# Acid-Catalyzed Peptide Bond Hydrolysis of Recombinant Human Interleukin 11

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Recombinant human interleukin 11 (rhIL-11) is a multispectrum cytokine that plays an important role in megakaryocytopoiesis and platelet production. Probing rhIL-11 chemical reactivity in aqueous solution is an important initial step in developing a dosage form for rhIL-11 clinical trials. This report documents rhIL-11 degradation kinetics at 50°C in solutions adjusted to pH 3.0 to 9.5. Stressed samples were analyzed by reverse-phase HPLC and degradation product peaks were isolated for structural characterization. The results show maximal stability in the region pH 6.5 to 7.0. Degradation product identification shows that the major reaction pathway in acidic solution involves peptide cleavage at aspartate$_{133}$–proline$_{134}$. In alkaline solution, protein disappearance proceeds via nonspecific loss to container surfaces. Degradation products at alkaline pH have not been identified.

KEY WORDS: interleukin 11 (IL-11); recombinant human IL-11; degradation; kinetics; products; aspartate–proline cleavage.

## INTRODUCTION

The hematopoietic growth factors are hormones that influence the proliferation and differentiation of various blood cells (1). Interleukin-11 is a novel cytokine with wide-spectrum biological activities that partially overlap with biological responses mediated by interleukin 6 (2–4). Recombinant human interleukin-11 (rhIL-11), Neumega, enhances megakaryocytopoiesis and increases platelet counts in normal and immunosuppressed animals (5–8). Clinical trials are under way to characterize rhIL-11 biological activity further.

Surveying chemical reactivity under stress conditions is an important first step in the overall effort to develop a suitable parenteral dosage form for any protein biopharmaceutical. As recombinant DNA technology provides ever more novel therapeutic proteins, the literature describing protein drug stability testing continues to grow (9–23). Reviews of common protein and peptide degradation modes are also available (24–26).

Here we describe the first of several studies aimed at determining the overall mechanisms of rhIL-11 instability. Specifically, this report describes the pH dependence of rhIL-11 degradation kinetics in aqueous solution at 50°C as determined by reverse-phase HPLC. Although the focus is on determining reaction rates, degradation product identification also provides mechanistic information about rhIL-11 chemistry in acidic aqueous solution.

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## MATERIALS AND METHODS

### Materials

rhIL-11 was produced at Genetics Institute as a 177-amino acid, monomeric protein expressed by *Escherichia coli* (Fig. 1). Various analytical methods demonstrated >98% purity for the protein stock solutions employed. rhIL-11 stock solutions and control samples were maintained at −80°C throughout the study. rhIL-11 sample solutions were prepared at 5 mg/mL in a pH-adjusted polybuffer system (2 m*M* each citrate, succinate, histidine, imidazole, HEPES, TRIS, and glycine). Samples were incubated at 50 ± 2°C at 0.5 mL in poly(propylene) Eppendorf tubes. Samples were withdrawn at timed intervals and analyzed by reverse-phase HPLC (below).

### Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)

HPLC runs used Waters 600E pumps and system controller with a WISP Model 700 autoinjector. Detection was with a Model 490E detector (280 nm), and quantitation with Model 860 Expert software. The analysis used a Vydac $C_4$ 4.6 × 50-mm, 5-μm column and a 2-mL/min flow rate. Gradient chromatography used 0.1% trifluoroacetic acid as buffer A and 0.1% trifluoroacetic acid in 90% aqueous acetonitrile as buffer B. A linear gradient of 10 min from 30 to 70% B was used. Samples for analysis were injected neat (20 μL) in triplicate without dilution or further workup.

### Thermal Precipitation

Samples were dialyzed into pH-adjusted polybuffer at 0.1 mg/mL [rhIL-11]. A 0.5-mL aliquot of each solution was transferred to a fluorescence cuvette and placed in the sample holder of an SLM/Aminco 8000C fluorescence spectrophotometer. A Neslab RTE-110 circulating temperature controller was used to ramp the cuvette temperature from 20 to 100°C at 1°C/min. Cuvette temperatures were determined with a Physitemp MT-23/5 thermocouple. Right-angle light scattering was monitored at 320 nm (excitation and emission wavelength) during the temperature ramp.

### Degradation Product Identification

rhIL-11 was maintained for 2 hr at 50°C in pH 2 trifluoroacetic acid solution. The sample was chromatographed (RP-HPLC) and degradation product fractions were collected.

