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Short communication

Factors affecting cleavage at aspartic residues in model decapeptides

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ABSTRACT

The aqueous stability of four decapeptides was investigated in an acetate buffer at different pHs (4.0–5.5) and temperatures (25, 40, and 60 °C). The four decapeptides share the following common sequence—Tyr-Ala-Arg-**Asp-Aaa**-Pro-Leu-Gly-Tyr-Thr, where Aaa represents Gln, Pro, Lys, or Leu. The major degradation pathway was found to be the cleavage at Asp-Aaa. The degradation process fits well the first-order kinetics. The cleavage of the decapeptide containing Asp-Pro was faster than that of other three decapeptides. A strong pH dependence of cleavage was observed for all decapeptides, especially when pH was <5. Three out of four decapeptides showed a clear Arrhenius temperature dependency whereas Asp-Pro-containing peptide did not.

1. Introduction

It is well known that proteins as potential pharmaceuticals are far more challenging for development than small-molecule drugs due to their chemical and physical instabilities. Unlike small molecules, proteins possess not only primary but also higher order structures. Any structural modifications could potentially affect their therapeutic efficacy and/or immunogenicity. Thus, it is important to understand their degradation pathways in order to design a rational formulation and/or dosage form to minimize the degradation.

Since multiple degradation pathways are often observed during protein preparations and storage, development of a protein formulation and/or dosage form is frequently the result of balancing these multiple degradation pathways.

Common chemical degradation pathways in proteins include deamidation, oxidation, and bond cleavage (hydrolysis). Among them, hydrolysis has not been widely studied in details, partly due to the complex nature of proteins. Nevertheless, literature data indicate that the hydrolytic events can easily occur at solvent-exposed Asp or Asn (following deamidation) residues in proteins, such as rhM-CSF [1], bFGF [2], and insulin [3]. Given the prevalence and severity of this degradation pathway, it seems necessary to understand the factors affecting the rate of hydrolysis under different conditions, so that a protein drug can be formulated with a minimal rate of hydrolysis.

However, due to the complexity of protein structures and sophisticated analytical tools for charactering the structural changes in proteins, direct investigation of factors affecting the hydrolytic stability of a protein molecule is very difficult. Rather, the hydrolytic stability of certain peptide bonds can be probed easily with model peptides. With such a peptide model, many chemical degradation pathways have been examined in detail, including Asn deamidation under the influences of salts, buffers, ionic strength and solvents [4–7], Asp isomerization [8,9], deamidation of Asn in a solid state [10,11], hydrolysis of succinimide [12] and His or Met oxidation [13–15].

Recently, we observed a major hydrolytic degradation at the Asp-X bond in a protein during storage. We are not aware of any literature data showing such a major hydrolytic event in protein. We suspect that the adjacent amino acid strongly affect the hydrolytic rate. In order to understand the effect of adjacent amino acid on the rate of hydrolysis, we synthesized four decapeptides and evaluated their hydrolytic stability in an aqueous solution. The four decapeptides share the following common sequence—Tyr-Ala-Arg-**Asp-Aaa**-Pro-Leu-Gly-Tyr-Thr, where Aaa represents Gln, Pro, Lys, or Leu. Other factors affecting peptide bond stability at Asp residues were also evaluated, including effect of pHs (4.0–5.5) and temperatures (25, 40, and 60 °C). Any pH higher than 5.5 was not investigated in this study because the original protein drug candidate was found to undergo deamidation rapidly at pH ≥6. The major degradation pathway of these model peptides was confirmed to be the cleavage at Asp-Aaa, which fits well the first-order kinetics. The cleavage of the decapeptide containing Asp-Pro was faster than that of other three decapeptides. A strong pH dependence of cleavage was observed for all decapeptides. Three out of four decapeptide

