective* amount of water (i.e., activity of water) available for the hexapeptide in the predominantly crystalline matrix (mannitol) is significantly higher than in the amorphous (lactose) system. Water dissolved in an amorphous solid can act as a plasticizer to greatly increase the free volume of the solid by reducing hydrogen bonding between adjoining molecules of the solid, with a corresponding reduction in its glass transition temperature, T_g (27–30). If the amount of water taken up is sufficient to plasticize the local region to a point where $T_g < T$

(the operating temperature), molecular mobility can be high enough to support enhanced chemical reactivity (21).

Relationship Between Solid State Kinetics and Solution Kinetics of the Asp-hexapeptide

One striking difference between the kinetics of degradation in solution (13) and in the solid state was the product distribution. In the solid state, lyophilization successfully suppressed the hydrolysis of the Asp-Gly amide bond (formation of tetrapeptide) and the breakdown of the cyclic intermediate which generates the isoAsp-hexapeptide product, since both of these reactions involve water as a participant. Thus, while the formation of tetrapeptide prevailed over the formation of cyclic imide at acidic pH values in solution, the trend was apparently reversed in the solid state under similar acidic conditions. The cyclic imide was observed to be the major product at all pH values (3.5 to 6.5) except the basic pH (8.0) in solid state. In contrast, the cyclic imide instantaneously degraded in solution to produce the isoAsp-hexapeptide as the predominant product at near neutral to alkaline pH such that the cyclic imide intermediate itself was not detectable under these conditions. More importantly, the maximum stability occurred at pH > 7.0 in solution, whereas the effect of pH was not statistically significant in solid state. However, it was apparent that the cyclic imide intermediate is susceptible to base-catalyzed hydrolysis since its stability decreased with increasing pH in solution as well as in the solid state.

SUMMARY AND CONCLUSIONS

Factorial studies of the type employed here have proven useful in formulation development, since they allow statistical analysis of multiple variables in one single experiment. They also provide relatively rapid identification of significant formulation variables for a given system. In this case, a "designed" experiment successfully delineated the influence of each variable and any two-variable interaction on the chemical stability of the Asp-hexapeptide. The results from these types of experiments may be useful for the optimization of formulations of therapeutic peptides.

This study indicated that the pH of the starting solution did not have a statistically significant impact on the mean degradation rate constant. Increasing the residual moisture content and/or temperature of the lyophilized formulations was deleterious to the chemical stability of the Asp-hexapeptide, especially if mannitol was used as the bulk excipient. Lactose, an amorphous bulking agent, imparted a stabilizing effect to the peptide drug at the moisture level and temperature ranges studied.

The general degradation patterns in the solid state proved to be similar to those observed in solution. There were no new degradation pathways observed in the solid state. However, the product distribution differed significantly from that seen in an aqueous medium. The removal of water by lyophilization suppressed certain hydrolytic pathways, specifically the breakdown of the cyclic anhydride and cyclic imide intermediates, such that the predominant pathway became the intramolecular formation of cyclic imide at all pH values except for pH 8.0. In general, these findings reinforced our proposed reaction mechanisms for the degradation of the Asp-hexapeptide in solution (13).

ACKNOWLEDGMENTS

C.O. acknowledges the financial support provided by the Parenteral Drug Association Pre-Doctoral Fellowship, Abbott Laboratories, and National Institute of General Medical Sciences Biotechnology Traineeship. The authors also acknowledge the following individuals from Abbott Laboratories for their invaluable contributions to the project: Dr. Madhup Dhaon, Dr. John Quick, Jerry Sutherland, Dr. Alex Puko, Brian Moon, Mary Jo Leveque, and Karen Papp.

This paper was presented at the Seventh Annual Meeting of the American Association of Pharmaceutical Scientists, San Antonio, TX, 1992.