Isolated fraction volumes were reduced on a Savant SpeedVac concentrator and diluted 1:10 with 50 m*M* sinapinic acid solution in 30% aqueous acetonitrile. A 0.5-μL aliquot of the mixture was dried on a sample probe and analyzed by laser-desorption time-of-flight mass spectrometry versus bovine trypsinogen as the external molecular mass calibrant.

## RESULTS

Figure 1 shows the rhIL-11 amino acid sequence. The molecule isolated from the *E. coli* expression system begins

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P G P P P G P P R V S P D P R A E L D S 20

T V L L T R S L L A D T R Q L A A Q L R 40

D K F P A D G D H *N* L D S L P T L A M S 60

A G A L G A L Q L P G V L T R L R A D L 80

L S Y L R H V Q W L R R A G G S S L K T 100

L E P E L G T L Q A R L D R L L R R L Q 120

L L M S R L A L P Q P P P D P P A P P L 140

A P P S S A W G G I R A A H A I L G G L 160

H L T L D W A V R G L L L L K T R L 178

**Fig. 1.** rhIL-11 amino acid sequence. Potential chemical reactivity sites include methionines (bold), asparagine (italics), glutamine (underlined), and aspartate–proline couples (shaded).

with $Gly_2$. Note the absence of cysteine residues and consensus sequences for N-linked glycosylation. rhIL-11 is monomeric and highly basic (p*I* >10.5), with an apparent (by gel electrophoresis) molecular weight of 22 kDa. Potential sites for chemical reactivity in the rhIL-11 protein include the following:

- two methionines (possible oxidation sites), at positions 59 and 123;
- a single asparagine (possible deamidation site) at position 50,
- seven glutamines (possible deamidation sites), at positions 34, 38, 68, 88, 198, 120, and 130; and
- two aspartyl–prolyl couples (possible peptide bond cleavage sites) at positions 12–13 and 133–134.

As an initial probe of the predominant pathways for rhIL-11, we incubated samples at 50°C in aqueous solutions adjusted to pH 3 through 9.5. At timed intervals, samples were withdrawn and analyzed by RP-HPLC as shown below.

### Chromatographic Profiling of rhIL-11 Degradation

Figure 2 overlays representative RP-HPLC traces for rhIL-11 samples maintained at 50°C and pH 9.0. The figure shows time-dependent loss of rhIL-11 but concomitant degradation product accumulation is not evident. In control experiments (data not shown) sodium dodecyl sulfate solution rinses of container surfaces showed intact protein recovery. Thus, it appears that rhIL-11 degrades in alkaline solution by denaturation and/or precipitation at the solution–container interface. This degradation pathway was not further characterized.

At 50°C and acidic pH, rhIL-11 disappears rapidly and RP-HPLC analysis shows degradation product accumulation. Figure 3 overlays representative RP-HPLC traces for an rhIL-11 sample maintained at pH 3.5 for timed intervals as long as 4 days. Figure 3 clearly shows rhIL-11 loss and concomitant accumulation of a single major peak.

**Table I.** Pseudo-First-Order Observed Rate Constant ($k_{obs}$) Values for rhIL-11 Hydrolysis

| Solution pH | Intercept (mg/mL)[a] | $k_{obs}$ ($days^{-1}$) Slope[a] | $k_{obs}$ ($days^{-1}$) SE[a] | Squared correlation coefficient[a] |
|---|---|---|---|---|
| 3.00 | 4.31 | 0.843 | 0.0996 | 0.960 |
| 3.50 | 4.52 | 0.485 | 0.0384 | 0.982 |
| 4.00 | 4.48 | 0.289 | 0.0369 | 0.954 |
| 4.50 | 4.61 | 0.138 | 0.0232 | 0.922 |
| 5.00 | 4.60 | 0.0780 | 0.0242 | 0.776 |
| 5.50 | 4.59 | 0.0608 | 0.0182 | 0.788 |
| 6.00 | 4.61 | 0.0178 | 0.0052 | 0.795 |
| 6.50 | 4.61 | 0.0178 | 0.0052 | 0.750 |
| 7.00 | 4.61 | 0.0046 | 0.00055 | 0.972 |
| 8.00 | 4.60 | 0.0183 | 0.00085 | 0.998 |
| 9.00 | 4.51 | 0.0329 | 0.0059 | 0.887 |
| 9.50 | 4.61 | 0.0585 | 0.0090 | 0.955 |

[a] By least-squares linear regression analysis according to Eq. (1).