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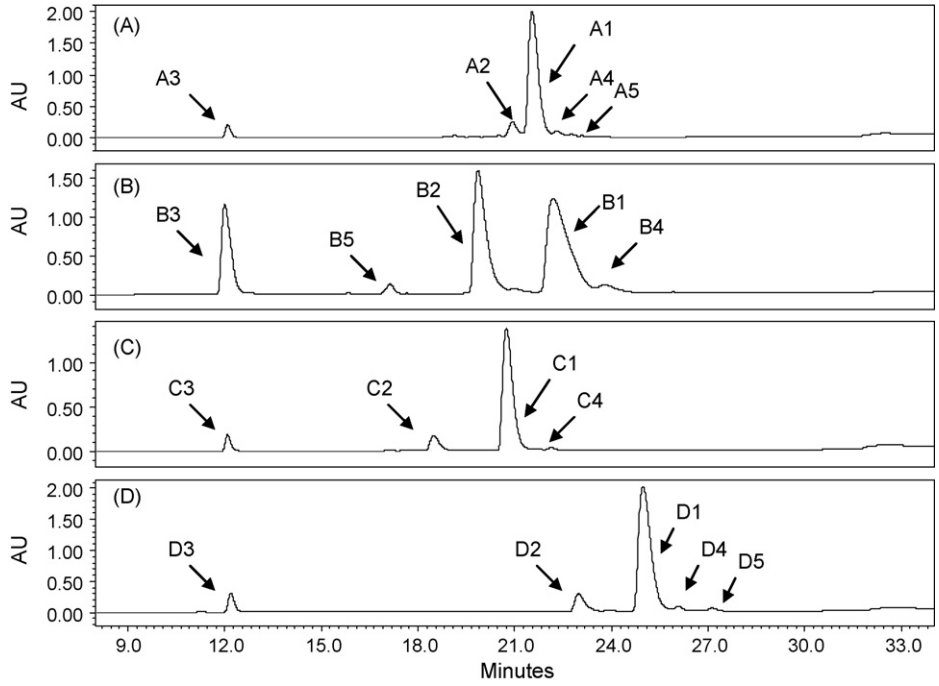

Fig. 1. Reversed HPLC analysis profiles of stability samples for the four model peptides. Samples were incubated at pH 4.5 and 40 °C for 8 weeks (peptides A, C and D) and 2 weeks (peptide B).

degradation followed a clear Arrhenius temperature dependency whereas Asp-Pro-containing peptide did not.

2. Materials and methods

2.1. Materials

Four peptides, Tyr-Ala-Arg-**Asp-Gln**-Pro-Leu-Gly-Tyr-Thr (A), Tyr-Ala-Arg-**Asp-Pro**-Pro-Leu-Gly-Tyr-Thr (B), Tyr-Ala-Arg-**Asp-Lys**-Pro-Leu-Gly-Tyr-Thr (C), and Tyr-Ala-Arg-**Asp-Leu**-Pro-Leu-Gly-Tyr-Thr (D) as lyophilized powder were provided by Pfizer Global Research and Development, Chemistry group with purity greater than 95%. Sodium acetate trihydrate, sodium chloride, Polysorbate 80 (PS80) are NF, EP, JP grade. Trifluoroacetic acid is HPLC grade. They were purchased from J.T. Baker. Acetic acid glacial extra pure was from EM Science. Acetonitrile, HPLC grade, was from Sigma–Aldrich. Water-for-injection was from Baxter. Deionized water $\geq$18 MΩ cm was generated using Milli-Q water purification system from Millipore. All materials were used as-received.

2.2. Sample preparation and stability studies

Formulation buffers with pH 5.5, 5.0, 4.5 and 4.0 were prepared by mixing appropriate amounts of 20 mM sodium acetate with 20 mM acetic acid. To mimic the composition of the protein formulation, sodium chloride and polysorbate 80 were also added to the buffer to a final concentration of 0.82% and 0.02% (w/v), respectively.

Stability samples were prepared by dissolving 4 mg of each peptide in 2 mL of the above formulation buffers with pH from 4.0 to 5.5. The pH of the solutions was verified after the peptides were completely dissolved. These solutions were aliquot into type I glass vials, capped, sealed and placed in 25, 40, and 60 °C stability chambers for up to 10 weeks. Samples were pulled at 2-, 4-, 6-, 8-, and 10-week intervals for analysis.

2.3. HPLC and LC/MS analysis

High-performance liquid chromatographic (HPLC) analysis was performed using Agilent 1100 series (Agilent Technologies Inc., California, USA). Five microliters of each peptide solution was injected to a Zobax C18 reversed-phase column (2.1 mm × 150 mm, 5 μm) and eluted at a flow rate 0.2 mL/min with mobile phase A of 0.1% TFA in water and mobile phase B of 0.085% TFA in acetonitrile. The column temperature was kept at ambient. A multi-step gradient was applied as following: 0–25% B in 20 min, 25–40% B in 7 min, 40–100% B in 8 min, and 100–0% B in 5 min. Elution profile was monitored by UV at 214 nm. The HPLC was also coupled with a Q-ToF micro™ mass spectrometer from Waters-Micromass, previously calibrated with myoglobin. The mass spectra were acquired and analyzed using MassLynx 4.0 software.

