# The Stability and Degradation Pathway of Recombinant Human Parathyroid Hormone: Deamidation of Asparaginyl Residue and Peptide Bond Cleavage at Aspartyl and Asparaginyl Residues

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***Purpose.*** The stability of recombinant human parathyroid hormone (rhPTH) was examined under acidic to alkaline conditions; its degradation pathways were elucidated from resultant products.

***Methods.*** Degradation assay was performed in the pH range 2 to 10 at 40, 50 and 60°C. The approximate molecular mass and pI values of the degradation products were estimated by electrophoresis. FAB-MS peptide mapping and amino acid composition analysis were used to determine these structures. The amount of each respective product was determined by HPLC.

***Results.*** At pH2, eight degradation products were found: 1-30rhPTH, 1-74rhPTH, 1-71rhPTH, 1-56rhPTH, 1-45rhPTH, 46-84rhPTH, 31-84rhPTH and Asp76-rhPTH; these were mainly as a consequence of peptide bond cleavage of the amide bond of Asp. At pH9, five products were found: isoAsp16-rhPTH, Asp16-rhPTH, Asp57-rhPTH, Asp76-rhPTH, 17-84rhPTH; the main degradation pathway was deamidation of Asn via a cyclic imide intermediate. Degradation products resulting from cleavage at Asp were increased in proportion to the extent that pH was lowered below 5. As pH was increased above 5, so were products resulting from deamidation of Asn. Correspondingly, levels of intact rhPTH were at a peak at pH5.

***Conclusions.*** Degradation of rhPTH under acidic conditions predominantly occurs by cleavage at Asp, whereas, above pH5, deamidation of Asn is the more prominent. rhPTH is most stable at pH5.

**KEY WORDS:** human parathyroid hormone; deamidation; cleavage; asparaginyl; aspartyl.

## INTRODUCTION

Human parathyroid hormone (hPTH), a peptide of 84 amino acid residues (1) secreted from the parathyroid gland, is the principle homeostatic regulator of the level of blood calcium through its actions on kidney and bone (2). hPTH and hPTH active fragments are reportedly potentially effective for the treatment of osteoporosis because they induce an anabolic response in bone (3). Since the cloning and expression of the hPTH gene are established (4), studies which address this peptide's therapeutic potential are expected to increase.

When developing bioactive peptides such as hPTH for medical use, consideration of their chemical stability under conditions of isolation or storage are required. Also, information about degradation products and pathways is important in optimizing formulations. The stability of peptides is affected by a number of degradation pathways, the most prominent of which can occur via oxidation of Met (5,6), deamidation of Asn, and cleavages of peptide bond at Asp (7–17).

Within rhPTH, there are five Asn, five Asp and two Met residues; all of these are potential sites for degradation. However, only the degradation through Met oxidation has previously been reported (5,6); to our knowledge, no other degradation pathway has been studied. In this study, therefore, we examined the stability of the intact peptide and identified the respective degradation products at pH levels which range from acid (pH2) to alkaline (pH10).

For the identification of degradation products, enzyme digestion and subsequent fast atom bombardment mass spectrometry (FAB-MS) were used. We previously reported that when intact rhPTH is digested with either trypsin or V-8 protease, six and eight peptide fragments are produced, respectively, each with a characteristic molecular weight that corresponds to its primary structure (18). Moreover, FAB-MS accurately detected an increase of one mass unit caused by conversion of Asn to Asp. Taken together, in combination with V-8 protease digestion, it is possible to make a distinction between Asp and iso-Asp residues (8,18).

## MATERIALS AND METHODS

### Production and Purification of rhPTH and rhPTH Variants

Using the method of Gardella (4), rhPTH and its authentic degradation products were produced in *E. coli* as respective fusion proteins with human growth hormone linked by a recognition sequence for thrombin; intact and variant rhPTH were then released by cleavage with thrombin. The products were subsequently purified by reversed-phase HPLC (RP-HPLC).

### Degradation of rhPTH

To examine the stability of peptides at each respective pH, 220 μg/ml rhPTH was incubated in 20mM borate-citrate-phosphate universal buffer, pH2-10, for 72h at 40, 50 and 60°C. For identification of the degradation products, 1 mg/ml rhPTH was incubated either at pH9 for 43h at 60°C or at pH2 for 29h at 60°C.