### rhIL-11 Degradation Kinetics

Least-squares regression of rhIL-11 concentration at any pH versus incubation interval permits calculating the rate constant for rhIL-11 degradation as

$$\ln([\text{rhIL11}]_t \div [\text{rhIL11}]_0) = \text{Intercept} - k_{obs} \times t \quad (1)$$

where the subscripts 0 and $t$ indicate rhIL-11 concentrations determined initially and at incubation interval $t$, respectively, and $k_{obs}$ is the pseudo-first-order observed rate constant for rhIL-11 degradation at a specific solution pH.

Figure 4 plots rhIL-11 concentration data versus incubation interval for pH 3.0, 4.5, and 7.0. The figure shows good adherence to Eq. (1) to long conversions (>2 half-lives) and increasing $k_{obs}$ values with decreasing pH in acidic solution. Table I summarizes all the kinetic data for rhIL-11 degradation at 50°C.

Figure 5 plots $\log(k_{obs})$ versus pH for rhIL-11 degradation in acidic, neutral, and alkaline solutions. The figure shows a minimum near pH 6.5 to 7.0, indicating optimal rhIL-11 stability (with respect to the chemistry detected by RP-HPLC) at neutral pH.

To describe the pH dependence of rhIL-11 degradation, we can express $k_{obs}$ values as

$$k_{obs} = k_0 + k_H \times [H^+] + k_{OH} \times [OH^-] \quad (2)$$

where $k_0$ is the pseudo-first-order rate constant for sponta-

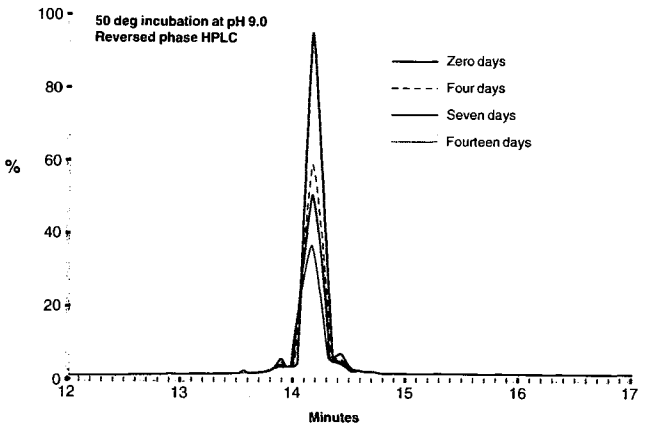


**Fig. 2.** rhIL-11 loss at 50°C and pH 9.0 as determined by RP-HPLC. Overlaying chromatograms for samples maintained for 0, 4, 7, and 14 days. rhIL-11 elutes at 14.2 min.

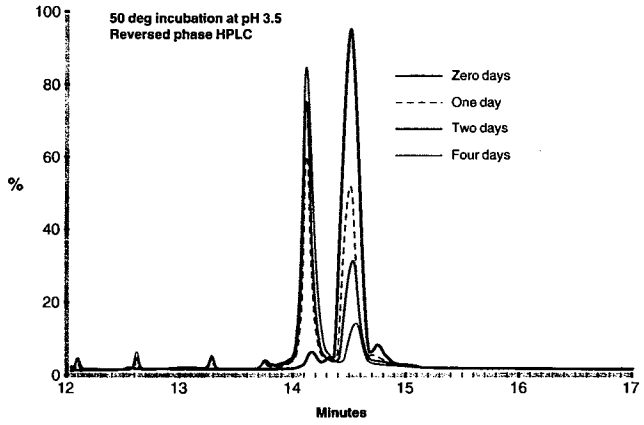


Fig. 3. rhIL-11 degradation at 50°C and pH 3.5 as determined by RP-HPLC. Overlaying chromatograms for samples maintained for 0, 1, 2, and 4 days. Degradation product elutes at 14.2 min and rhIL-11 elutes at 14.6 min.

neous reaction, and $k_H$ and $k_{OH}$ are bimolecular rate constants for acid- and base-catalyzed rhIL-11 degradation, respectively.

Least-squares regression of $k_{obs}$ versus $10^{-pH}$ and $10^{-(14-pH)}$ according to Eq. (2) gives the following best-fit parameters:

$k_0 = 0.00733\ day^{-1}$,
$k_H = 3210$ L/mol-day, and
$k_{OH} = 2218$ L/mol-day.

