PEGylation Prevents the N-Terminal Degradation of Megakaryocyte Growth and Development Factor

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Purpose. Determine the effect of PEGylation on *in-vitro* degradation for recombinant human Megakaryocyte Growth and Development Factor (rHuMGDF) in the neutral pH range.

Methods. Degradation products were characterized by cation-exchange HPLC, N-terminal sequencing and mass spectrometry.

Results. The main route of degradation was through non-enzymatic cyclization of the first two amino acids and subsequent cleavage to form a diketopiperazine and des(Ser, Pro)rHuMGDF. This reaction was prevented by alkylation of the N-terminus by polyethylene glycol (PEG).

Conclusions. PEGylation of proteins is commonly performed to achieve increased *in-vivo* circulation half-lives. For rHuMGDF, an additional advantage of PEGylation was enhanced *in-vitro* shelf-life stability.

KEY WORDS: cytokine; rHuMGDF; PEGylation; diketopiperazine; stability.

INTRODUCTION

Mpl ligand is a recently discovered hematopoietic factor that specifically regulates platelet production (1–3). This cytokine, also known as thrombopoietin or megapoietin, functions by inducing progenitor cells to form megakaryocytes and to stimulate megakaryocytes to differentiate into platelets. The search for a specific regulator of platelet production lasted almost 40 years (4,5). The receptor for Mpl ligand is a member of a super-family of cytokine receptors with extensive amino acid homologies that include the receptors for erythropoietin, granulocyte colony-stimulating factor, and interleukin-3 (6,7). The ligand for the Mpl receptor has recently been cDNA cloned, expressed by heterologous gene expression, and purified to homogeneity. Mpl ligand is currently being developed for pharmaceutical applications for regulating and restoring platelet counts (8). Potential indications include the prevention and treatment of thrombocytopenia associated with chemotherapy, irradiation, and bone-marrow transplantation.

The cDNA for the human Mpl ligand encodes for a primary translation product of 353 amino acids consisting of a 21-amino acid signal peptide and a mature protein of 332 amino acids that is composed of two domains (9). The amino-terminal domain shows 25% sequence identity (50% similarity) to the red blood cell stimulating cytokine, erythropoietin (9). Both Mpl ligand (N-terminal domain) and erythropoietin have four cysteines of which three are conserved, including the first and last, that in erythropoietin form an essential disulfide bond. Based on sequence analogy with erythropoietin, the N-terminal domain of Mpl ligand is predicted to adopt a four α-helical conformation similar to that of erythropoietin and other related cytokines (9).

The biological activity of the Mpl ligand resides in the N-terminal domain (9). Recombinant forms of truncated Mpl ligand that are missing the C-terminal domain are competent for receptor binding and the proliferation and maturation of megakaryocytes *in-vitro* (9). The C-terminal domain has no sequence similarity to any known protein and its function remains to be elucidated. However, the C-terminal domain is highly glycosylated and may act to increase the half-life of circulating Mpl ligand (10). A truncated version of the native Mpl ligand containing the N-terminal domain, specifically referred to as megakaryocyte growth and development factor (MGDF), is the form of Mpl ligand used throughout this report. MGDF was derived from recombinant DNA technology and the human version is designated as rHuMGDF.

The *in-vivo* circulating half-life of rHuMGDF was increased by covalent attachment of polyethylene glycol (PEG) at the N-terminal amine, without diminishing its biological activity (11–13). We have investigated the shelf-life stability of both rHuMGDF and PEG-rHuMGDF under identical conditions. The principal degradation route of the rHuMGDF in neutral pH solutions was observed as a new peak by cation-exchange chromatography which was not present for PEG-rHuMGDF. This paper will describe the characterization of this main degradation reaction and its inhibition by PEGylation.

