Characterization of Asparagine Deamidation and Aspartate Isomerization in Recombinant Human Interleukin-11

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Purpose. The aim of this study was to investigate asparagine (Asn) deamidation and aspartate (Asp) isomerization and to measure the content of isoaspartate (isoAsp) in recombinant human interleukin-11 (rhIL-11).

Methods. The rhIL-11 control and heat stressed samples were characterized with trypsin and endoproteinase Asp-N peptide mapping, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), reversed-phase high performance liquid chromatography (RP-HPLC), electrospray ionization mass spectrometry (ESI MS) and capillary electrophoresis (CE). The total isoAsp content and bioactivity were also assessed.

Results. Stress of rhIL11 at 30°C for 6 weeks in liquid resulted in significant isomerization of Asp^{45} and Asp^{47}. Isomerization of Asp^{51} and deamidation of Asn^{49} were also detected at low levels. The stressed rhIL-11 molecule contained 0.3 mol of isoAsp per mol of protein, compared to only 0.007 mol/mol of protein in the control.

Conclusions. Asp and Asn residues, located in a loop structure of rhIL-11, undergo isoAsp formation under stressed conditions.

KEY WORDS: interleukin-11; isomerization; deamidation; isoaspartate.

INTRODUCTION

Protein drugs require comprehensive and well-designed purity and structural characterization as well as stability and biologic function analyses. Enzymatic or non-enzymatic modifications, which may occur at every step during the process, can potentially alter charge and/or size homogeneity, and subsequently, the bioactivity of protein drugs. A number of molecular alterations have been documented in literature: aggregation, oxidation, proteolytic cleavage, disulfide bond scrambling, glycosylation, deamidation and isomerization (1–4). These modifications are often related to the decrease, and sometimes complete loss of biologic activity of protein pharmaceuticals. IsoAsp formation is an important pathway for protein degradation and may have significant impact on protein structure and/or function (1–7). It is known that isoAsp can be produced by Asn deamidation and Asp isomerization (8,9), which usually occur at the sequences of Asn/Asp followed by glycine, serine or histidine, especially in regions with flexible conformations (10–13).

Interleukin-11 (IL-11) is a multifunctional cytokine in the IL-6 type subfamily of long-chain helical cytokines. Recombinant human IL-11 (rhIL-11) was licensed to prevent severe chemotherapy-induced thrombocytopenia. Based on molecular modeling, the location of exon/intron boundaries in the IL-11 genomic structure and limited proteolysis studies (14), it was proposed that IL-11 adopts a four-helix bundle folding (Fig. 1). Selective chemical modification combined with deletion and site-directed mutagenesis experiments indicated that in rhIL-11, several surface residues and the carboxyl terminus of rhIL-11 are involved in receptor binding (14,15). In the present study, the effects of heat stress on rhIL-11 in liquid formulation were studied. We investigated Asn deamidation and Asp isomerization in rhIL-11 with a variety of techniques. The total isoAsp content was also assessed with a non-radioactive method (16).

MATERIALS AND METHODS

Materials

The rhIL-11 used in this study was manufactured and purified at Wyeth BioPharma Genetics Institute Campus in Andover, MA, USA. The control sample was a 14.5 mg/ml rhIL-11 solution in buffer containing 10 mM sodium phosphate, 0.3 M glycine, pH 7.0 and stored at –80°C. The stressed sample was prepared by diluting the control sample to 5.2 mg/ml with the above buffer and heat stressed at 30 °C for six weeks. Trypsin treated with L-(tosylamido 2-phenyl) ethyl chloromethyl ketone (TPCK) was purchased from Worthington Biochemical Corporation. Endoproteinase Asp-N was from Boehringer Mannheim Biochemica. Trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA) were from Pierce. Pre-cast 10% to 20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) Sepragel (14 × 18 cm) and Daiichi Silver Stain Kit were from Integrated Separations System. Vydac C_{18} column (4.6 × 250 mm, 5 μM, 300 Å) and Vydac C_4 column (4.6 × 250 mm, 5 μM, 300 Å) were from the Nest Group. Jupiter C_{18} column (4.6 × 250 mm, 5 μM, 300 Å) was from Phenomenex. IsoAsp-δ-sleep-inducing peptide (isoAsp-DSIP, WAGGβDASGE) was from Bachem (Torrance, CA, USA). ISOQUANT protein deamidation detection kit containing solutions of protein isoaspartyl methyltransferase (PIMT) and *S*-adenosyl-L-methionine (SAM) was from Promega (Madison, WI, USA). *S*-adenosyl-L-homocysteine (SAH) was obtained from Sigma (St. Louis, MO, USA). Partisil 10 SCX column (85-Å, 10-μm particle size, 4.6 × 250 mm) was from HiChrom.

HPLC Equipment

Unless otherwise stated, the HPLC system used in this study consisted of a Waters Model 600-MS pump system and a Model 490E programmable multiwavelength UV/Vis detector.

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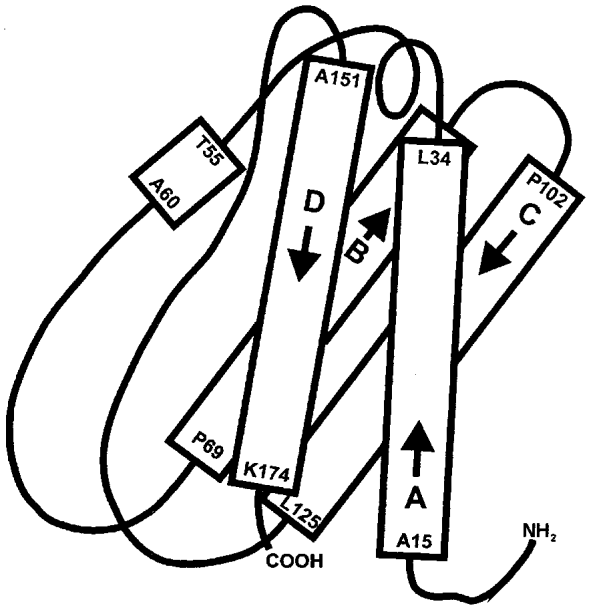
ABBREVIATIONS: Asn, asparagine; Asp, aspartate; isoAsp, isoaspartate; rhIL-11, recombinant human interleukin-11; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; RP-HPLC, reversed-phase high performance liquid chromatography; ESI MS, electrospray ionization mass spectrometry; CE, capillary electrophoresis; SCX, strong cation exchange; TFA, trifluoroacetic acid; HFBA, heptafluorobutyric acid; SAH, *S*-adenosyl-L-homocysteine; isoAsp-DSIP, isoAsp-δ-sleep-inducing peptide; PIMT, protein isoaspartyl methyltransferase; SAM, *S*-adenosyl-L-methionine.