Figure 5 shows the best-fit line (on a logarithmic scale) for $k_{obs}$ calculated according to Eq. (2) and demonstrates reasonable adherence to the kinetic model.

### Thermal Precipitation

Upon thermal stressing, rhIL-11 precipitates from solution as demonstrated by increased right-angle light scattering (RALS). Monitoring RALS signal intensity for rhIL-11 solutions during a linear temperature ramp permits determination of $T_p$, the midpoint temperature for thermal precipitation.

Figure 6 shows $T_p$ values as a function of solution pH and demonstrates that precipitation midpoint temperatures are between 75 and 90°C over the pH range 5.0 to 11.0. Although other explanations are possible, the most likely mechanism underlying the observed temperature-dependent precipitation is thermal denaturation followed by rapid (that is, non-rate-limiting) aggregation and precipitation. If so, the kinetic determinations (described in the preceding section) at 50°C represent reactions of the native and not the denatured rhIL-11 protein.

### rhIL-11 Degradation Product Identification

The degradation product peak eluting at 14.2 min in Fig. 2 was collected and characterized by N-terminal sequence analysis and mass spectrometry. Two peptide fragments were identified in the peak, namely, the rhIL-11 peptides corresponding to residues 1–133 and 134–177. Identifying the degradation products therefore establishes peptide bond cleavage at the $Asp_{133}$–$Pro_{134}$ couple as the predominant degradation pathway for rhIL-11 at 50°C in acidic aqueous solution.

## DISCUSSION

Developing parenteral formulations for protein biopharmaceuticals frequently begins with surveying protein degradation pathways under stress conditions. Using RP-HPLC as an analytical probe, we have profiled rhIL-11 degradation at 50°C over the pH range 3 to 9.5. Under these conditions, rhIL-11 loss kinetics are consistent with acid- and base-catalyzed degradation reactions and show a reaction rate minimum at neutral pH.

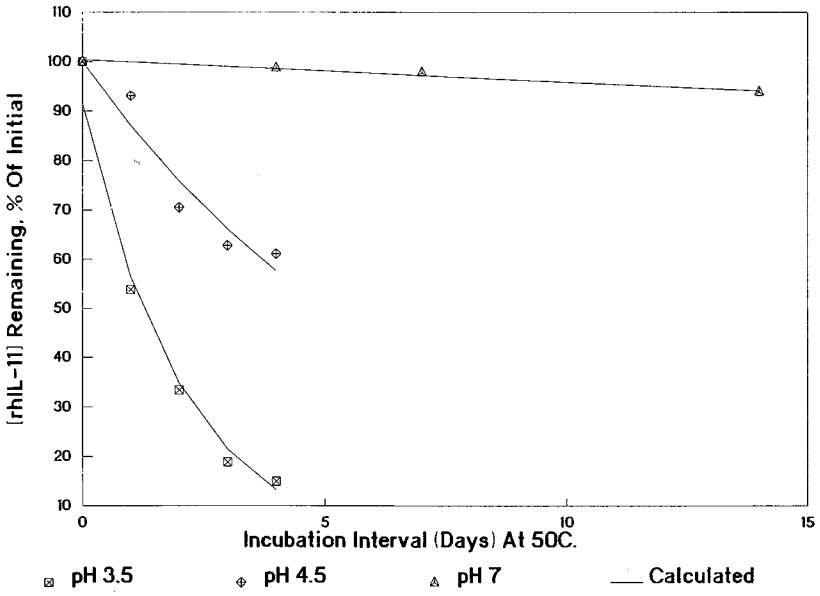


Fig. 4. Kinetic plots of rhIL-11 fraction remaining versus incubation interval at 50°C and various pH levels. rhIL-11 determinations by RP-HPLC. Solid lines calculated according to Eq. (1). See Table I for $k_{obs}$ values.