3. Results and discussion

3.1. Chromatographic separation and identification of degradation products

Chromatographic analysis of aged stability samples using reversed-phase HPLC with a gradient elution is shown in Fig. 1. Peaks A1, B1, C1, and D1 are the intact decapeptides. Although Asp can also form *iso*-Asp degradation product, this method does not differentiate these two isomers. During storage, each decapeptide generated two major and one or two minor degradation products in addition to the parent peptide. All major degradation products were baseline separated from their parent peptides.

Online monitoring of each peaks with mass spectrometer during the HPLC elution indicated that almost all the peaks are derived from the parent peptides. One major degradation product was present in all the stability samples (peaks A3, B3, C3, and D3) and was identified to have a *m*/*z* value of 524.2. This value corresponds to the cleaved tetrapeptide from the N-terminus—Tyr-Ala-Arg-**Asp**. The peaks A2, B2, C2 and D2 have *m*/*z* of 678.3, 647.2, 678.4 and 663.3, and correspond to the remaining amino acid sequence of the

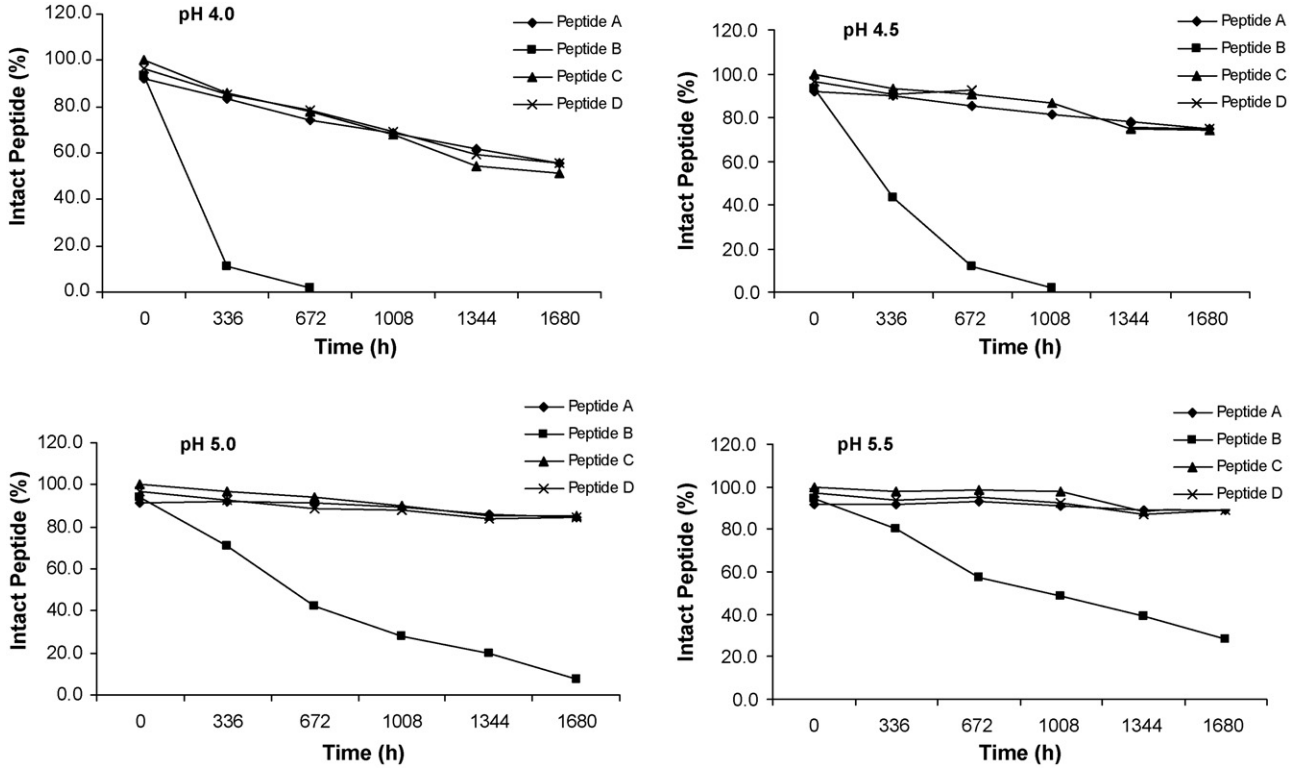

Fig. 2. Primary sequence impact on the rate of cleavage at pHs from 4.0 to 5.5. All the samples used for this analysis were stored at 40 °C for up to 10 weeks. Samples were withdrawn every 2 weeks for analysis.