REFERENCES

1. J. Geigert. Overview of the stability and handling of recombinant protein drugs. *J. Parenter. Sci. Tech.* 43:220–224 (1989).
2. L. E. Kirsch, R. M. Molloy, M. Debono, P. Baker, and K. Farid. Kinetics of the aspartyl transpeptidation of daptomycin, a novel lipopeptide antibiotic. *Pharm. Res.* 6:387–393 (1989).
3. B. A. Johnson, J. M. Shirokawa, K. S. Hancock, M. W. Spellman, L. J. Basa, and D. W. Aswad. Formation of isoaspartate at two distinct sites during in vitro aging of human growth hormone. *J. Biol. Chem.* 264:14262–14271 (1989).
4. J. Bongers, E. P. Heimer, T. Lambros, Y. E. Pan, R. M. Campbell, and A. M. Felix. Degradation of aspartic acid and asparagine residues in human growth hormone-releasing factor. *Int. J. Peptide Protein Res.* 39:364–374 (1992).
5. K. Patel and R. T. Borchardt. Chemical pathways of peptide degradation. II. Kinetics of deamidation of an asparaginyl residue in a model hexapeptide. *Pharm. Res.* 7:703–711 (1990).
6. K. Patel and R. T. Borchardt. Chemical pathways of peptide degradation. III. Effect of primary sequence on the pathways of deamidation of asparaginyl residues in hexapeptides. *Pharm. Res.* 7:787–793 (1990).
7. F. Araki, H. Nakamura, N. Nojima, K. Tsukumo, and S. Sakamoto. Stability of recombinant human epidermal growth factor in various solutions. *Chem. Pharm. Bull.* 37:404–406 (1989).
8. H. Yamada, T. Ueda, R. Kuroki, T. Fukumura, T. Yasukochi, T. Hirabayashi, K. Fujita, and T. Imoto. Isolation and characterization of 101-β-lysozyme that possesses the β-aspartyl sequence at aspartic acid-101. *Biochemistry* 24:7953–7959 (1985).
9. B. N. Violand, M. R. Schlittler, P. C. Toren, and N. R. Siegel. Formation of isoaspartate 99 in bovine and porcine somatotropins. *J. Protein Chem.* 9:109–117 (1990).
10. A. R. Friedman, A. K. Ichhpurani, D. M. Brown, R. M. Hillman, L. F. Krabill, R. A. Martin, H. A. Zurcher-Neely, and D. M. Guido. Degradation of growth hormone releasing factor analogs in neutral aqueous solution is related to deamidation of asparagine residues. *Int. J. Peptide Protein Res.* 37:14–20 (1991).
11. M. J. Humphrey and P. S. Kingrose. Peptides and related drugs: A review of their absorption, metabolism, and excretion. *Drug Metab. Rev.* 17:283–310 (1986).
12. D. W. Mendenhall. Stability of parenterals. *Drug Dev. Ind. Pharm.* 10(8/9):1297 (1984).
13. C. Oliyai and R. T. Borchardt. Chemical pathways of peptide degradation. IV. Pathways, kinetics, and mechanism of degradation of an aspartyl residue in a model hexapeptide. *Pharm. Res.* 10:95–102 (1993).
14. T. Oguchi, K. Terada, K. Yamamoto, and Y. J. Nakai. Study of freeze-drying in drug-additive binary system. *J. Pharmacobio. Dyn.* 9:s–10 (1986).
15. J. T. Carstensen. Stability of solids and solid dosage form. *J. Pharm. Sci.* 63:1–14 (1974).
16. J. T. Carstensen. Effects of moisture on the stability of solid dosage forms. *Drug Dev. Ind.* 14:1927–1969 (1988).

17. T. R. Kovalcik and J. K. Guillory. The stability of cyclophosphamide in lyophilized cakes: Part I. Mannitol, lactose, and sodium bicarbonate as excipients. *J. Parenter. Sci. Technol.* **42**:29–37 (1988).
18. M. J. Pikal, K. M. Dellerman, M. L. Roy, and R. M. Riggin. The effects of formulation variables on the stability of freeze-dried human growth hormone. *Pharm. Res.* **8**:427–436 (1991).
19. R. G. Strickley, G. C. Visor, L. Lin, and L. Gu. An unexpected pH effect on the stability of moexipril lyophilized powder. *Pharm. Res.* **6**:971–975 (1989).
20. R. Pearlman and T. Nguyen. Pharmaceutics of protein drugs. *J. Pharm. Pharmacol.* **44**:178–185 (1992).
21. C. Ahlneck and G. Zografi. The molecular basis of moisture effects on the physical and chemical stability of drugs in the solid state. *Int. J. Pharm.* **62**:87–95 (1990).
22. M. J. Pikal, A. L. Lukes, and J. E. Lang. Thermal decomposition of amorphous beta lactam antibacterials. *J. Pharm. Sci.* **66**:1312–1316 (1977).
23. R. Hüttenrauch. Modification of starting materials to improve tabletting properties. *Pharm. Ind.* **45**:435–440 (1983).
24. R. Hüttenrauch. Fundamentals of pharmaceutics. *Acta Pharm. Technol.* **34**:1–10 (1988).
25. J. A. Hersey and I. Krycer. Biopharmaceutical implications of technological change. *Int. J. Pharm. Technol. & Prod. Manuf.* **1**:18–21 (1980).
26. R. Huttenrauch, S. Fricke, and P. Zielke. Mechanical activation of pharmaceutical systems. *Pharm. Res.* **2**:302–306 (1985).
27. F. Franks. *Water—A Comprehensive Treatise,* Plenum Press, New York, 1982.
28. H. Levine and L. Slade. Water as a plasticizer: Physicochemical aspects of low-moisture polymeric systems. In F. Franks (ed.), *Water Science Reviews,* Cambridge University Press, Cambridge, 1987, pp. 79–185.
29. H. Levine and L. Slade. Thermomechanical properties of small carbohydrate-water glasses and "rubbers". *J. Chem. Soc. Faraday Trans.* **84**:2619–2633 (1988).
30. L. Slade and H. Levine. Non-equilibrium behaviors of small carbohydrate-water systems. *Pure Appl. Chem.* **60**:1841–1864 (1988).