### Enzyme Digestion

Either rhPTH or its degradation products were dissolved in 1% ammonium bicarbonate (pH7.8) to a concentration of 1mg/ml. TPCK-trypsin (COOPER Biomedical, Malvern, PA, USA) or *Staphylococcus aureus* V-8 protease (Boehringer Mannheim, Mannheim, Germany) was added and incubated at 37°C for 16h at a substrate–enzyme ratio of 50:1.

### High-Performance Liquid Chromatography

RP-HPLC was performed with the Waters HPLC system (Waters Japan, Tokyo, Japan), which consisted of a pump (type 600), an automatic sample injector (WISP 712), a UV detector

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(type 490), a data module (type 740), and a YMC-Pack ODS A column (150 × 6.0 mm i.d., YMC, Kyoto, Japan). Analysis of stability, evaluation of the amount of degradation products produced under different individual conditions of pH, and, isolation of degradation products at pH9, were performed under the following conditions. Solvent A was 0.1% trifluoroacetic acid (TFA) in water, and solvent B was 0.1% TFA in acetonitrile/water (60/40, v/v); the column was equilibrated at 60% A/40% B, eluted with 60% A/40% B to 50%A/50% B for 10 min and then with 50%A/50%B to 40%A/60% B over 40 min at a flow rate of 1.0 ml/min. A detection wavelength of 215 nm and column temperature of 50°C were used. To isolate degraded products at pH2, the same HPLC conditions were used except for that of the gradient; the initial concentration of 70% A/30% B was changed to 50%A/50%B for 29 min, then to 40% A/60% B for 40 min.

Cation exchange HPLC (CE-HPLC) was also used to isolate degradation products. A TSKgel CM-2SW column (250 × 4.6 mm i.d., Tosoh, Tokyo, Japan) was fitted to the same HPLC system as above and separation was carried out at room temperature. Solvent A was acetonitrile/10 mM sodium phosphate buffer pH6.0 (20/80, v/v), and solvent B was solvent A containing 0.5M sodium chloride. Samples were eluted with a linear gradient of 100%A to 50% A/50% B for 50 min at 1 ml/min.

To fractionate peptide fragments produced by trypsin digestion, another RP-HPLC system, a GILSON Microanalytical system (GILSON, Villiers-le-Bel, France) equipped with Vydac 218TP510 ODS column (250 × 2.1 mm i.d., Vydac, Hesperia, CA, USA), was used. The sample was eluted by a linear gradient of 0 to 40% acetonitrile containing 0.1% TFA for 80 min at 0.2ml/min. Eluates were monitored at 220 nm, and fractionated peptides were subjected to peptide sequence analysis.

### Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a PhastSystem with PhastGel High Density (Pharmacia Biotech, Tokyo, Japan). Gels were visualized by silver staining. Isoelectric focusing (IEF) in a 5% acrylamide gel containing 5% ampholine (pH3–10) was also performed on a PhastSystem. Gels were stained with Coomassie Brilliant Blue R-250.

### Mass Spectrometry

Mass spectra were obtained with a 70-250SEQ double focusing mass spectrometer (Micromass, Manchester, UK), equipped with a xenon FAB ion source. A resolving power of 2000 was employed at an acceleration potential of 8kV. The matrix used was thioglycerol containing a small amount of TFA. Between 1 and 2 μl of a sample was added to the matrix.

### Amino Acid Composition and Peptide Sequence Analysis

To analyze amino acid composition, the degraded products (approximately 1nmole each) were hydrolyzed in 6M hydrochloric acid containing 1% phenol for 24h at 110°C. Subsequent derivation to phenylthiocarbamyl amino acid and quantitative analyses were performed with a PICO-TAG amino acid composition analysis system (Waters Japan, Tokyo, Japan).

Automated peptide sequence analysis was carried out with a model 476A protein sequencer (Applied Biosystems, Tokyo, Japan).

## RESULTS

### pH-Stability of rhPTH

Aliquots of rhPTH were individually incubated under different pH conditions at 40, 50 and 60°C for 72h and subsequently analyzed by RP-HPLC. Fig. 1(a) shows the residual ratio of peak areas with the same retention time as intact rhPTH. The ratio decreased with the rise of temperature. The curves obtained show a maximum at pH5 at all temperatures examined.