G P P P G P P R V S P D P R A E L D S T V L L T R 25
T1 T2 T3
S L L A D T R Q L A A Q L R D K F P A D G D H N L 50
T4 T5 T6
D S L P T L A M S A G A L G A L Q L P G V L T R L 75
T7 T8
R A D L L S Y L R H V Q W L R R A G G S S L K T L 100
T9 T10 T11 T12
E P E L G T L Q A R L D R L L R R L Q L L M S R L 125
T13 T14 T15 T16 T17
A L P Q P P P D P P A P P L A P P S S A W G G I R 150
T18
A A H A I L G G L H L T L D W A V R G L L L L K T 175
T19 T20 T21
R L
T22

Fig. 1. Primary sequence and four-helix bundle structure of rhIL-11. Peptides expected from trypsin cleavage are underlined and labeled with T1 to T22.

Tryptic Peptide Mapping

Control and stressed rhIL-11 solutions were adjusted to a 1-ml volume with 50 mM Tris-HCl, pH 8.2 and digested with trypsin at an enzyme to substrate ratio 1:20. The samples were incubated under argon at 37°C for 20 h. The digestion was quenched by addition of 5 μl neat TFA. Each digest was then injected onto a Vydac C_{18} column. The peptides were eluted with a acetonitrile gradient using 0.1% (w/v) TFA as solvent A and 0.1% (w/v) TFA in 95% (v/v) acetonitrile as solvent B, at a flow rate of 1.0 ml/min. Gradient conditions were as follows: 0–5 min, 5% B; 5–32 min, 5–45% B; 32–34 min, 45% B; 34–37 min, 100% B. The column was re-equilibrated between runs by using a 1 min gradient of 100% B to 5% B, followed by a 20 min isocratic elution with 5% B.

NH_2-Terminal Sequencing

Automated Edman degradation was performed using an ABI Model 476A liquid-pulsed sequencer equipped with an on-line ABI Model 757 detector.

Electrospray Ionization Mass Spectrometry

Analysis of RP-HPLC fractions was performed on a VG Platform mass spectrometer equipped with electrospray ionization, operating in the positive ion mode of analysis. For LC/MS analysis, the effluent from a Vydac C_{18} column was split so that a flow rate of 25 μl/min was introduced to the mass spectrometer.

Preparative RP-HPLC of Stressed rhIL-11

To isolate degradation products from the heat stressed rhIL-11 for ESI MS and NH_2-terminal sequencing analysis, a Vydac C_4 column was used. The column was equilibrated in 0.1% (w/v) TFA (solvent A). Upon injection, the column was eluted with a 20% to 70% linear gradient of 0.1% (w/v) TFA in 95% (v/v) acetonitrile (solvent B) at a flow rate of 0.75 ml/min, over the course of 100 min. Fractions containing degradation products were collected manually for subsequent analysis.

SDS-PAGE

The rhIL-11 samples were diluted to 2 μg/μl and mixed with an equal volume of sample buffer (31 mM Tris-HCl, pH 6.8, 2.5% (w/v) SDS, 5% (v/v) glycerol and 0.001% (w/v) bromophenol blue), at ambient temperature. Samples were analyzed using pre-cast 10% to 20% SDS-PAGE gel with a protein load of 50 μg/lane. The electrophoretic buffer contained 0.19 M glycine, 25 mM Tris and 0.1% (w/v) SDS. The gel was run at 45 mA (constant current) for 3 h. After electrophoresis, the gel was silver stained with a Daiichi Silver Stain Kit.

Asp-N Peptide Mapping

Asp-N solution at 0.02 mg/ml in 50 mM Tris-HCl, pH 7.5 was added to the samples to an enzyme to substrate ratio of 1:56. The reaction was incubated at 37°C for 20 h and quenched with 5 μl of HFBA and 3 μl of TFA. The digest was injected onto a Vydac C_{18} column and peptides were eluted with a linear gradient using 4 mM HFBA, 6 mM TFA as solvent A and 4 mM HFBA, 6 mM TFA in 95% (v/v) acetonitrile as solvent B, at a flow rate of 0.75 ml/min. Gradient conditions were: 0 to 10 min, 5% B; 10 to 55 min, 5% to 35% B; 55 to 85 min, 35% to 50% B; 85 to 100 min, 50% to 65% B; 100 to 105 min, 65% to 90% B; 105 to 115 min, 90% B; 115 to 116 min, 5% B.

Isocratic Elution of Tryptic Peptides

An HP1050 HPLC system (Hewlett Packard) coupled with a Jupiter C_{18} column was used for isocratic elution analysis of rhIL-11 tryptic peptides. Mobile phases A and B were 70:30 and 60:40 water/acetonitrile (v/v), respectively, each containing 0.1% (v/v) TFA. The samples were run at 10% B in an isocratic mode for 60 min. The flow rate was 1 ml/min. The column temperature was kept at 40°C.

