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Research Article

Major Degradation Products of Basic Fibroblast Growth Factor: Detection of Succinimide and Iso-aspartate in Place of Aspartate[15]

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The degradation products of basic fibroblast growth factor (bFGF) were isolated by ion exchange HPLC (HP-IEC) and characterized. The predominant product at pH 5 was a succinimide in place of aspartate[15] as determined by LC/MS, N-terminal sequencing, and susceptibility to degradation at pH >6.5. The rate of appearance of the succinimidyl-bFGF at 22 °C was comparable to that reported for small peptides, consistent with a high flexibility predicted for asp[15]-gly. Tryptic mapping together with [^{3}H]-methylation indicated that iso-aspartate was formed at the position of asp[15]. Size exclusion HPLC indicated the presence of intact and truncated dimers and trimers which associated through disulfide linkages. Two truncated monomer forms were found that co-eluted by HP-IEC; the cleavages were determined to be at asp[28]-pro and asp[15]-gly using LC/MS and N-terminal sequencing. These degradation products which occurred at sites that are away from receptor or heparin binding domains of bFGF remained bioactive in a cell proliferation assay.

KEY WORDS: protein; degradation; succinimide; iso-asp; ion exchange HPLC; LC/MS.

INTRODUCTION

Identification of the degradation products of protein pharmaceuticals is important in optimizing formulations and defining product constituents over the course of their shelf lives. A number of well-defined pathways for protein degradation have been reviewed (1,2). The major aging processes include aggregation/precipitation, oxidation of cysteines and methionines, deamidation of asparagines, peptide bond cleavages at aspartate residues, and aspartate isomerization to iso-aspartate. Deamidation at asparagine residues, which occurs at neutral to alkaline pH's, involves formation of a succinimide intermediate followed by loss of ammonia (1–5). In addition to this modification, peptide chain cleavage at aspartate residues occurs at acidic pH with the highest rate seen when asp precedes proline (5,6). Isomerization to iso-aspartate residues (with insertion of a methylene group into the peptide chain) involves the formation of a succinimide intermediate with loss of a water molecule (7–9). Formation of this cyclic intermediate is a result of the attack of the deprotonated peptide bond nitrogen of the n+1 position onto the β-carbonyl carbon of the ionized aspartate side chain with a pH optimum of 4-5 (4,8–10). Whereas such a mechanism has long been known in small peptides, only in recent years has a stable succinimide intermediate in proteins been identified (8–10).

In this report we describe identification of the major degradation products of human basic fibroblast growth factor (bFGF). bFGF is a globular single chain protein of 155 amino acids (11) which has 12 antiparallel β-strands that are folded into a β-barrel structure with three-fold axis of symmetry (12). There are four free thiols, two of which are solvent-accessible and two are buried. The protein also has two solvent-exposed methionines, 7 aspartates, and 5 asparagines, all of which are potential sites of degradation. Three of the aspartates and one of the asparagines are followed by glycine and are located in regions of the protein that are predicted to have high flexibility; both of these conditions are thought to favor succinimide formation (1,8–9). We report here that other than aggregation and precipitation (13), the predominant degradation product of bFGF at pH 5 is a succinimide at asp[15] which is not detectable at pH 6.5. Other degradation products include species cleaved at asp[28]-pro and at asp[15]-gly, as well as disulfide-linked dimers and trimers.

MATERIALS

Recombinant human bFGF was produced at Scios Nova. Ammonium sulfate and disodium ethylenediaminetet-

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raacetic acid (EDTA) were from Sigma. Dithiothreitol (DTT) was from Research Organics. Spectrograde trifluoroacetic acid (TFA) from Pierce, and HPLC grade acetonitrile (ACN) from Baxter. TPCK-treated trypsin was from Sigma. Protein L-isoaspartyl methyltransferase (PIMT) was prepared according to Henzel et al. (14). Bovine adrenal cortex capillary endothelial cells (ACE) at passage levels 6 through 9 were obtained from in-house cell bank. Gelatin was purchased from Sigma (G-2500). Bioassay medium (DMEM: HAM'S F-12, 1:1, 2mM glutamine) was from Gibco and 1% bovine calf serum from Hyclone.