In Fig. 2, typical RP-HPLC profiles which were incubated at pH2, 60°C and pH9, 60°C are shown. At pH2, eight degradation products which are referred to by the prefix "A" followed by a designated number (A1–A8) were found (Fig. 2(a)). At pH9, five products referred to with the prefix B followed by a designated number (B1–B5) were found (Fig. 2(b)). Some of the respective quantities at each pH at 60°C are shown in Fig. 1(b). The degraded products A1, A3–A5 were observed below pH5, whereas above this pH, B1-B3 were observed. This indicates that rhPTH is stable at pH5. Similar degradation profiles were observed in the HPLC analysis at 40°C and 50°C. Thus, the identification of these degradation products, which were generated under acidic and alkaline conditions, was carried out at pH2, 60°C and pH9, 60°C as follows.

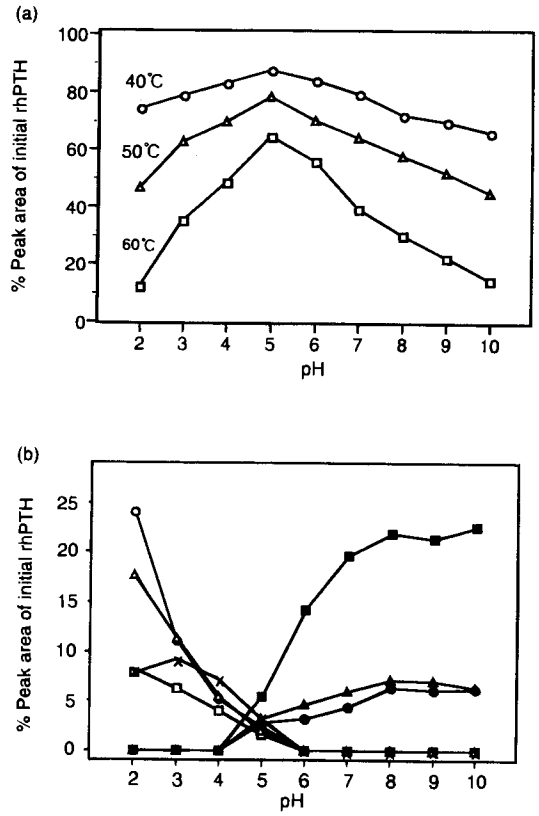


**Fig. 1.** Profiles of pH-stability and appearance of intact and degradation products of rhPTH. (a) profile of a component equivalent to intact rhPTH, (b) appearance of some degradation products at 60°C. Degraded product A1 (○), A3 (□), A4 (X), A5 (△), B1 (●), B2 (■), and B3 (▲).