Direct Measurement of IsoAsp Content by SCX-HPLC

The isoAsp content in rhIL-11 was measured by a non-radioactive SCX-HPLC method, modified from a recently developed RP-HPLC assay (16). SAH stock solutions were made by dissolving SAH solid with purified water. The concentration of SAH stock solutions was determined by measuring absorbance at 260 nm and using a molar extinction coefficient of 15,400 $M^{-1}cm^{-1}$. The concentration of isoAsp-

DSIP solutions used for PIMT enzymatic reaction was determined at 280 nm using a molar extinction coefficient of 5540 $M^{-1}cm^{-1}$.

Methylation reactions were carried out by mixing 1 vol of the PIMT solution, 1 vol of SAM, and 1 vol of 5× reaction buffer provided in the ISOQUANT kit with 1 vol of purified water and 1 vol of rhIL-11 or isoAsp-DSIP solution. A blank was prepared using purified water instead of the test solution. The mixture was incubated at 30°C for 40 min. The reaction was stopped by adding 1 vol of stop solution supplied with the ISOQUANT kit.

HPLC analysis of SAH standards and methylation reactions was performed on a Waters Alliance system with detection at 260 nm. A Partisil 10 SCX column was used. Mobile phase A was 15% (v/v) acetonitrile in 0.01 M ammonium formate, pH 2.6. Mobile phase B was 15% (v/v) acetonitrile in 1.0 M ammonium formate, pH 4.3. The flow rate was 1.5 ml/min. The column was equilibrated at 100% buffer A. After the sample was injected, the column was washed with 100% A for 5 min followed by a step gradient of 0% to 30% buffer B over 5 min, 30% to 100% B over one min, and 100% to 0% B over 4 min. The column was then equilibrated for additional 5 min in 100% A before the next injection.

The SAH peak in the methylation reaction of each sample was then integrated. The SAH peak area corresponding to SAH produced in the blank reaction was subtracted from those of SAH produced in isoAsp-DSIP or rhIL-11 containing reactions when calculating the recovery. The isoAsp content was calculated based on the area of SAH peak in the sample and the SAH standard curve.

Biological Assay

The biologic activity of rhIL-11 control and heat stressed samples was assessed i*n vitro* by a proliferation assay using a mouse plasmacytoma cell line T10 (17).

RESULTS

Tryptic Peptide Map of rhIL-11

Trypsin digestion of rhIL-11 control sample was analyzed using an RP-HPLC system interfaced to an ESI mass spectrometer. For peptide identification, observed masses for each of the peaks in the tryptic map of rhIL-11 control sample were compared to the theoretical masses of expected fragments. Additionally, peptides were identified by NH_2-terminal sequencing. Results of both analyses are presented in Table I and summarized in Fig. 2 (A). IsoAsp is not reactive in the Edman degradation, therefore, a sequencing block at an Asn/Asp site provides an indirect evidence for the presence of isoAsp. Sequencing of fraction 30 revealed the sequence of the T6T7 peptide up to leucine^{50}, after which no signal was observed. The mass of this peptide was consistent with the full length T6T7, suggesting that this peptide underwent isomerization at Asp^{51}. Fraction 28 was determined to be T6T7 oxidized at Met^{58} (18).

Asn^{49} deamidation was detected by trypsin peptide mapping of heat stressed rhIL-11. While most of the peptides maintained the same elution positions and relative peak areas, an approximate 10% decrease in the T6T7 peptide (contains Asn^{49}) was observed. Additionally, two minor new peaks appeared adjacent to the T6T7 peptide [Peak 1 and Peak 2, Fig. 2 (B)], which were identified as T6T7-related by NH_2-terminal sequencing. Trypsin peptide mapping of the N49D mutant of rhIL-11, which represents one of the expected deamidation forms, showed that the elution position of the T6T7 peptide with aspartic acid replacing asparagine at position 49 corresponded to that of Peak 2 [Fig. 2(C)]. Therefore, Peak 2 likely represented T6T7 in which Asn^{49} was deamidated to Asp.

Characterization of Peaks 1 and 2 from the Trypic Map of Stressed rhIL-11

The tryptic peptides in peak 1 and 2 of stressed rhIL-11 sample were subjected to endoproteinase Asp-N digestion and ESI MS analysis. The peptides expected from Asp-N digestion are: D^{40}KFPA, D^{45}G, D^{47}HNL, and D^{51}SL…R^{74}. All peptides recovered from Asp-N digestion of peak 1, except one, had the expected amino acid sequence [Fig. 3(A)]. NH_2-terminal sequencing of this peptide gave the amino acid sequence DHNL. However, the peptide eluted 15 m later than the normal D^{47}-L^{50} peptide observed in the reference, indicating a more hydrophobic fragment. It was inferred that the residue following leucine in this peptide is isoAsp, resulting from isomerization of Asp^{51}. The modified peptide represents the sequence of T6T7, D^{47}HNL(βD)...R^{74}. No other unexpected amino acid sequences were detected in the Asp-N digest of peak 1. Consistent with Asp isomerization, the mass of Peak 1 determined by ESI MS (3,562.5 amu) was the same as that of unmodified T6T7 peptide [Fig. 3(B)].

The same procedure was used for Peak 2 identification. Again, an unexpected peptide was detected in the Asp-N digestion of peak 2 [Fig. 3(A)]. Two sub-fragments of the expected peptide were also detected due to overdigestion. Edman degradation of the unexpected peptide stopped after the sequence DH. The elution position of this peptide was identical to the elution position of a synthetic peptide D^{47}HβDL^{50}, indicating that Peak 2 contains a product of Asn^{49} deamidation. The sequence results confirmed that Asn was deamidated to isoAsp at position 49, since the β-aspartyl linkage is resistant to Edman degradation, and Asp-N can not cleave peptide bonds N-terminal to isoAsp (confirmed using the synthetic peptide D^{47}HβDL^{50}). NH_2-terminal sequencing of Peak 2 also detected normal Asp at position 49, which is another expected product of Asn deamidation. Deamidation of Asn^{49} to Asp created a new cleavage site for Asp-N within the D^{47}–L^{50} sequence. The resulting dipeptides DH and DL were not detected by peptide mapping of the Asp-N digest of Peak 2, since such small fragments eluted with the solvent front. Consistent with deamidation of Asn^{49} to Asp/isoAsp, the molecular mass determined by ESI MS for Peak 2 (3,563.6 amu) was 1 mass unit higher then the mass observed for the unmodified T6T7 peptide (3,562.5 amu) (Fig. 3 (B)).