four peptides—**Gln**-Pro-Leu-Gly-Tyr-Thr (peptide A), **Pro**-Pro-Leu-Gly-Tyr-Thr (peptide B), **Lys**-Pro-Leu-Gly-Tyr-Thr (peptide C) and **Leu**-Pro-Leu-Gly-Tyr-Thr (peptide D), respectively. These indicated that all four decapetides have undergone cleavage at the C-terminal side of Asp-4.

In addition to the major degradation products, minor products were also identified. In peptide A stability sample, two small additional peaks (A4 and A5) were found to have *m*/*z* of 1020.5 and 653.3 and correspond to the peptide A losing N-terminal Tyr (Ala-Arg-**Asp-Gln-**Pro-Leu-Gly-Tyr-Thr) and Tyr-Ala-Arg-**Asp-Gln**. In peptide C stability sample, peak C4 has *m*/*z* of 1165.6 and corresponds to hydrated peptide C (Tyr-Ala-Arg-**Asp-Lys**-Pro-Leu-Gly-Tyr-Thr*H_2O). In peptide D stability sample, peaks D4 and D5 have *m*/*z* of 1062.5 and 1150.6 and correspond to peptide D losing C-terminal Thr (Tyr-Ala-Arg-**Asp-Leu**-Pro-Leu-Gly-Tyr) and hydrated peptide D (Tyr-Ala-Arg-**Asp-Leu**-Pro-Leu-Gly-Tyr-Thr*H_2O). The minor peaks were seen in peptide B—B4 and B5. B4 might be an artifact because it was not picked up by mass spectrometer. B5 has *m*/*z* of 1029, which does not match any of the components from the peptide B. Further investigation is on going. These minor components constitute less than 5% of the total degradation product, respectively for peptides A–D and their abundance did not change significantly over time. Therefore, the cleavage at the peptide bond of Asp-Aaa is the major degradation pathway for all four decapeptides under the conditions investigated.

The susceptibility to hydrolysis of Asp-Aaa peptide bond observed in this study has been reported by Kirsch et al. who investigated the degradation pathways of glucagon, a 29 amino acid peptide used for emergency treatment of insulin-induced hypoglycemia [16–18]. Glucagon contains three Asp and all of them were found to undergo cleavage at the C-terminal site of Asp peptide bond simultaneously under pH 2.5 and temperature stress condition of 60 °C [16–18]. The amino acids that followed Asp in glucagon are Tyr (Asp9-Tyr10), Ser (Asp15-Ser16) and Phe (Asp21-Phe22). Similarly, Oliyali and Borchardt [11] also found that a hexapeptide with amino acid of Val-Tyr-Pro-Asp-Gly-Ala under highly acidic condition (pH 0.3–3.0) decomposed predominantly via intramolecular cleavage of the Asp-Gly amide bond, forming fragment 1 Val-Tyr-Pro-Asp and fragment 2 Gly-Ala. Furthermore, Tsuda et al. [19] observed that under storage condition of 60 °C and pH lower than 4.0, the intestinal hormone secretin, which contains two Asp residues at positions 3 and 15 degraded into fragment peptides as a result of cleavage reaction at positions Asp3-Gly4 and Asp15-Gly16. The results from the current study, along with those aforementioned in the literature, suggest that Asp-Aaa bond is more susceptible to cleavage than other peptide bonds in solution phase.

Instability of Asp-Aaa bond in gas phase has also been reported. Martin and co-workers [20] investigated *E. coli* thioredoxin and recombinant human macrophage colony stimulating factor (rhM-CSF) cleavage in gas phase under tightly controlled condition using MALD-ToF mass spectrometer. The *E. coli* thioredoxin (108 amino acids) contains 11 Asp, followed by Lys, Asp, Ser, Thr, Leu, Gly, Phe, Glu, and Gln; whereas rhM-CSF (302 amino acids) contains 2 Asp, both followed by Pro. Their investigation showed that 10 of 11 Asp-Aaa bonds in *E. coli* thioredoxin and all 2 Asp-Pro in rhM-CSF undergone cleavages. Fragments from the Asp-Pro cleavages are the most abundant while distribution of other fragment ions is similar. Even though the studies performed by Martin et al. were in the gas phase, it provides valuable information regarding the instability of Asp-Aaa bond, especially the Asp-Pro in proteins susceptible to the cleavage.