MATERIALS AND METHODS

Materials

The N-terminal domain (1,9) of Mpl ligand, residues 1–163, was obtained by heterologous gene expression in *E. coli* (14,15) and was prepared by Amgen Inc. PEG-rHuMGDF was prepared at Amgen by the covalent attachment of polyethylene glycol (PEG) to the N-terminal amine of rHuMGDF, see Scheme 1 (11–13,16). All other reagents were of analytical grade or better, and de-ionized double-distilled water, Milli-Q-grade, was used throughout this study.

$$CH_3(-O-CH_2-CH_2)_n-O-CH_2CH_2CH_2-NH-MGDF$$

Scheme 1. PEGylation at the α-amino group by alkylation, resulting in a secondary amine linkage.

Methods

Stability

rHuMGDF and PEG-rHuMGDF were formulated at 0.5 mg/ml in 10 mM phosphate, 150 mM NaCl, and pH 7. A mixture containing equal concentrations of rHuMGDF and PEG-rHuMGDF was also prepared in the same buffer system. Samples were filtered through a 0.2 μm nylon filter and dis-

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pensed into sterile type I, 3 cc glass vials at 0.5 ml volumes, stoppered with silicon coated rubber stoppers, and stored at −80°C, 29°C, and 37°C.

Cation-Exchange Chromatography

A Hewlett Packard 1050 Liquid Chromatography System, with a TSKgel SP-5PW cation-exchange column, a flow rate at 0.7 ml/min, absorbance monitored at 215 nm, and protein loaded at 20 μg per injection were used for analysis. Chromatography consisted of an isocratic mobile-phase for 10 min of 25 mM sodium chloride, 20 mM sodium phosphate, 15% glycerol (v/v) at pH 7.1, followed by a 44 min linear gradient that reached a final mobile-phase condition of 500 mM sodium chloride, 20 mM sodium phosphate, 15% glycerol (v/v) at pH 7.1. Main peak purity was determined by dividing the area of the main peak by the total area of all integrated peaks. For the mixture formulation, composed of rHuMGDF and PEG-rHuMGDF, only the corresponding peaks related to each individual component were used to determine peak purity. In all cases, the recovery of protein eluted was also compared to a standard and was typically 95% with a standard deviation of ±10%.

Mass Spectrometry

(LC-MS)

A Sciex API 100 mass spectrometer with an ion-spray interface was used for the molecular weight determination of degraded species. Analyses were performed on a Vydac C_{18}, 90 Å, 2.1 × 250 mm, 5 μm, Pharmaceutical reversed-phase column with a flow rate of 0.2 ml/min. Peaks were eluted by an isocratic mobile phase of 0.1% TFA (w/v), in water for 2 min, followed by a 20 min linear gradient of 0% to 27% acetonitrile, containing 0.1% TFA (w/v). The column effluent was split approximately 1:10 and directed to the Sciex API 100 system. The data were obtained by scanning from 100 to 600 Da for peptides and 100 to 2950 Da for protein with a scan time of 4 s and a step size of 0.1 Da. In addition, the elution profile was monitored by UV absorbance at 215 and 280 nm. The molecular mass of the protein was determined on a Finnigan Mat TSQ 7000 triple quadrupole mass spectrometer fitted with an electrospray source. The mass spectrometer was interfaced directly to the column effluent of a Hewlett Packard 1090 HPLC.

N-Terminal Sequencing

Amino acid sequence from the N-terminus was determined by Edman degradation chemistry using either a Hewlett Packard G1005A sequencer or an ABI Procise 494 HT sequencer.

Preparation of PEG-rHuMGDF

A five-fold molar excess of methoxypolyethylene glycol aldehyde (average molecular weight 20 kDa) was mixed with a solution containing rHuMGDF (>1 mg/ml), 100 mM sodium phosphate, pH 5, 20 mM sodium cyanoborohydride, and 4°C. After 16 hr, size-exclusion HPLC analysis, using a Superdex 200HR 10/30 column with a mobile-phase of 0.1 M sodium phosphate, pH 6.9, and a flow rate of 0.7 ml/min, indicated that 90% of the protein had been modified. The PEGylated conjugate was diluted to 1 mg/ml and adjusted to pH 4. The conjugate was isolated using ion-exchange HPLC developed with a linear gradient from 0% to 25% of 20 mM sodium phosphate, pH 7.2, and 15% glycerol initially and 1 M NaCl, 20 mM sodium phosphate, pH 7.2, and 15% glycerol as the second buffer. MonoPEGylated protein fractions were identified by SDS-PAGE and appropiate fractions were pooled, concentrated, and sterile filtered.