Peaks 1 and 2 constituted approximately 5% and 3%, respectively, of the total T6T7 peak area. Thus, the decrease of peptide T6T7 observed in the peptide map of the heat stressed rhIL-11 can be explained by Asn^{49} deamidation and Asp^{51} isomerization.

Analysis of Fractions from Preparative RP-HPLC of Stressed rhIL-11

In RP-HPLC analysis of rhIL-11 stressed samples using a Vydac C_4 column, three new peaks showed up [Fig. 4(A)] and

Table I. Identification of Tryptic Peptides of rhIL-11 Control

Peak number	N-terminal sequence	Mass observed	Mass calculated[a]	Identification
1	LR	287.2	287.4	T8 peptide (L75-R76)
2	LDR	402.3	402.5	T14 peptide (L111-R113)
3	QTIASN	632.5	632.7	Bovine Trypsin (Q218-N223)
4	AGGSSLK	618.4	618.7	T12 peptide (A92-K98)
5	RAGGSSLK	774.6	774.9	T11T12 peptide (R91-K98)
6	AGGSSLKR	774.6	774.9	T12 peptide + R (A92-R99)
7	VSPDPR	669.5	669.7	T2 peptide (V9-R14)
8	SAASLNSR	804.6	804.9	Bovine Trypsin (S92-R99)
9	GPPPGPPR	773.7	773.9	T1 peptide (G1-R8)
	NKPGVYTK (minor)	905.9	906.1	Bovine Trypsin (N201-K208)
10	TRL	388.3	388.5	T21T22 peptide (T175-L177)
11	LLR	400.3	400.5	T15 peptide (L114-R116)
	LLRR (minor)	556.6	556.7	T15T16 peptide (L114-R117)
12	SGIQVR	658.5	658.8	Bovine Trypsin (S44-R49)
13	QLAAQLR	798.6	798.9	T5 peptide (Q33-R39)
14	SLLADTR	774.6	774.9	T4 peptide (S26-R32)
15	SSGTSYPDVLK	1152.8	1153.3	Bovine Trypsin (S126-K136)
16		781.7	781.9	<Q T5 peptide[b] (Q33-R39)
	HVQWLRR (minor)	993.8	994.2	T10T11 peptide (H85-R91)
17	HVQWLR	837.7	838.0	T10 peptide (H85-R90)
18	TLEPELGTLQAR	1327.2	1327.5	T13 peptide (T99-R110)
19	AELDSTVLLTR	1217.0	1217.4	T3 peptide (A15-R25)
20	RLQLLMR	1016.0	1016.3	T16T17 peptide (R117-R124)
	LQLLMR (minor)	859.7	860.1	T17 peptide (L118-R124)
21	GLLLLK	655.7	655.9	T20 peptide (G169-K174)
22	SIVHPSYNSNTLNNDI. .K	2273.2	2273.6	Bovine Trypsin (S70-K89)
23	LALPQPPPDP. . .R	2599.5	2600.0	T18 peptide (L125-R150)
24	ADLLSYLR	949.5	950.1	T9 peptide (A77-R84)
25	LRADLLSYLR	1219.2	1219.5	T8T9 peptide (L75-R84)
26	SAGALGALQLPGVL. . .R	1522.8	1523.8	T7 peptide clip (S59-R74)
27	AAHAILGGLH. . .R	1913.9	1914.2	T19 peptide (A151-R168)
28	DKFPADGDHNL. . . R	3579.5	3579.1	T6T7 peptide +16 u (D40-R74)
29	AILGGLHLTLDWAVR	1634.6	1635.0	T19 peptide clip (A154-R168)
30	DKFPADGDGNL (βD). . .	3562.6	3563.1	T6T7 peptide[c] (D40-R74)
31	DKFPADGDHNLDSLP. . .R	3562.6	3563.1	T6T7 peptide (D40-R74)

[a] Average mass, takes into account the natural relative abundance of all isotopes.
[b] N-terminal pyroglutamic acid, no sequence information obtained.
[c] Asp^{51} isomerized T6T7.

were collected for further analysis. In SDS-PAGE, several bands with low molecular weight were observed [Fig. 4(B)]. Peak 3 also contained intact rhIL-11, as shown by SDS-PAGE. This is due to the partial overlapping of peak 3 and the peak corresponding to unmodified rhIL-11. NH_2-terminal sequencing of peak 2 and 3 revealed sequences of GPPP-GPPR… and LDSLPTLA…, respectively. Subsequent analysis of these peaks with ESI MS indicated that they corresponded to deamidation related peptide bond cleavage at Asn^{49} [Fig. 4(C)]. Peak 2 is fragment 1–49, while peak 3 is fragment 50–177. Peak 1 had a molecular mass of 19,091 Da, as determined by ESI MS (data not shown). However, no NH_2-terminal sequence data could be obtained for this peak, and it proved to be resistant to enzymatic cleavage with trypsin (data not shown).

Analysis of the T6T7 Peptide

The rhIL-11 molecule contains eleven Asp residues (Fig. 1), which are potential sites for isomerization. Isomerization of Asp^{51} was detected in the heat-stressed sample and is described earlier. To identify if any additional residues are susceptible to isomerization, peptides containing Asp from trypsin digestion of stressed rhIL-11 were then isolated and analyzed by CE, ESI MS and NH_2-terminal sequencing, since no modification of any of these peptides was apparent by peptide mapping (Fig. 2). Out of 8 peptides containing Asp, only peptide T6T7 (contains four Asp residues, at positions 40, 45, 47 and 51) appeared to be modified. CE analysis of the T6T7 peptide indicated that in the stressed sample, approximately 25% of this peptide was found in a modified form, compared to that approximately 2% was detected in the starting material [Fig. 5(A)]. The molecular mass of the modified T6T7 peptide from the stressed material determined by ESI MS was the same as the mass of the unmodified counterpart from either the stressed sample or the control (data not shown), consistent with Asp isomerization.