3.2. Degradation kinetics and rate comparison

The storage stability of the four model peptides was evaluated at different pHs for up to 10 weeks. The change in intact peptide concentration with time at different pHs, as monitored by reversed-phase HPLC, is shown in Fig. 2. The curves fitted well the first-order degradation kinetics (linear relationship between ln *C* and time) as shown in Fig. 3. The selected apparent rate constant derived from

Table 1
Apparent degradation rate constant at pH 4.5.

Peptide	T (°C)	k ($\times 10^4$ h^{-1})	T (°C)	k ($\times 10^4$ h^{-1})	T (°C)	k ($\times 10^4$ h^{-1})
A	25	0.22	40	1.26	60	7.45
B	25	4.70	40	38.0	60	79.6
C	25	0.62	40	1.87	60	9.70
D	25	0.40	40	1.68	60	8.80

the slope of these curves for each peptide is listed in Table 1. The first-order degradation kinetics was also observed by others for a Asp-containing oligopeptide secretin in aqueous solution [19].

The relative rates of peptide cleavage at specified condition vary depending on the amino acid following Asp. The Asp-Aaa cleavage of peptides A, C and D occurred at comparable rates, whereas cleavage of peptide B was much more rapid at all pHs. The rate difference was over 11 times at pH 5.5 and 40 °C and 14 times at pH 4 and 40 °C. The Asp is followed by Pro in peptide B, whereas the Asp is followed by Gln, Lys and Leu in peptides A, C and D, respectively. A brief review of the literatures showed that the labile character of Asp-Pro bonds has been reported by others in solution phase during studies of amino acid sequence of various proteins [21] as well as in gas phase in the study of protein characterization as mentioned above [20].

Several mechanisms have been proposed in the past on the cleavage of Asp-Aaa bonds. Extensive investigation of a Asp-containing hexapeptide by Oliyai and Borchardt [11] using nuclear magnetic resonance (NMR) and kinetic solvent isotope effect techniques indicated that the most likely degradation mechanism, as shown in Fig. 4, involves acid-catalyzed intramolecular nucleophilic attack of the aspartyl carboxylic acid side chain on the $n+1$ peptide bond carbonyl center, resulting in the formation of a cyclic anhydride intermediate. This intermediate undergoes hydrolysis to give the cleaved products. Such cleavage mechanism was also corroborated by Kirsch et al. [17,18] during their investigation of Glucagon degradation pathways.

Although the side chain of aspartic acid participation in the rapid cleavage of Asp-Aaa bonds is evident based on above analysis, the role of proline in the degradation pathway has not been described and should also be considered since Asp-Pro peptide bond is much more labile than Asp-Gln, Asp-Gly and Asp-Leu found in our study and others [20]. It is obvious that the basicity of the secondary amine group in proline is higher than that of the primary amine group, as reflected by its highest pK_a. The proline nitrogen in the peptide bond linkage would have greater basicity than those of other three amino acids and easily undergoes protonation under acidic condition. The increased basicity of the nitrogen in proline relative to nitrogen in other amino acids might help the cleavage reactions in two steps. First, the lone pair of electron on the nitrogen may polarize further the carbonyl C–N bond and make the carbonyl carbon more susceptible to nucleophilic attack. Second, the increased basicity of nitrogen may promote protonation of nitrogen of the intermediate. Thus, Asp-Pro peptide bond has the highest cleavage rate. This is consistent with the mechanism proposed by Martin et al. for gas-phase cleavage of Asp-Pro bond of rhM-CSF [20].