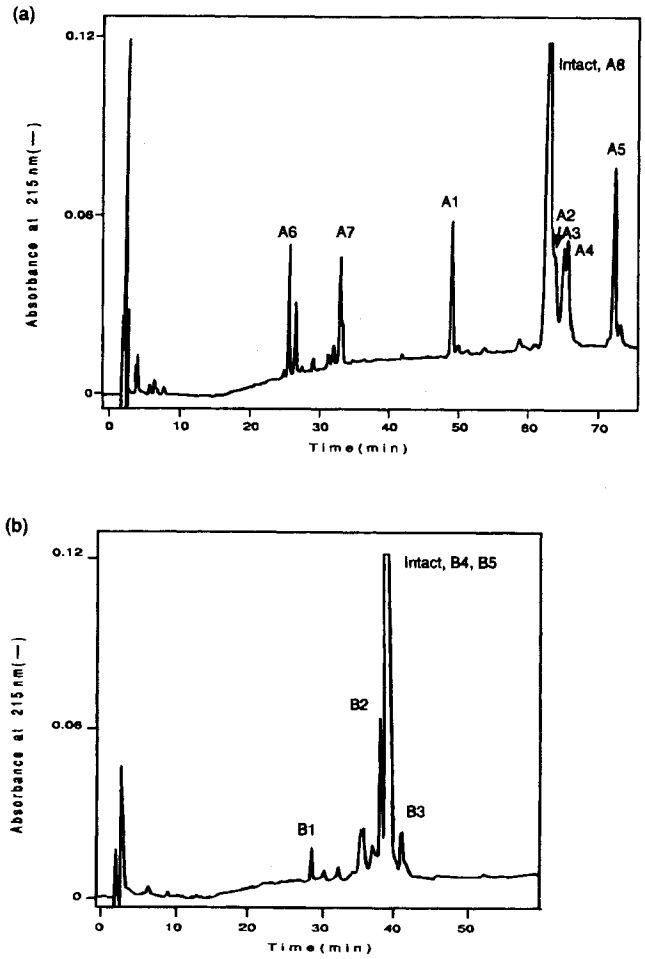


**Fig. 2.** HPLC chromatograms for the degradation of rhPTH at (a) pH2, 60°C and (b) pH9, 60°C.

## Isolation and Determination of Degradation Products at pH9

rhPTH was incubated at pH9 for 43h at 60°C and its degradation products were isolated by a combination of CE-HPLC and RP-HPLC. Five degradation products, B1-B5, were eluted according to their respective retention times, as shown in Fig. 2(b). Although degradation products B4, B5 and intact rhPTH were inseparable by RP-HPLC (Fig. 2(b)), separation was achieved through CE-HPLC.

As estimated by SDS-PAGE, B1 had a molecular mass of approximately 7.5 kDa, whereas, all other degradation products appeared similar in size to intact rhPTH (Table I). The isoelectric points (pI) of products B2-B5 were all at 8.1 compared to 9.0 for the intact peptide (Table I). From these results, we predicted that the product B1 occurred as a consequence of cleavage, and B2-B5 were deamidated forms of rhPTH.

To confirm their structure, fractionated products were digested by V-8 protease or trypsin and subsequently analyzed by FAB-MS, respectively. A typical digestion profile is shown in Fig. 3. V-8 protease digested the amide bond of Gly38 and Gly47, as previously reported (18). Analysis of the degradation product B1 revealed a peptide fragment consisting of amino acids 17–22 but without the preceding sequence, 1–6. Other peptide fragments were consistent with intact rhPTH (Table II(a)). In addition, the calculated molecular weight of 17–84 rhPTH was 7622.7 (average mass) which was similar to the value derived from SDS-PAGE. B1 was therefore, 17–84 rhPTH, as a consequence of cleavage of the amide bond of Asn16.

**Table I.** Estimated Molecular Mass and pI Value for Degradation Products of rhPTH by SDS-PAGE and IEF

| | Molecular mass (kDa) | pI |
|---|---|---|
| Intact | 10 | 9.0 |
| A1 | 2.2 | 8.2 |
| A2 | 7.5 | 7.2 |
| A3 | 7.0 | 7.2 |
| A4 | 6.5 | 9.4 |
| A5 | 3.0 | 8.1 |
| A6 | 4.5 | 8.5 |
| A7 | 6.0 | 8.4 |
| A8 | 10 | 8.1 |
| B1 | 7.5 | 9.0 |
| B2 | 10 | 8.1 |
| B3 | 10 | 8.1 |
| B4 | 10 | 8.1 |
| B5 | 10 | 8.1 |

After trypsin digestion of the degradation products B2 and B3, an increase of one mass unit was observed in a peptide fragment consisting of residues 14–20 (Table II(b)). Similarly, a difference of one mass unit was found after V-8 protease digestion for peptide fragments consisting of residues 5–19 and 5–22 (Table II(a)). Furthermore, V-8 protease digestion revealed two new peptide fragments for B3: V9 consisted of residues 5–16 and V10 consisted of residues 17–22. V9 had one mass unit of increased molecular weight than that calculated for the intact peptide. These data strongly suggests that B3 was a deamidated form of rhPTH where Asn16 had been converted into Asp; this allows V-8 protease digestion at this position. In the case of B2, it was indicated that the Asnl6 was converted

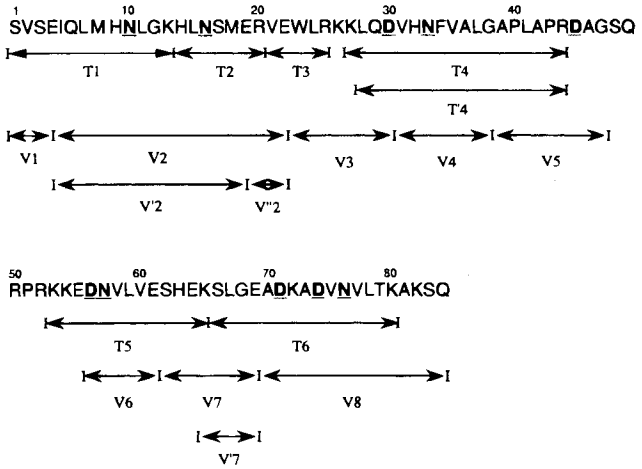


**Fig. 3.** Amino acid sequence of intact rhPTH. Tm and Vm show tryptic and V-8 protease peptides observed in FAB-MS, respectively. Positions of Asn (N) and Asp (D) are underlined.