To determine the nature and the site(s) of the modification, the T6T7 peptide from stressed rhIL-11 sample was subdigested with Asp-N. All of the resulting peptides except one corresponded to those obtained for the control [Fig. 5(B)]. Edman degradation resulted in the sequence $D^{40}KFPA^{44}$.

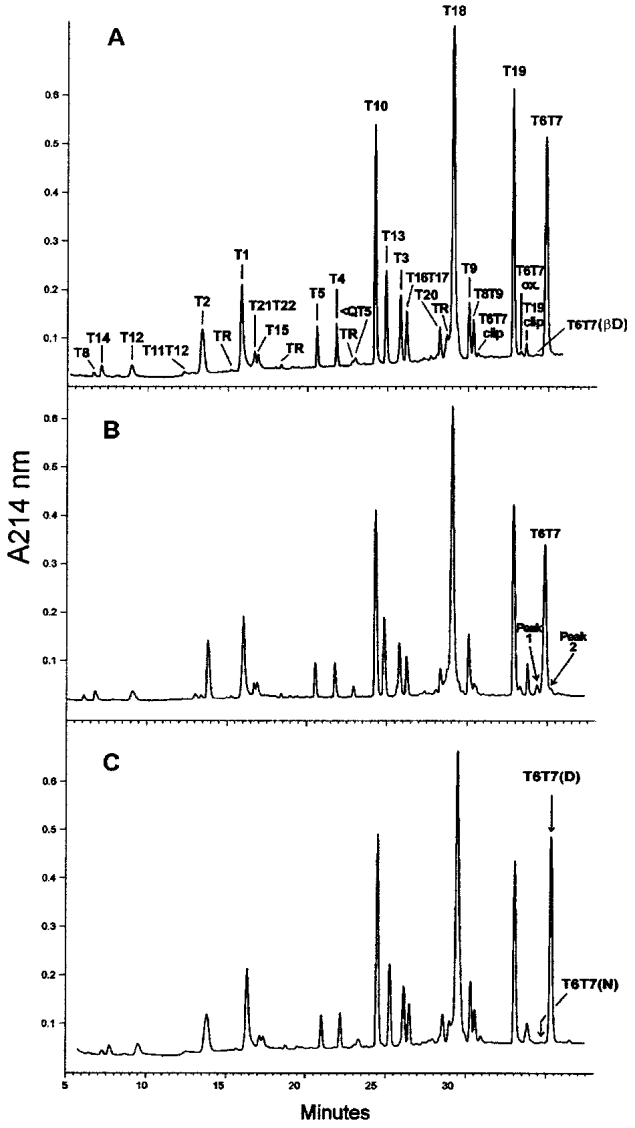


Fig. 2. Tryptic peptide map of rhIL-11. (A) The rhIL-11 control sample. Peptides were identified with both LC/ESI-MS and NH_2-terminal sequencing. Peptides from rhIL-11 are labeled. Trypsin autodigestion are labeled with TR. The T6T7 peptide containing isoAsp is labeled as T6T7(βD). (B) Stressed rhIL-11. Unmodified T6T7 peptide is indicated, and its modified forms are indicated as Peak 1 and Peak 2. (C) N49D mutant. Peptide T6T7 containing Asp at position 49 as T6T7(D). The elution position of peptide T6T7 containing Asn at position 49, T6T7(N) is also indicated.

However, the mass of this peptide (1228.3 amu) determined by ESI MS corresponded to the amino acid sequence D^{40}-L^{50} (data not shown). These results suggested the presence of isoAsp at positions 45 and 47. The peak area of the modified D^{40}-L^{50} peptide constituted approximately 24% of the total peak area. Taken together, these results indicated that under stress conditions, Asp^{45} and Asp^{47}, and to a lesser extent Asp^{51}, are susceptible to the isomerization reaction. No isomerization was observed for Asp^{40}.

Isocratic Analysis of T6T7 Peptide

The trypsin peptide mapping method described above could not resolve the major isomerized forms of the T6T7 peptide. Therefore, an isocratic RP-HPLC assay was developed to separate these T6T7 isoforms. Fig. 6 illustrates the profiles of trypsin digested rhIL-11 control and stressed samples under the isocratic condition. For rhIL-11 control [Fig. 6(A)], the main peak, peak 4, could be the unmodified T6T7 peptide. A small, partially resolved shoulder, peak 3, corresponded to modified T6T7. For the heat stressed sample, five significant peaks were resolved. Peaks 1, 2 and 5 were new modifications of T6T7 arising from heat stress conditions.

Amino acid sequence was determined for the major peak collected from the control sample (peak 4, Fig. 6) and for peaks 1 to 5 from heat stressed sample. Peak 4 yielded a complete T6T7 sequence through all potential modification sites, indicating that this peak corresponded to unmodified T6T7. Edman degradation of peak 1 stopped at residue 45. This information, combined with the chromatographic (early elution) behavior of peak 1, suggested that it might be a tri-isoAsp T6T7 variant, with isoAsp at positions 45, 47 and 51. Peak 2 co-migrated with peak 3 upon CE analysis (data not shown), but was resolved from peak 3 upon isocratic RP-HPLC analysis. These results implied that peaks 2 and 3 might be two di-isoAsp forms of the T6T7 peptide. The amino acid sequence of peak 2 stopped at position 47, suggesting it may represent the di-isoAsp form with isoAsp at positions 47 and 51. There was no signal observed after Ala^{44} for peak 3, suggesting its identification as a major di-isoAsp variant, with modified residues 45 and 47. The area of peak 3 was much larger than that of peak 2. This is consistent with the results described in the earlier sections that under stress conditions, Asp^{45} and Asp^{47}, and to a lesser extent Asp^{51}, are susceptible to the isomerization reaction. However, unequivocal identifications of peaks 2 and 3 would require further analysis. The results for peak 5 were more complex. The unmodified sequence was detected, however, the yields were low for all three Asp sites. In addition, a low yield was observed for Asn^{49} indicating partial deamidation. These results suggest that peak 5 may be composed of various isomerized forms that are in addition to deamidation at Asn^{49}.