METHODS

bFGF was isolated from an E. coli expression system essentially as described by Thompson et al. (11). The purified material, with methionine 1 truncated, was 95–98% monomeric by various HPLC methods. A solution of 2 mg/ml bFGF was made either in a citrate-EDTA, pH 5 buffer or a phosphate-EDTA, pH 6.5 buffer. After storage of the pH 5 solutions at 25°C for 13 weeks, 8 ml sample was filtered through 0.2 μm Acrodisc filters to remove precipitates (~25% of total protein). The protein content of the solution was determined by UV spectroscopy. Absorption spectra from 200 to 500 nm were obtained on a Cary 3 spectrophotometer (zeroed at 500 nm) against appropriate reference controls. An $E^{0.1\%}$ (277 nm) of 0.84 $ml.mg^{-1}.cm^{-1}$ was used to determine bFGF concentration.

12 mg aged protein (filtered soluble fraction) was applied to an ion exchange HPLC (HP-IEC) column (poly-Cat A poly-aspartate anion exchange column, 7.5 mm i.d. × 75 mm length; 5 μm particle size; Poly-LC Inc.). Samples were eluted with a linear gradient at 3.4 mM/min ammonium sulfate. Fifty fractions (~1 ml) were collected on ice while monitoring sample absorbance at 288 nm to prevent detector saturation. The protein concentration of each fraction was then determined, the salt and protein concentrations were equalized, and the samples were re-analyzed by HP-IEC shortly after isolation using a 6.8 mM/min gradient. The remainder of the samples were aliquoted and stored at −70°C until further analysis.

Size exclusion HPLC (HP-SEC) was conducted on a Bio-sil TSK-125 column (7.8 mm i.d. × 250 mm length) using 100 mM phosphate, 1 mM EDTA, 1 M NaCl as the mobile phase. Molecular weight was calculated using a reference protein mix (Sigma) containing γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa).

The exact molecular mass was determined by LC/electrospray MS using a Finnigan TSQ700 system. 20-50 pmol sample was injected onto a C18 reverse phase fused silica column (250 μ i.d. × 20 cm length) at 2 μL/min and eluted using an acetonitrile gradient (2%/min). The electrospray needle was operated at a voltage differential of 3—4 kV and a sheath flow of methoxyethanol was used. Scan range was set at 500–2000 and data were acquired every 3 sec. Mass was calculated using Finnigan MAT Biomass Data Reduction software. Cytochrome C (12,361 amu) was used as an external standard for mass calibration.

N-terminal sequencing on 0.5–1 nmol samples was performed on an ABI 477A sequencer using PTH derivatization and standard ABI cycles. Samples were desalted with water and dried on a pro-spin cartridge (from ABI) prior to analysis.

Tryptic digestion of bFGF was done on 0.5 mg/ml protein at pH 8 and 37°C. Initial digestion was made at a 1:100 ratio of trypsin:bFGF for 2 hrs, then the same amount of trypsin was added and incubation was continued for another 2 hr. Samples for peptide mapping were quenched with 2% TFA. Samples for iso-asp determination were quenched with 4 mM phenylmethylsulfonylfluoride (PMSF). Peptide mapping was conducted on a Vydac C18 reversed phase column using a flow rate of 1 ml/min and a complex acetonitrile/TFA gradient (starting at 2% ACN for 2 min followed by 0.4% ACN/min up to 14 min, 0.25%/min up to 18 min, 0.5%/min up to 46 min, 0.25%/min up to 54 min, 1.4%/min up to 76 min and then down to 2% ACN at 80 min). Peptide identity had previously been determined by sequencing and amino acid composition (Hawes et al., personal communication).