3.3. Impact of formulation pH on the rate of degradation

The pH dependency of the cleavage is clearly seen in Fig. 5. The rate constant for all four peptides investigated increased with decreasing pH. This increase is more dramatic when pH is <5, especially for peptide B. For every 0.5 pH unit decrease, the increase of cleavage rate constant is in the range of 1.4–2.7 times at 40 °C. This supports that the formation of cleavage products is acid catalyzed. As discussed earlier, this pH depen-

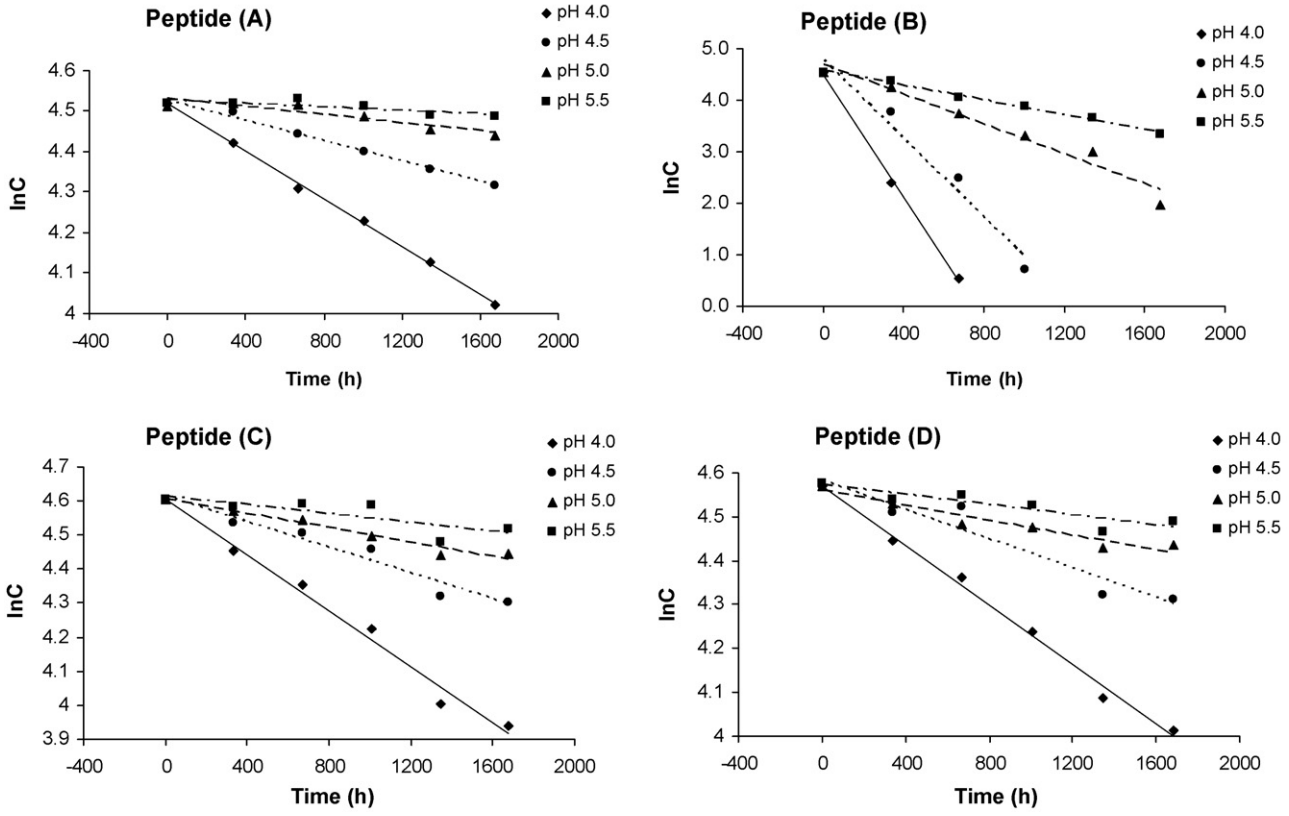

Fig. 3. First-order plot for peptides A–D at pHs 4.0, 4.5, 5.0 and 5.5. All the samples were stored at 40 °C for up to 10 weeks. Samples were withdrawn every 2 weeks for analysis.