Peptide Mapping of rHuMGDF and PEG-rHuMGDF

A tryptic digest of both rHuMGDF and PEG-rHuMGDF was done to verify the identity of each protein. Each protein was dialyzed into 100 mM sodium phosphate, pH 7.8, and 0.2 mg/ml. The trypsin solution at 0.5 μg/μl was prepared immediately before use and added to each protein sample to an enzyme:substrate ratio of 1:40 by weight. Digestion was carried out at 37°C for 18 hours then promptly analyzed with a gradient HPLC system. The chromatography consisted of a 2% to 92% linear gradient over 90 min of acetonitrile in water with 0.1% (v/v) trifluoroacetic acid. The flow rate was 0.2 mL/min at 40°C using a YMC C-8 bonded silica column (0.2 × 25 cm), particle size 5 μm, 300 Å pore size. The peptide maps of the two protein samples are shown in Fig. 1. The two maps are the same, within experimental error, except for the PEG-

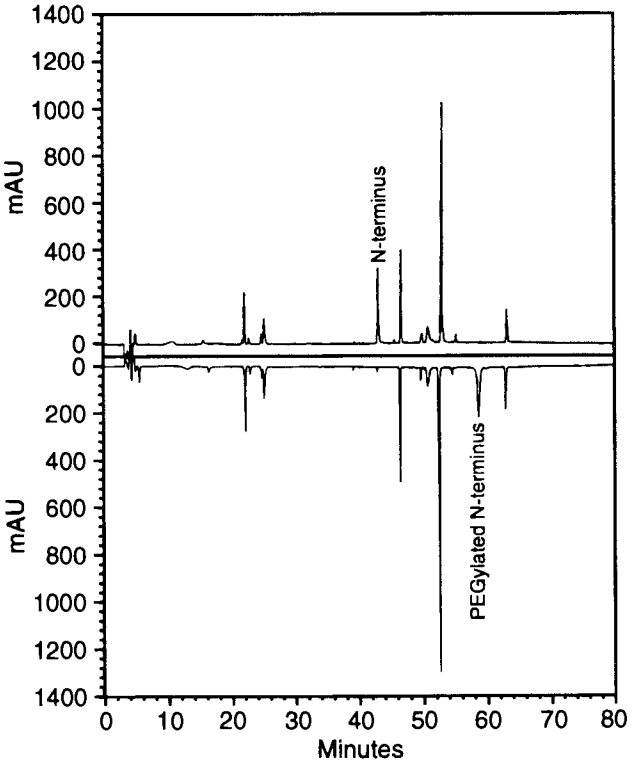


Fig. 1. Representative reversed-phase HPLC chromatograms of a tryptic digest for both rHuMGDF (top chromatogram) and PEG-rHuMGDF (bottom chromatogram) protein samples. The bottom chromatogram demonstrates the loss of peak at 43 min, which represents the tryptic peptide containing N-terminus, and the presence of a peak at 59 min, which corresponds to the tryptic peptide containing PEGylated N-terminus.

rHuMGDF sample is missing the peak that elutes at 43 min and contains a new peak that elutes at 59 min. The peak that eluted at 43 min (Fig. 1 upper chromatogram) was collected and had a mass of 2420 Da that corresponds to the N-terminal tryptic peptide (expected mass of 2420 Da). The peak that eluted at 59 min (Fig. 1 lower chromatogram) was collected and determined by sequence analysis and mass spectrometry to be consistent with the N-terminal tryptic peptide with an attached PEG group. The tryptic peptide maps show that only the N-terminal peptide contains the PEGylated moiety.