**Table II.** Mass Values of Enzymatic Digests of Degradation Products of rhPTH

(a) V-8 Protease Digests

| Peptide | Position | Theoretical mass | Observed mass value ($MH^+$) | | | |
|---|---|---|---|---|---|---|
| | | | Intact | B1 | B2 | B3 |
| V1 | 1–4 | 421.2 | 421.2 | n.d.[a] | 421.2 | 421.2 |
| V2 | 5–22 | 2149.1 | 2149.2 | n.d.[a] | **2150.0**[b] | **2150.0**[b] |
| V'2 | 5–19 | 1764.9 | 1765.0 | n.d.[a] | **1766.1**[b] | **1766.0**[b] |
| V"2 | 20–22 | 403.2 | 403.2 | 403.2 | 403.2 | 403.2 |
| V3 | 23–30 | 1086.6 | 1086.7 | 1086.8 | 1086.9 | 1086.9 |
| V4 | 31–38 | 856.5 | 856.4 | 856.6 | 856.6 | 856.6 |
| V5 | 39–47 | 867.5 | 867.4 | 867.5 | 867.5 | 867.5 |
| V6 | 56–61 | 688.3 | 688.3 | 688.4 | 688.4 | 688.5 |
| V7 | 62–69 | 886.4 | 886.4 | 886.5 | 886.5 | 886.5 |
| V'7 | 65–69 | 533.3 | 533.2 | 533.3 | 533.3 | 533.4 |
| V8 | 70–84 | 1587.9 | 1587.9 | 1588.1 | 1588.1 | 1588.2 |
| V9 | 5–16 | 1417.8 | n.d.[a] | n.d.[a] | n.d.[a] | **1419.1**[b] |
| V10 | 17–22 | 750.4 | n.d.[a] | 750.4 | n.d.[a] | 750.5 |

(b) Tryptic Digests

| Peptide | Position | Theoretical mass | Observed mass value ($MH^+$) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Intact | B2 | B3 | B4 | B5 | A8 |
| T1 | 1–13 | 1455.8 | 1456.1 | 1456.1 | 1456.1 | 1456.1 | 1456.1 | 1456.1 |
| T2 | 14–20 | 886.4 | 886.6 | **887.7**[b] | **887.8**[b] | 886.8 | 886.8 | 886.4 |
| T3 | 21–25 | 702.1 | 702.5 | 702.6 | 702.7 | 702.7 | 702.7 | 702.4 |
| T4 | 27–44 | 1946.1 | 1946.0 | 1946.0 | 1946.0 | 1946.0 | 1946.0 | 1946.1 |
| T'4 | 28–44 | 1818.0 | 1818.0 | 1818.0 | 1818.0 | 1818.0 | 1818.0 | 1818.1 |
| T5 | 53–65 | 1554.8 | 1555.1 | 1555.0 | 1555.0 | **1556.1**[b] | 1555.2 | 1555.1 |
| T6 | 66–80 | 1559.8 | 1559.8 | 1560.1 | 1560.1 | 1560.1 | **1561.1**[b] | **1561.1**[b] |

[a] "n.d." represents not detected.

[b] Values have 1 mass unit increment against theoretical mass values.

into iso-Asp residue and thus prevented V-8 protease digestion at this position (8). We further substantiated these observations as follows: In comparisons of degradation product elution profiles with that of the authentic Asp16-rhPTH variant using RP-HPLC, elution of B3 was at a similar position, whereas, that of B2 was different. We also isolated a tryptic peptide fragment at the position 14–20 from B3 using RP-HPLC and examined its amino acid sequence. We found this to be: His-Leu-*Asp*-Ser-Met-Glu-Arg. Together with the FAB-MS analysis, we confirmed that Asn had changed to Asp at position 16. We concluded that degradation products B2 and B3 contained iso-Asp and Asp at position 16, respectively.

After FAB-MS spectroscopy of trypsin digested degradation products B4 and B5, an increase of one mass unit was also observed in the peptide fragments at positions 53–65 and 66–88, respectively (Table II(b)). The elution profiles of B4 and B5 from both RP- and CE-HPLC were similar to that of authentic Asp57- and Asp76-rhPTH variant, respectively. Thus, the degraded product B4 was determined to be Asp57-rhPTH and B5, Asp76-rhPTH.