IsoAsp Content in rhIL-11

IsoAsp can result from both Asn deamidation and Asp isomerization. In heat stressed rhIL-11, isoAsp was produced mainly from isomerization of Asp^{45} and Asp^{47}, and to a lesser extent, Asn^{49} deamidation and Asp^{51} isomerization. The total isoAsp amount in rhIL-11 control and stressed samples was determined by a non-radioactive assay (16). Chromatographic profiles of the enzymatic reactions are shown in Fig. 7. The accuracy of the method was determined by using the isoAsp-DSIP control peptide. In a reaction containing 67 pmol of isoAsp-DSIP, 69 pmol (103%) of SAH was detected. The rhIL-11 control sample contained approximately 0.007 mole of isoAsp per mole of protein. After being stressed at 30°C for 6 weeks, rhIL-11 contained 0.3 mole of isoAsp per mole of protein. This is in good agreement with CE and peptide mapping data, which indicated that stressed rhIL-11 was approximately 25% di-isoAsp substituted at positions 45 and 47, 3% deamidated at Asn^{49} and 5% isomerized at Asp^{51}.

Biological Assay

The T10 proliferation assay showed that the stressed rhIL-11 had a specific activity of $1.2 \pm 0.16 \times 10^6$ U/mg, a 20%

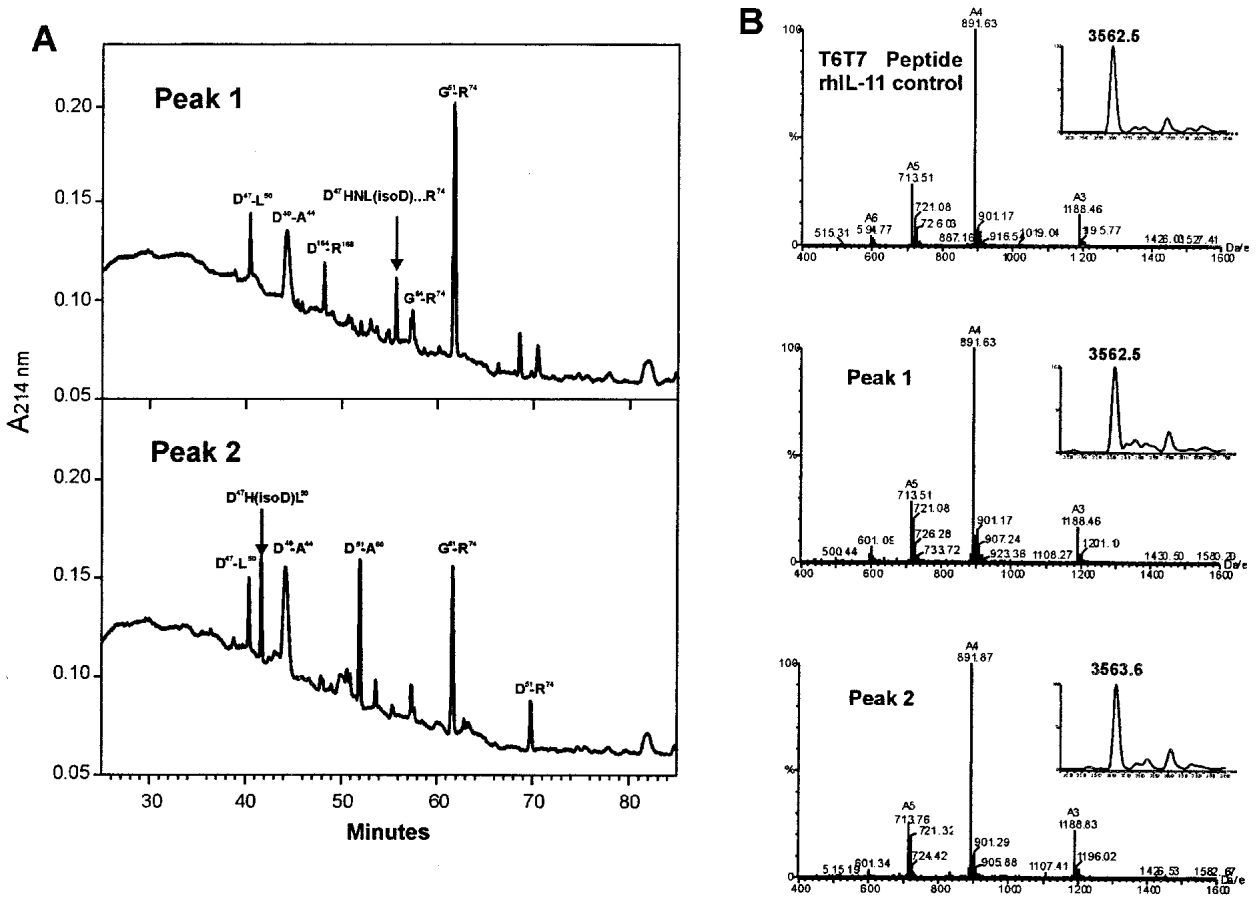


Fig. 3. Asp-N peptide mapping and ESI MS of Peak 1 and Peak 2 in Fig. 2. (A) Asp-N digest of Peak 1 with an arrow indicating a peptide containing isoAsp from isomerization of Asp^{51} (top panel), and Asp-N digestion of Peak 2 with an arrow indicating a peptide containing isoAsp at position 49 resulting from deamidation of Asn (bottom panel). (B) ESI mass spectra of the unmodified T6T7 collected from rhIL-111 control sample (top), Peak 1 (middle) and Peak 2 (bottom). The mass charge states are shown above each peak and the insets show spectra obtained from deconvluted data derived from the multiply charged ions.