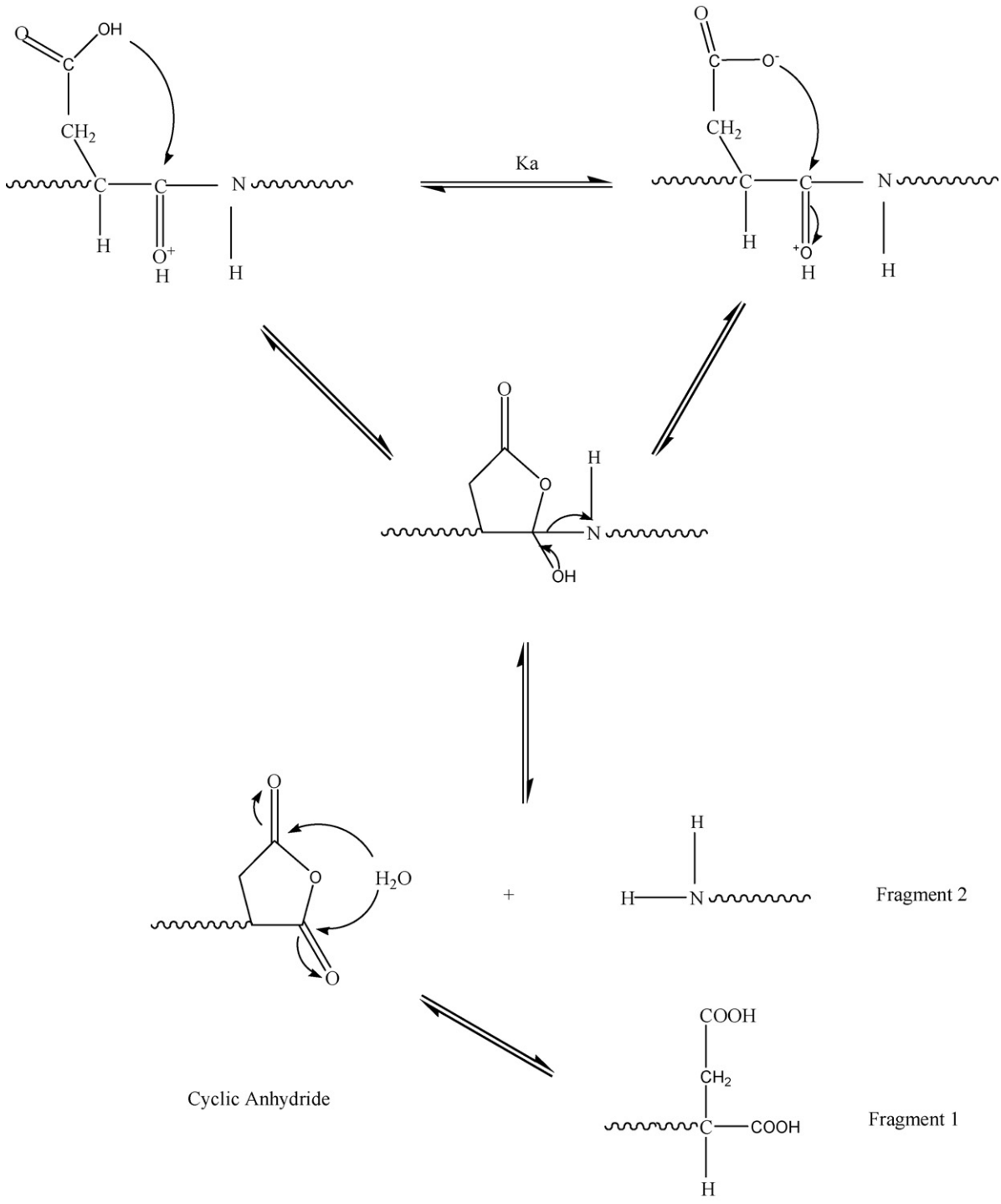

Fig. 4. Asp-Aaa cleavage mechanism proposed by Oliyai et al. [11] involves acid-catalyzed intramolecular nucleophilic attack of the aspartyl carboxylic acid side chain on the $n+1$ peptide bond carbonyl center, resulting in the formation of a cyclic anhydride intermediate. This intermediate undergoes hydrolysis to give the cleaved products.

dency of the cleavage was also observed in glucagon Asp-Aaa peptide bond cleavage reported by Kirsch et al. [16–18] and Asp-containing hexapeptide reported by Oliyai and Borchardt [11]. The cleavage mechanism, as discussed previously, is believed to be acid-catalyzed intramolecular nucleophilic attack of the aspartyl carboxylic acid side chain on Asp-Aaa peptide bond carbonyl center, which results in the formation of a cyclic anhydride intermediate. This intermediate undergoes hydrolysis and yields the cleavage product [18].