In summary, degradation products B1–B5 were 17–84rhPTH, isoAsp16-rhPTH, Asp16-rhPTH, Asp57-rhPTH and Asp76-rhPTH, respectively.

## Separation and Determination of Degraded Products at pH2

After incubation at pH2 for 29h at 60°C, the rhPTH degradation products were isolated in a similar manner as those at pH9. Eight degradation products (A1–A8) were fractionated. The elution positions for A1–A8 by RP-HPLC are shown in Fig. 2(a). To achieve adequate separation, the RP-HPLC condition was adjusted, so that the retention of intact rhPTH was prolonged as compared to the retention under the condition used to separate alkaline degradation products (Fig. 2(b)). Under this condition, A2 and A8 could not be clearly resolved on this chromatogram, so these degradation products were subsequently separated using CE-HPLC.

From the results of SDS-PAGE analysis, A1–A7 exhibited a smaller molecular mass than that of intact rhPTH (Table I). Despite similar molecular mass as intact rhPTH, A8 had a lower pI value, suggesting that this product was the deamidated form of rhPTH (Table I).

From the amino acid composition analysis (Table III), the structure of each degraded product was determined as follows: A1, 1–30 rhPTH; A2, 1–74 rhPTH; A3, 1–71 rhPTH; A4, 1–56 rhPTH; A5, 1–45 rhPTH; A6, 46–84 rhPTH; and A7, 31–84

**Table III.** Amino Acid Composition of Degradation Products of rhPTH

| Amino acid | A1 | A2 | A3 | A4 | A5 | A6 | A7 |
|---|---|---|---|---|---|---|---|
| Asx | 3.0(3) | 8.7(9) | 7.9(8) | 6.1(6) | 4.9(5) | 4.4(5) | 6.6(7) |
| Glx | 5.0(5) | 10.0(10) | 10.0(10) | 7.0(7) | 5.0(5) | 6.0(6) | 6.0(6) |
| Ser | 2.5(3) | 4.7(6) | 5.3(6) | 3.6(4) | 2.6(3) | 3.6(4) | 3.4(4) |
| Gly | 1.1(1) | 3.8(4) | 4.3(4) | 3.3(3) | 2.1(2) | 2.2(2) | 3.1(3) |
| His | 2.1(2) | 3.6(4) | 4.1(4) | 3.2(3) | 2.8(3) | 1.2(1) | 2.0(2) |
| Arg | 1.8(2) | 4.7(5) | 4.6(5) | 4.8(5) | 2.7(3) | 2.2(2) | 3.3(3) |
| Thr | 0.0(0) | 0.2(0) | 0.2(0) | 0.0(0) | 0.0(0) | 1.1(1) | 0.9(1) |
| Ala | 0.2(0) | 5.6(6) | 4.8(5) | 3.9(4) | 2.9(3) | 4.2(4) | 6.4(7) |
| Pro | 0.0(0) | 2.7(3) | 2.9(3) | 3.1(3) | 2.1(2) | 1.1(1) | 3.0(3) |
| Tyr | 0.0(0) | 0.0(0) | 0.0(0) | 0.0(0) | 0.0(0) | 0.0(0) | 0.0(0) |
| Val | 2.1(2) | 6.0(6) | 6.3(6) | 4.2(4) | 4.0(4) | 4.2(4) | 5.7(6) |
| Met | 2.1(2) | 1.9(2) | 1.5(2) | 2.1(2) | 2.1(2) | 0.0(0) | 0.0(0) |
| Cys | 0.0(0) | 0.0(0) | 0.0(0) | 0.0(0) | 0.0(0) | 0.0(0) | 0.0(0) |
| Ile | 1.0(1) | 0.9(1) | 1.0(1) | 1.0(1) | 1.1(1) | 0.0(0) | 0.0(0) |
| Leu | 4.8(5) | 8.3(9) | 8.4(9) | 7.0(7) | 6.7(7) | 3.1(3) | 5.0(5) |
| Phe | 0.2(0) | 0.9(1) | 1.0(1) | 1.0(1) | 1.0(1) | 0.3(0) | 1.0(1) |
| Lys | 2.3(3) | 6.6(7) | 5.1(6) | 4.5(5) | 2.2(3) | 6.2(6) | 5.7(6) |
| Total residues | 30 | 74 | 71 | 56 | 45 | 39 | 54 |
| Proposed peptide | 1–30 | 1–74 | 1–71 | 1–56 | 1–45 | 46–84 | 31–84 |

The number in parentheses are the number of amino acid residue from the proposed peptide.

rhPTH. All these products were produced as a result of cleavage of the amide bond of Asp. Cleavage occurred at all Asp residues of intact rhPTH.