N-terminal sequencing of PEG-rHuMGDF (see Results, Table I) shows the absence of Ser (the first amino acid) for the first cycle. The absence of Ser in the first sequencing cycle was due to the attached PEG-moiety at the α-amino group that remained intact through the sequencing reaction and was unrecoverable. The sequencing result demonstrates that PEG-rHUMGDF contains a PEG-moiety attached at the N-terminus.

RESULTS

Stability

The stability of rHuMGDF and PEG-rHuMGDF was monitored by several different analytical methods including SDS-PAGE, size-exclusion HPLC, reversed-phase HPLC, and cation-exchange HPLC. At neutral pH, the principal degradation product for rHuMGDF was des(Ser, Pro)rHuMGDF which was detected by cation-exchange liquid chromatography. The formation of other degradation products were not significantly altered by PEGylation.

The shelf-life stability of rHuMGDF, PEG-rHuMGDF, and a mixture of both proteins were compared by cation-exchange chromatography at different temperatures (Figs. 2 and 3). The main degradation species of rHuMGDF at elevated temperatures eluted immediately after the main peak with an elution time of 37 minutes (Fig. 2a). PEG-rHuMGDF treated identically did not contain a similar degradation species (Fig. 2b). The loss of main peak at 29°C and 37°C as measured by cation-exchange chromatography for rHuMGDF and PEG-rHuMGDF are shown in Fig. 3. PEG-rHuMGDF demonstrates significantly less degradation than rHuMGDF. The small amount of degradation observed for PEG-rHuMGDF was a result of degradation reactions other than the N-terminal cleavage reaction. Degradation profiles for each protein were similar whether the formulation contained the individual proteins or from a mixture formulation that contained both proteins (Fig. 3). Therefore, any differences in the stability of rHuMGDF and PEG-rHuMGDF were inherent to the proteins and were not due to other components that may be present in the different preparations.

Characterization

Degraded samples of rHuMGDF were produced by incubation at elevated temperatures for several weeks to a few months and analyzed by cation-exchange HPLC (Fig. 2), reversed-phase LC/MS (Fig. 4), and N-terminal sequencing (Table I). N-terminal sequencing was done on both rHuMGDF and PEG-rHuMGDF stored for two weeks at −80°C and 37°C. The sequence obtained for the first six amino acids of the control sample (−80°C) was SPAPPA, corresponding to intact rHuMGDF. The sequence for the heat stressed rHuMGDF (37°C) resulted in two sequences with two N-terminal amino acids, Ser corresponding to intact rHuMGDF (57% of sample) and Ala corresponding to des(Ser, Pro)rHuMGDF (43% of sample). The ratios of the sequences were consistent with the percentages of main and degraded peaks observed by cation-exchange HPLC. Only a single sequence was obtained for PEG-rHuMGDF for both the −80°C and 37°C samples and had the expected sequence except for the absence of the N-terminal amino acid. The residue of the first cycle for the PEG-rHuMGDF was unrecoverable due to the N-terminally attached PEG moiety. For a sample stored at 29°C for 21 days, the peak eluting immediately after the main peak on cation-exchange HPLC (Fig. 2a) was collected and determined to have a mass of 17,249 Da by mass spectrometry, which is the mass expected for des(Ser, Pro)rHuMGDF (17248 Da). A sample degraded for four months at 37°C contained a reversed-phase LC/MS