For quantitative measurements of iso-aspartyl content, bFGF or its fragments were methylated for 30 min at 30°C and pH 6.8 in the presence of PIMT. Reactions were carried out using 5 μM bFGF, 1 μM PIMT, and 50 μM [^{3}H-methyl]AdoMet. Methylation reactions were terminated by adding an equal volume of a buffer containing 0.4 M sodium borate, pH 10, 5% (w/v) sodium dodecyl sulfate, and 2.2% methanol. Methyl incorporation was determined using a methanol diffusion assay as described previously (15). Methylation reactions of PMSF-quenched digests destined for RP-HPLC analysis were performed for 20 min at 30°C and pH 6.1, terminated by adding 1/10th volume of 85% phosphoric acid, and kept frozen at −15°C. Upon thawing, they were immediately injected onto RP-HPLC. Fractions were collected and subjected to liquid scintillation counting in order to evaluate methyl incorporation of individual tryptic fragments. Fractions for post-column methylation were dried down in 5% glycerol and resuspended in 150 μl diffusion assay buffer, pH 6.8. 20 μl portions were tested for methyl accepting capacity using methanol diffusion assay as described above.

Biological activities of the purified fractions were assessed in a proliferation assay using ACE cells (16,17). Briefly, 10^3 cells/well were seeded in 190 μl of assay medium in 96 well plates. Three-fold dilutions of the test fractions and a bFGF reference standard were prepared in phosphate buffered saline containing 0.2% gelatin and 10 μl were added to each well to produce a titration curve ranging from 378 ng/ml to 0.006 ng/ml protein. Cultures were incubated at 37°C in 10% CO_2 for 72 h and processed as described (17). The ED_{50} (50% maximum effective dose) was calculated using a four parameter fit to the dose-response curve (18). The biological activity of each test fraction was expressed as a percentage of the ED_{50} of the bFGF reference sample.

RESULTS

HP-IEC profiles: By HP-IEC, fresh bFGF was ~98% main peak (Fig. 1A). Incubation in citrate buffer, pH 5 at 25°C resulted in the appearance of a major component which eluted ~1.4 min later than the main peak (Fig. 1B). Moreover, there was an increase in a number of minor components that eluted at ~16 min and ~17–19 min later than main

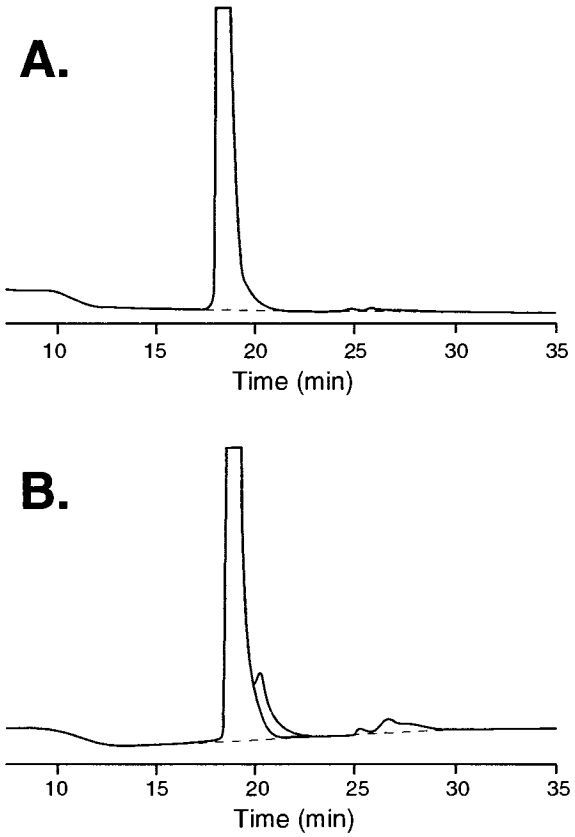

Figure 1: HP-IEC profiles of bFGF. A) fresh sample; B) sample aged at pH 5 (25°C, 13 weeks). A 6.8 mM/min ammonium sulfate gradient at pH 6 was used.

peak (Fig. 1B). The major degradation product was detected as early as 1 week at 4°C; by 13 weeks at 25°C, this component was 12% of soluble protein. However, bFGF incubated at pH 6.5 did not show this product (not shown).