Acid-catalyzed hydrolysis can be divided into two categories—general and specific types [22]. General acid–base catalysis is characterized by a unit change in log rate constant with a unit change in pH in the catalytic regions. In this study, the maximum increase in rate constant was 5.4 times when the pH was dropped below 5. It seems that specific acid catalysis (mediated by hydronium ion) would be applicable to the cleavage reaction of these decapeptides. However, other factors need to be considered in interpreting the results. We know that small Asp-containing peptides undergo reversible isomerization through succinimide formation, which is also strongly pH-dependent in the region studied [9]. In addition, although secondary structure of a peptide of this size is generally not present in an aqueous solution, the

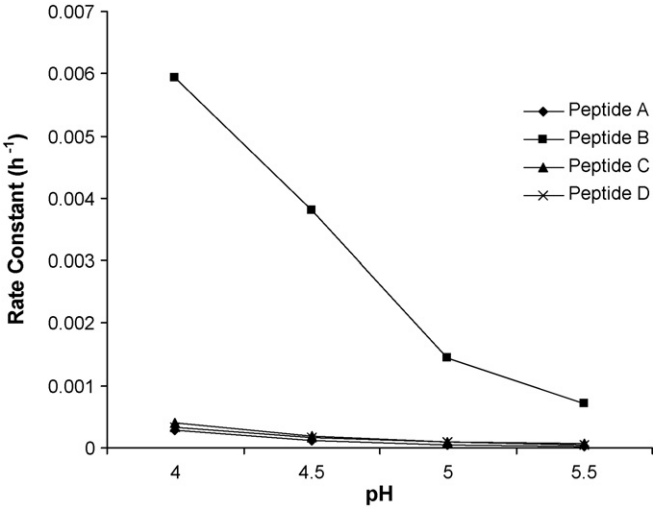

Fig. 5. Profiles of rate constant as function of pH. All the samples were stored at 40 °C for up to 10 weeks. Samples were withdrawn every 2 weeks for analysis.

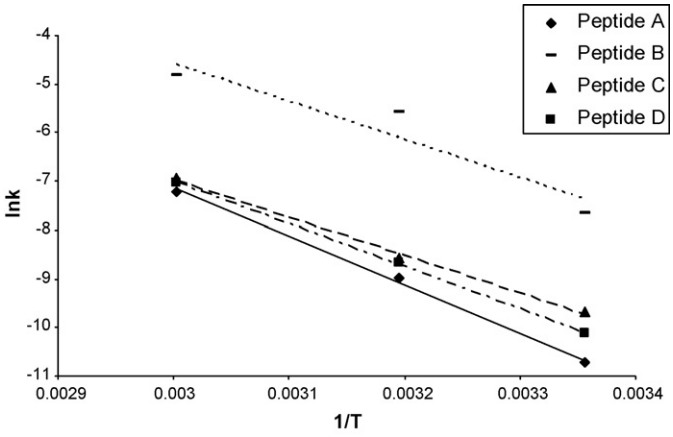

Fig. 6. Arrhenius plot of decapeptide degradation at pH 4.5. All the samples used in this analysis were in pH 4.5 formulation buffer and stored up to 10 weeks. Samples were withdrawn every 2 weeks for analysis.

steric and inhibitory effect of other amino acids on the hydrolytic event cannot be ruled out.

3.4. Effect of temperature on the rate of degradation

The cleavage of the model peptides occurs very rapidly at pH 4.0. In a trial experiment, the model peptides completely degraded at this pH at 60 °C at the second stability sampling point (data not shown). Therefore, the temperature dependency of the storage stability was evaluated at pH 4.5 for the four model peptides. The observed rate constant at different storage temperatures of 25, 40, and 60 °C was shown in Fig. 6. Peptides A, C, and D demonstrated a clear Arrhenius temperature dependency within this temperature range, whereas a large deviation was observed for peptide B. The arch-shaped deviation has been suggested to be due to a change in degradation mechanism with temperature [22]. This might be true for peptide B as a few unknown degradation products were not simply hydrolytic products.

4. Conclusion

The major degradation pathway of the four model peptides in the pH range of 4.0–5.5 in the temperature range of 25–60 °C was found to be the cleavage at Asp-Aaa. The degradation process fits well a first-order kinetics for all four peptides. The cleavage of the decapeptide containing Asp-Pro was the fastest among the four model decapeptides. A strong pH dependence of cleavage was observed for all decapeptides, especially peptide containing Ala-Pro. The degradation was much more rapid at lower pHs, especially when pH was <5. Three model decapeptides showed a clear Arrhenius temperature dependency, whereas Asp-Pro-containing peptide did not, suggesting change of degradation mechanism with temperature.

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