Table I. Percent Residue Recovery from N-Terminal Sequencing of rHuMGDF and PEG-rHuMGDF Incubated for 2 Weeks at −80°C or 37°C

	rHuMGDF				PEG-rHuMGDF			
Cycle	−80°C 1°[a] (%: AA)[b]	−80°C 2° (%: AA)	37°C 1° (%: AA)	37°C 2° (%: AA)	−80°C 1° (%: AA)	−80°C 2° (%: AA)	37°C 1° (%: AA)	37°C 2° (%: AA)
1	19: S	—	26: S	20: A	26: P	—	23: P	—
2	7: P	—	*29: P*	*29: P*	29: A	—	29: A	—
3	9: A	—	19: A	14: P	25: P	—	23: P	—
4	6: P	—	18: P	18: A	24: P	—	22: P	—
5	6: P	—	14: P	ND	24: A	—	23: A	—
6	4: A	—	18: A	24: D	ND	—	ND	—
7	ND[c]	—	ND	20: L	24: D	—	21: D	—
8	4: D	—	24: D	ND	31: L	—	25: L	—
9	4: L	—	18: L	14: V	21: R	—	19: R	—

[a] Refers to primary and secondary sequences as determined from recovery data.
[b] AA refers to amino acid. Automated N-terminal sequencing by Edman degradation was performed with approximately 10 pmol of protein for rHuMGDF at −80°C, PEG-rHuGMDF at −80°C and 37°C and 40 pmol of rHuMGDF at 37°C. Percentages are calculated as recovery from the estimated load.
[c] Not determined.

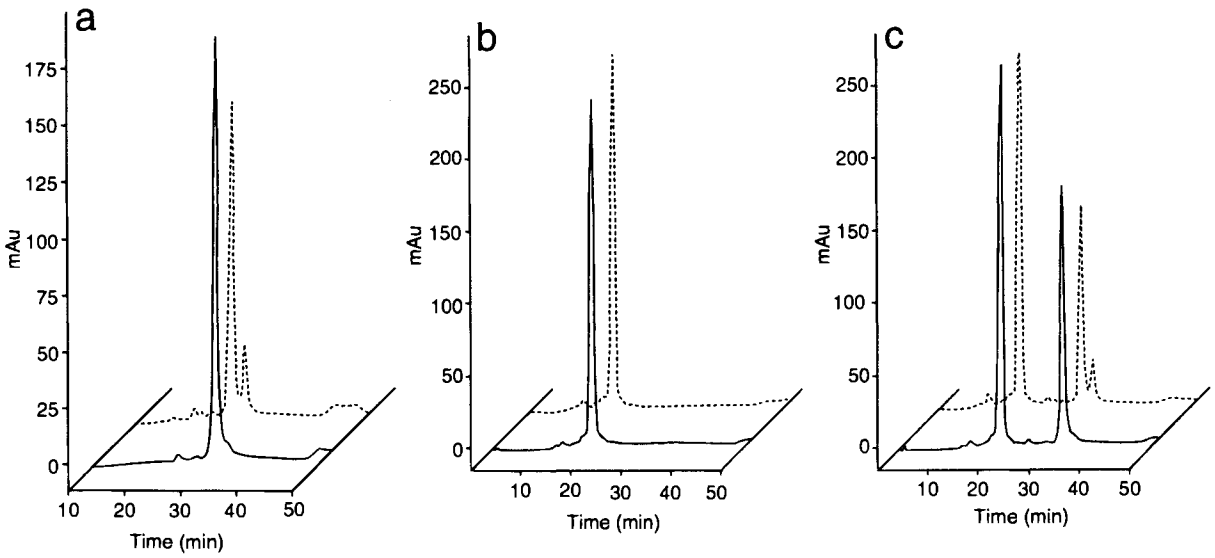


Fig. 2. Representative cation-exchange HPLC chromatograms of a) rHuMGDF, b) PEG-rHuMGDF, and c) a mixture formulation of both proteins. Proteins were in 10 mM phosphate, 150 mM NaCl, pH 7 and stored for 21 days. The solid lines represents samples stored at −80°C, and the dashed lines represents samples stored at 29°C. rHuMGDF eluted at 35 min, and PEG-rHuMGDF eluted at 22 min in both individual and mixture formulated samples. Elution of des(Ser, Pro)rHuMGDF was 37 min.

peak that eluted at 15 min (see Fig. 4, dashed-chromatogram) with a mass of 185.1 Da which corresponds to the expected mass of SerPro-diketopiperazine (184.2 Da). The majority of the protein from this sample (4 months at 37°C) had precipitated and was removed by filtration prior to analysis. However the SerPro-diketopiperazine was soluble and eluted at 15 min by reversed-phase LC/MS.