Monomer-multimer Distribution by HP-SEC: In order to identify bFGF degradation products, aged bFGF was fractionated by HP-IEC (Fig. 2A). By HP-SEC, the apparent molecular weights of fresh monomeric and dimeric bFGF were calculated to be 13.5±0.4 kDa (which is less than the theoretical value of 17.1 kDa) and 29.6±0.5 kDa, respectively. Fractions 2 through 8 all eluted at a position with an apparent molecular weight of 13.7 kDa, suggesting that they were monomeric bFGF (F5 shown in Fig. 2B). F10–14 (~1.4 min post-main peak) also eluted as a monomer (F12 shown in Fig. 2B). F22–24 (~6.5 min post-main peak) eluted as a truncated monomer missing ~3700 Da (F23 shown in Fig. 2B). F27–33 eluted where the dimers should elute (F27 shown in Fig. 2B). The disulfide linkage of the dimers was previously confirmed by a) their dissociation to monomers in the presence of 20–30 mM DTT, b) their increased concentration at higher pH, and c) their absence in the cysteine-to-serine mutant (19). F34–39 contained trimer and truncated dimer forms (not shown).

Identification of asp^{28}-pro cleaved and asp^{15}-gly cleaved bFGF: The sequence of full length expressed bFGF is shown in Fig. 3. The methionine at position 1 of the final purified bFGF (designated bFGF(2-155)) was removed. bFGF(2-155) has a predicted mass of 17,124 Da. By LC/MS

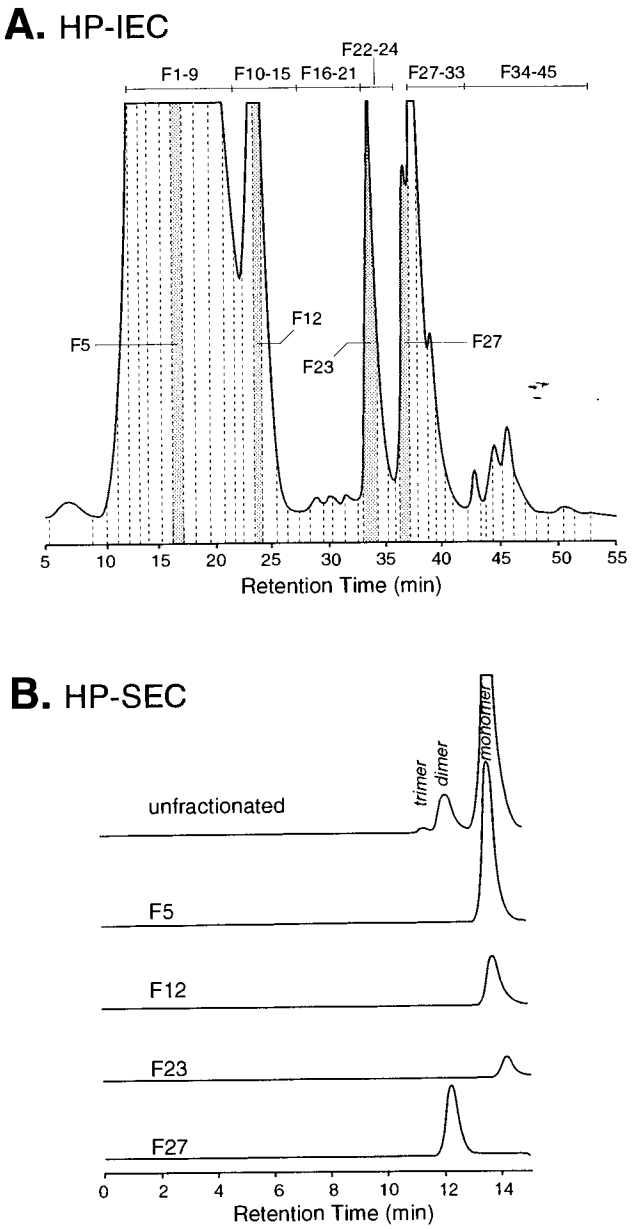

Figure 2: Fractionation of degraded bFGF by HP-IEC (A) and characterization by HP-SEC (B). 12 mg bFGF in citrate-EDTA, pH 5 was aged at 25°C for 13 weeks, filtered and fractionated by HP-IEC. See Methods for details.