For degradation product A8, an increment of one mass unit was observed between residues 66–80 by FAB-MS peptide mapping (Table II(b)). A8 had a similar character to that of the authentic Asp76-rhPTH variant, as determined by HPLC, and was designated Asp76-rhPTH.

In summary, the degradation products at pH2 in the order of A1–8 were concluded to be: 1–30 rhPTH, 1–74 rhPTH, 1–71 rhPTH, 1–56 rhPTH, 1–45 rhPTH, 46–84 rhPTH, 31–84 rhPTH and Asp76-rhPTH.

## DISCUSSION

In this study, we examined rhPTH degradation under pH2–10, at 40–60°C. In this range of temperatures at each pH, similar degradation products were observed upon HPLC analysis. Because hPTH has an essentially flexible higher-order structure in aqueous solution at room temperature (19,20), temperature effects on the structure and the degradation pathway would be expected to be small. At pH9, the Asn residues of rhPTH were deamidated to Asp and iso-Asp. Generally, deamidation of Asn residues can take place either via direct hydrolysis or via the formation of a cyclic imide intermediate, which is favored at conditions of neutral to alkaline pH. Both Asp and iso-Asp residues can be produced via the cyclic imide pathway, whereas iso-Asp cannot be formed from direct hydrolysis (8–11). Therefore, the appearance of the iso-Asp form strongly suggested that the degradation pathway of rhPTH occurred via the formation of a cyclic imide intermediate. Also at this pH, 17–84 rhPTH was produced by cleavage of the amide bond of Asn16. This could have resulted from cyclic imide formation of an Asn residue (12,13).

At a pH ranging between 5 and 10, the deamidated products of Asn16 were iso-Asp and Asp, which remained in a relatively constant ratio of 3:1 throughout (Fig. 1(b): isoAsp16, B2; Asp16, B3). Similar isoAsp16/Asp16 ratios have been observed in other studies of small peptides (8,10,12). Since rhPTH consists of 84 amino acid residues, the size of the peptides was presumed to have no significant effect on the ratio of these products.

Under neutral to alkaline conditions, a C-flanking residue of Asn has been shown to have significant effects on the deamidation rate in peptides; practically, Leu, Val and Pro have much slower rates than those of Gly and Ser (9,11,12,14–16). In rhPTH, the C-flanking residues of Asn are: Asn10-Leu, Asn16-Ser, Asn33-Phe, Asn57-Val, Asn76-Val, respectively (Fig. 3). Therefore, significantly different deamidation rates might be expected to be observed.

At a pH below 5, seven products from the cleavage of amide bond of Asp were found (A1–A7). Cleavage of the amide bond of Asp is thought to occur via two mechanisms: one is by acid-catalyzed amide hydrolysis of the peptide bond; the other is through the formation of a cyclic anhydride intermediate (17,21). In this cleavage, the Asp-Pro sequence reportedly undergoes a particularly high hydrolysis rate (17,22), but, this sequence is absent in rhPTH so no significant difference in the hydrolysis rate would be expected. The quantity of A1 (1–30 rhPTH) and A5 (1–45 rhPTH) was much larger than A3 (1–71 rhPTH) and A4 (1–56 rhPTH) (Fig. 1(b)). The increased quantity of these shorter peptides may have resulted through a degradation of both intact rhPTH as well as A3 or A4. Our measurements reflect only the consequence of 72h incubation and do not address the exact hydrolysis rate.

Under acidic conditions, the predominant deamidation pathway is direct hydrolysis (8), and none of the primary sequence affects degradation (23). In this study, Asp-76-rhPTH was the only deamidated product found after incubation at pH2. A presumed reason that other deamidated products were not found was that the yield of deamidated products was very low under these conditions. In practice, the amount of isolated Asp76-rhPTH was 1/5–1/10 the amount of the other cleaved products.

In rhPTH degradation, two major pathways were revealed; one occurred under acidic conditions via hydrolysis of the amide bond of Asp, whereas, a neutral to alkaline environment favored the other, i.e., deamidation through cyclic imide intermediate formation at Asn. The 'cut off' boundary for each degradation pathway was at pH5, where rhPTH is most stable.

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