A synthetic hexapeptide (SPAPPA) that corresponds to residues at the N-terminus of rHuMGDF, was formulated in 10 mM phosphate, 150 mM NaCl, pH 7 and was monitored under the same stability conditions as rHuMGDF and PEG-rHuMGDF. The synthetic hexapeptide degraded into two components that eluted earlier than the main peak (Fig. 5, dashed chromatogram) when analyzed by LC/MS. The main peak (elution at 24 min) represented the starting peptide SPAPPA with a mass of 540 Da, and is in agreement with the calculated mass of 540 Da. The peak eluting at 18 min was confirmed as the des(Ser, Pro)APPA peptide with a mass of 355 Da and the peak eluting at 15 min had a mass of 185 Da, and is in agreement with the expected mass of SerPro-diketopiperazine (187 Da).

DISCUSSION

Enhanced Stability as a Result of PEGylation

PEGylation of proteins is a common strategy to achieve an increased *in-vivo* circulation time and perhaps decreased

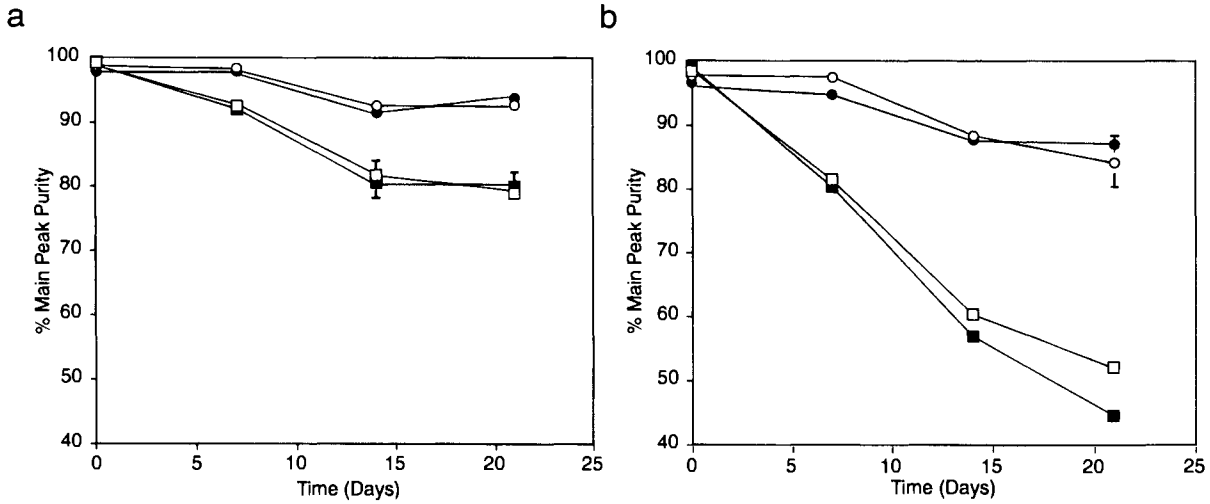


Fig. 3. Degradation profiles for rHuMGDF and PEG-rHuMGDF, a) at 29°C and b) at 37°C, as determined from cation-exchange HPLC. Graphs represent percent main peak detected over time. Proteins were stored in 10 mM phosphate, 150 mM NaCl, and pH 7; (-□-) rHuMGDF, (-○-) PEG-rHuMGDF and both (-■-) rHuMGDF, and (-●-) PEG-rHuMGDF from a mixture formulation. Error bars represent percent deviation of duplicate injections.