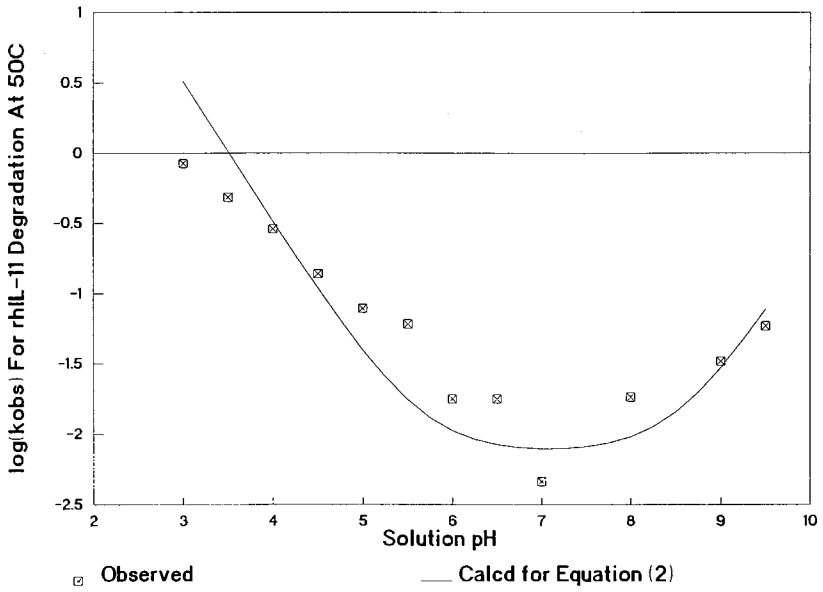


Fig. 5. pH–log(rate) plot for rhIL-11 loss at 50°C. Points are individual $k_{obs}$ values. The line is the best fit to $\log(k_{obs}) = \log[(k_0 + k_H*(10^{-pH}) + k_{OH}*10^{-(pOH)}]$.

Degradation product identification shows that hydrolytic cleavage at $Asp_{133}$–$Pro_{134}$ is the predominant rhIL-11 degradation pathway at 50°C in acidic solution. Although the literature documents peptide bond hydrolysis at aspartyl–prolyl couples as a facile degradation pathway for proteins (27–30), it is dangerous to employ generalized mechanisms to infer degradation pathway for novel proteins. It is therefore interesting to note that rhIL-11 hydrolysis favors reaction at the 133–134 position over hydrolysis at the nominally equivalent $Asp_{12}$–$Pro_{13}$ position. We observed a similar selectivity for Asp–Pro hydrolytic cleavage in recombinant human macrophage colony-stimulating factor stress studies (23).

There are two possible explanations for the Asp–Pro cleavage selectivity demonstrated for rhIL-11. Either protein tertiary structure strongly influences chemical reactivity or primary structure (VSPDP at $Asp_{12}$–$Pro_{13}$ versus PPPDP at $Asp_{133}$–$Pro_{134}$) drives the observed selectivity. The available data cannot distinguish between these two possibilities.

This report represents an introduction to the chemistry available to rhIL-11 under stress conditions. Further characterization awaits the outcome of ongoing experiments that

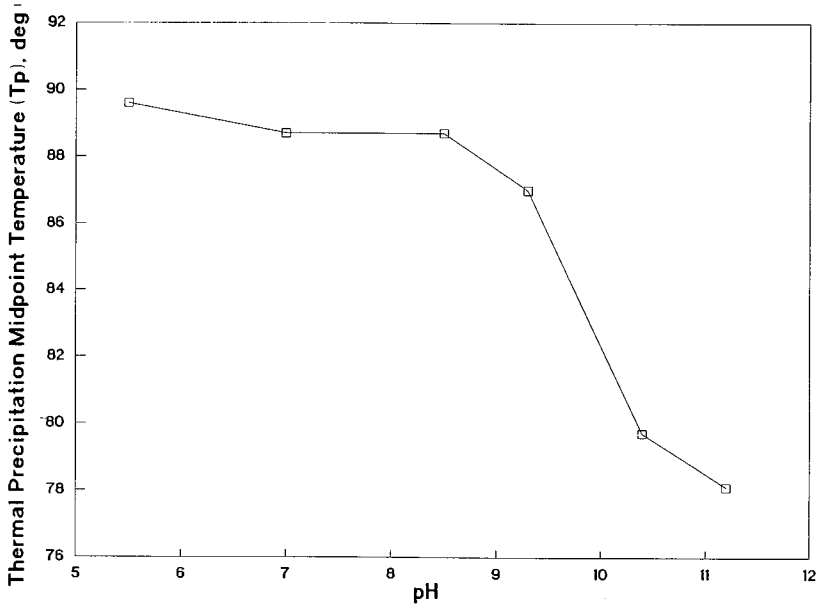


Fig. 6. rhIL-11 thermal precipitation midpoint temperature ($T_p$) value versus solution pH. $T_p$ values were determined by right-angle light-scatter monitoring of samples subjected to a linear temperature ramp.

examine degradation kinetics and products using multiple analytical techniques and stress plus realistic storage conditions.

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