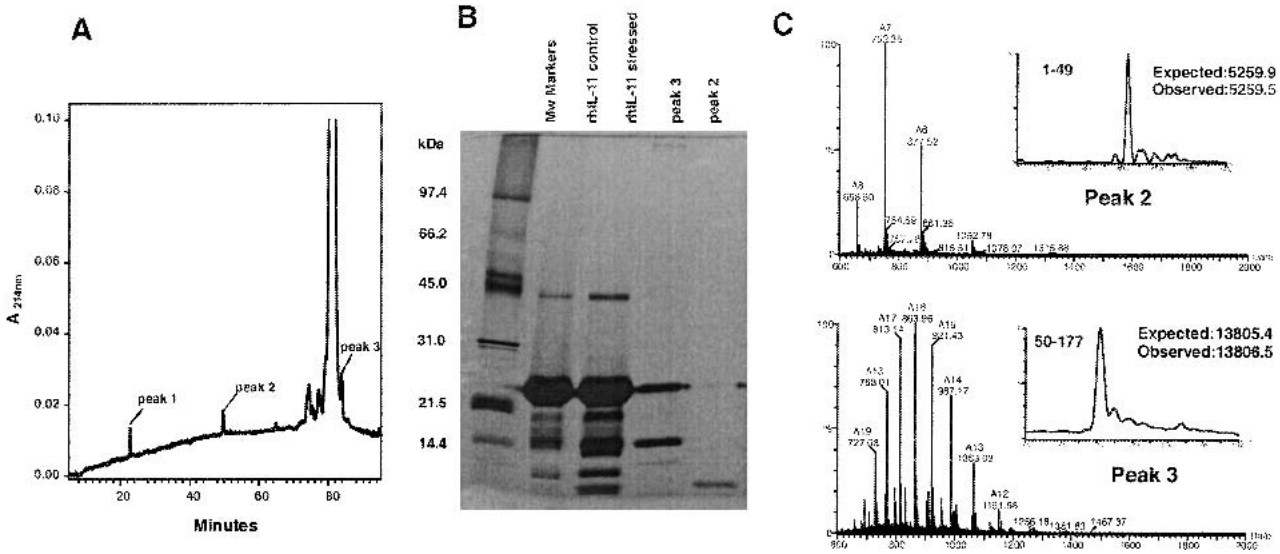


Fig. 4. Analysis of stressed rhIL-11 by RP-HPLC, NH_2-terminal sequencing, SDS-PAGE and ESI MS. (A) RP-HPLC of heat stressed rhIL-11. Injection amount was 60 μg. Peaks not present in rhIL-11 control sample are labeled and collected for Edman sequencing. (B) SDS-PAGE. The control and stressed rhIL-11 samples and peaks 2 and 3 from RP-HPLC were analyzed on the same gel. (C) Identification of the 1-49 (peak 2) and 50-177 (peak 3) fragments by ESI MS. The mass and charge states are shown above each peak. The insets show spectra obtained from deconvoluted data derived from the multiply charged ions.

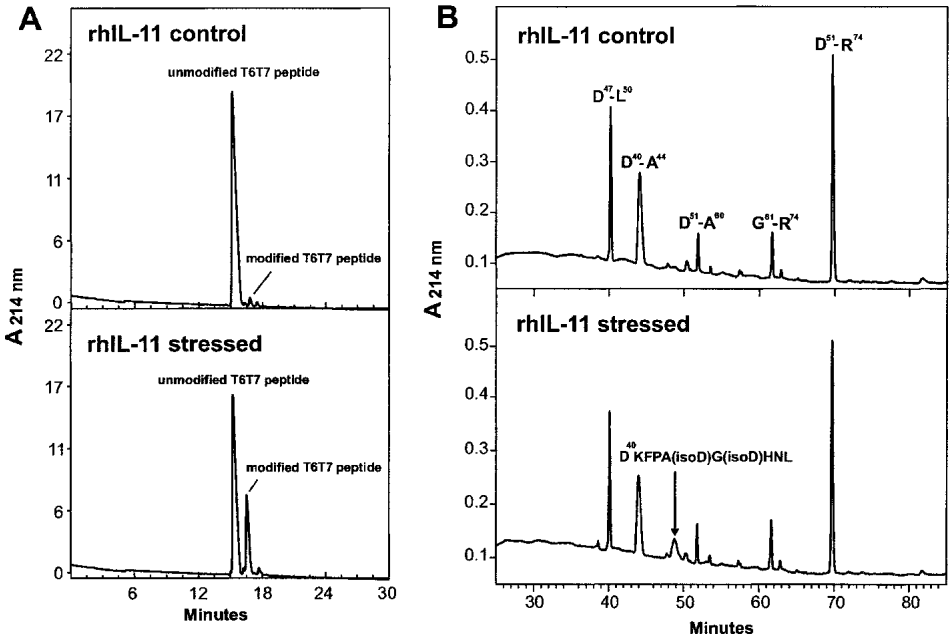


Fig. 5. CE and Asp-N peptide map of the T6T7 Peptide. (A) CE profile of the T6T7 peptide from rhIL-11 control (top) and stressed samples (bottom). (B) Asp-N peptide map of the T6T7 peptide from rhIL-11 control (top) and heat stressed samples (bottom). The arrow indicates the peptide that contains isoAsp at positions 45 and 47, resulting from isomerization of two Asp residues.

decline from $1.5 \pm 0.57 \times 10^6$ U/mg of the control. The T10 bioassay may be not sensitive in detecting low levels of degradation by-products, since the observed difference falls within the variability of the assay and is not considered as