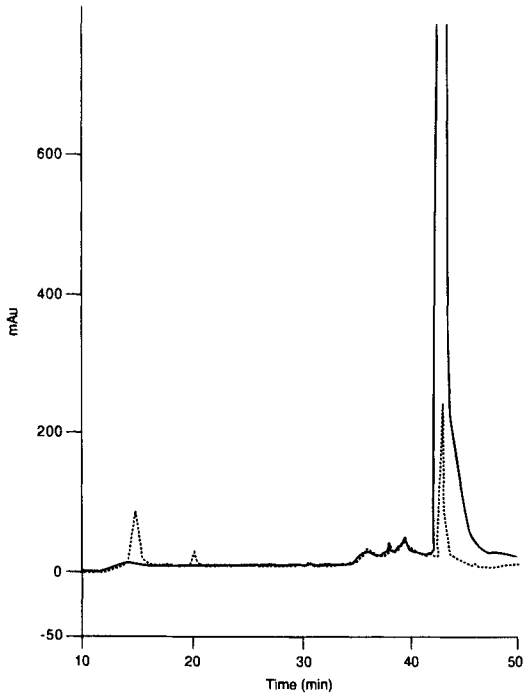


Fig. 4. Representative reversed-phase LC/MS of rHuMGDF in 10 mM phosphate, 150 mM NaCl, at pH 7 degraded for 4 months at 37°C. The degraded sample was filtered through a 0.45 μm Nylon filter to remove precipitates that were present. The chromatogram with a solid line represents rHuMGDF (eluted at 42 min) stored at −80°C, and the dashed line represents 37°C degraded rHuMGDF containing main peak (eluted at 42 min) along with SerPro-diketopiperazine (eluted at 15 min) with a mass of 185.1 Da.

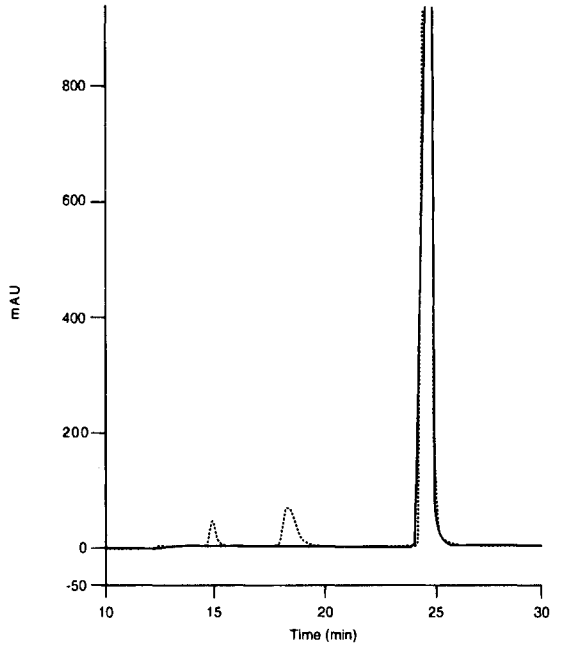


Fig. 5. Representative reversed-phase LC/MS chromatogram of hexapeptide (SPAPPA) stored in 10 mM phosphate, 150 mM NaCl, pH 7. The solid line represents peptide stored at −80°C and the intact peptide eluted at 45 min. The dashed line represents the peptide, degraded due to storage at 37°C. The peaks observed represent SPAPPA (24 min), des(Ser, Pro)APPA (18 min), and SerPro-diketopiperazine (15 min), with determined masses of 539.5 Da, 355.2 Da and 184.9 Da, respectively.

immunogenicity. rHuMGDF was specifically PEGylated at the α-amino group of residue one. PEGylation of rHuMGDF was accomplished by reductive alkylation resulting in a secondary amine linkage as illustrated in Scheme 1. PEGylation of rHuMGDF inhibited the degradation that was observed by cation-exchange HPLC (Fig. 2). To determine if the stability observed for PEG-rHuMGDF was inherently due to the attached PEG or a serendipitous result of other preparative procedures associated with the PEGylation attachment, a mixture formulation containing both PEG-rHuMGDF and rHuMGDF was tested. The results in Fig. 3 show an identical increased stability for PEG-rHuMGDF as a mixture or alone. Therefore, the increased stability of PEG-rHuMGDF is directly due to the presence of an N-terminally attached PEG moiety.

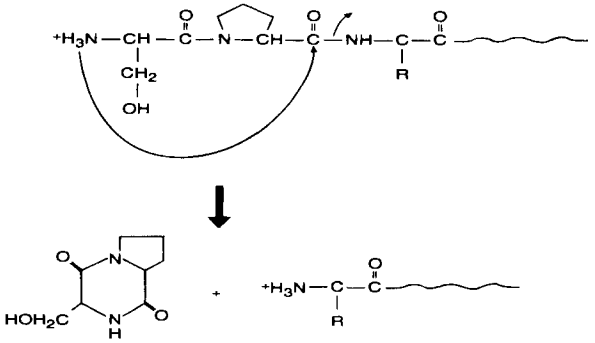
Characterization of Diketopiperazine Formation

In a neutral buffer system, rHuMGDF produces a degradation product that elutes slightly later than the rHuMGDF main peak, which was observed by cation-exchange HPLC (see Fig. 2a). This later eluting peak was shown to be composed of des(Ser, Pro)rHuMGDF, according to mass spectrometry analysis (see Results). Sequence analysis of degraded rHuMGDF contained two N-terminal sequences, and is consistent with the formation of des(Ser, Pro)rHuMGDF and (Ser, Pro)diketopiperazine (17). In addition, the existence of (Ser, Pro)diketopiperazine from degraded rHuMGDF was determined by LC/MS (see Results and Fig. 4).

The possibility exists, that degradation of rHuMGDF (Figs. 2–4) is due to the presence of an endoproteolytic contaminant from the *E. coli* purification rather than the proposed non-enzymatic cleavage reaction. However, when a synthetically prepared peptide (SPAPPA), that corresponds to the N-terminal region of rHuMGDF, was formulated and monitored under the same conditions, the results (Fig. 5) confirm the same specific cleavage between residues 2 and 3. The synthetic hexapeptide would not contain an endoproteolytic contaminant and therefore the N-terminal degradation reaction observed for rHuMGDF occurred by the chemical reaction illustrated in Scheme 2.

Diketopiperazine Formation of Proteins in General

In the field of peptide synthesis the formation of diketopiperazines has been extensively studied. However the formation of diketopiperazine in proteins is much less studied. Battersby



Scheme 2. Diketopiperazine formation and cleavage of the first two N-terminal amino acids (Ser and Pro) in rHuMGDF.

et al. (17) identified the formation of diketopiperazine and subsequent loss of the first two amino acids for human growth hormone. The amino acid sequence is critical to the rate of diketopiperazine formation. When the second amino acid from the N-terminus is proline, spontaneous formation may be expected of a diketopiperazine and cleavage of the polypeptide bond between residues two and three. The occurrence of proline as the second amino acid in rHuMGDF is the likely reason for the rapid diketopiperazine formation. A search through the protein data bank (Wisconsin Sequence Analysis Package, Genetics Computer Group) identified ~2000 proteins that contain proline as the second residue. This corresponds to ~4% of the protein population, about the same frequency of occurrence for proline in general. Therefore, no obvious physiological preference or selective mechanism for a labile penultimate proline residue is apparent.

We conclude that *in-vitro* degradation of rHuMGDF, in the neutral pH range through non-enzymatic cyclization of the first two amino acids and subsequent cleavage to form Ser,Pro diketopiperazine was prevented by alkylation of the N-terminus by polyethylene glycol (PEG). This conjugation of the α-amino group with PEG has the added advantage of increasing the *in-vitro* shelf-life of rHuMGDF.

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