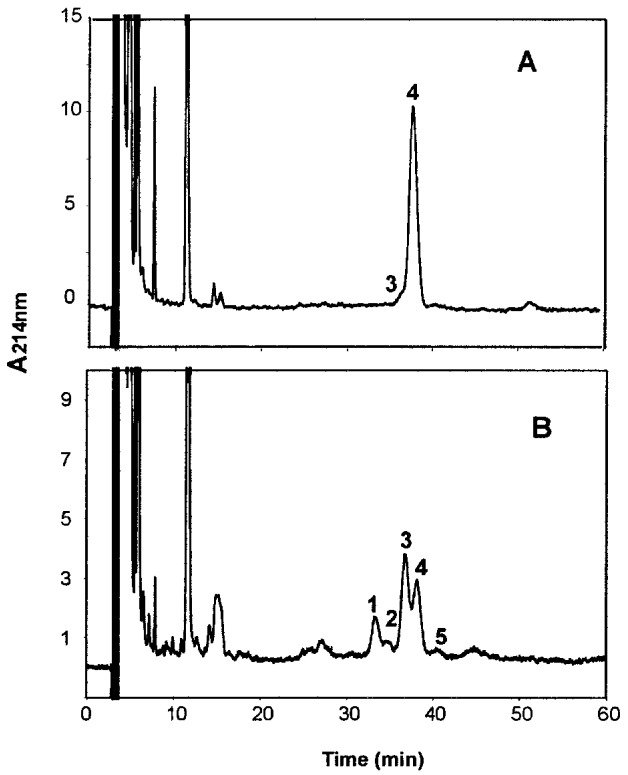


Fig. 6. Isocratic elution of rhIL-11 trypsin peptides. (A) The rhIL-11 control sample. (B) The rhIL-11 stressed sample. Five peaks were resolved in the T6T7 peptide region and were labeled from 1 to 5.

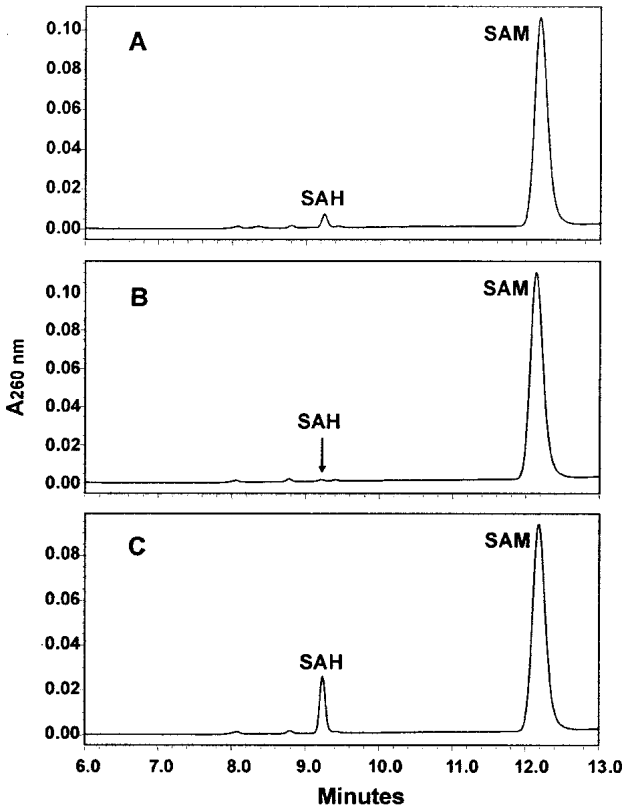


Fig. 7. SCX-HPLC profiles of PIMT enzymatic reactions. The injection volumes were 20 μl. SAH and SAM peaks are labeled. (A) isoAsp-DSIP. (B) The rhIL-11 control sample. (C) The rhIL-11 stressed sample.

statistically significant. Therefore, the effect of Asp isomerization and Asn deamidation on the bioactivity of rhIL-11 is not clear at this time.

DISCUSSION

Deamidation of Asn and isomerization of Asp can be major degradation pathways in proteins (1–7). While deamidation/isomerization does not alter the biologic potency of some protein pharmaceuticals (4,7,19,20), there are a number of cases in which deamidation of Asn and/or isomerization of Asp is related to a protein's decreased bioactivity (4,7,21–23). Whether Asn deamidation and Asp isomerization may result in loss of efficacy of protein drugs or not, these degradation reactions and their major product, isoAsp, could raise concerns to pharmaceutical industry and regulatory agencies. It has been reported that accumulation of isoAsp in cellular proteins leads to early death of PIMT-deficient mice (24), and recent findings suggest that isoAsp may play a role in autoimmunity (25). The conformation of proteins may be distorted by deamidation and isomerization (26,27). The dysfunctional isoAsp variants can be potential undesirable antagonists by competing with normal molecules in binding to therapeutic targets (7).

Characterization of Asn deamidation and Asp isomerization is challenging. Detection and quantification of isoAsp arising from Asp isomerization is particularly difficult, since only one peptide bond bears a subtle rearrangement without charge alteration or change in mass. In this study, Asn deamidation and Asp isomerization in rhIL-11 under stressed conditions were detected by using a variety of techniques. Asp^{45} and Asp^{47} were found to isomerize at a significant level while Asp^{51} isomerization was detected at a lower level. Deamidation of Asn^{49} occurred at a low level, and it caused peptide bond cleavage. The total amount of isoAsp in rhIL-11 measured by a non-radioactive HPLC method correlated well with the results from peptide mapping and CE experiments. All these Asp residues are followed by amino acids with small side chains (glycine, histidine, and serine). Asp^{40}, on the other hand, is followed by lysine, and was not detected to undergo isomerization. In addition, according to the proposed model of rhIL-11 tertiary structure (14), the region encompassing residues 35 to 68, where all the above Asp/Asn residues are clustered, is expected to form a flexible loop structure (Fig. 1). These structural features of rhIL-11 facilitate Asp isomerization and Asn deamidation (10–13). The techniques described in this paper enabled us to analyze Asp isomerization/Asn deamidation and subsequent isoAsp formation, and they should be of general applications to characterization of other protein pharmaceuticals.

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