(Fig. 4A), F5 consisted of bFGF(2-155) with a corrected mass of 17,127±3 amu, and a minor des-ala bFGF component (designated at bFGF(3-155)). The exact mass difference between F5 (using bFGF(2-155)) and a major component in F23 was 2596 amu (Fig. 4B). A minor component missing 1,334 amu was also detected in F23 by LC/MS (not shown). N-terminal sequencing revealed the presence of two components in nearly equal proportions: one beginning with prolys—arg and the other with gly-gly-ser. These data are consistent with co-elution of two cleavage products in F23: bFGF(29-155) which was cleaved at asp^{28}-pro site (missing 2596 Da) and bFGF(16-155) which was cleaved at asp^{15}-gly site (missing 1334 Da).

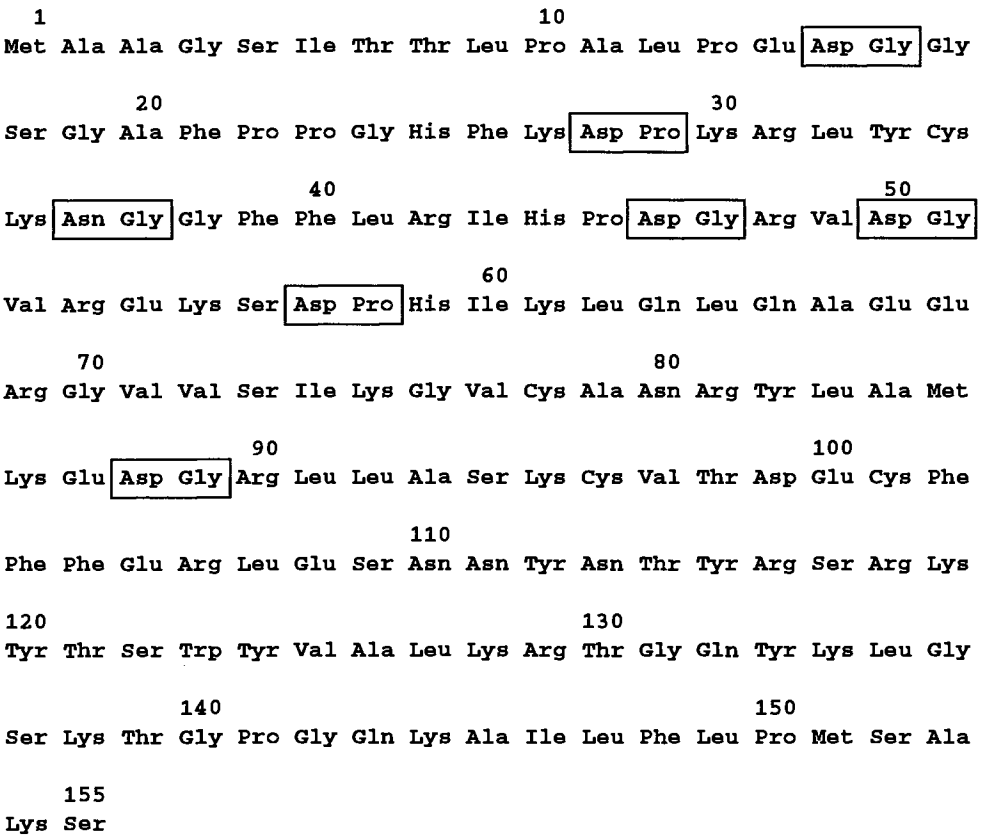

Figure 3: Primary sequence of bFGF. The positions of asp-pro, asp-gly, and asn-gly are highlighted. The final bFGF product does not contain methionine at position 1.

Identification of succinimide at aspartate[15]: N-terminal sequencing of F12 stopped at aspartate[15] (Table 1) which could have been due to either an iso-asp or a succinimide at this position (Fig.5). By LC/MS, a mass which was 18±2 amu smaller than F5 was obtained (Fig. 4C). The mass loss of one water equivalent, and the elution at a more basic position on the HP-IEC were consistent with the presence of a succinimide rather than iso-asp at aspartate[15].

Supporting evidence for the presence of succinimide at aspartate[15] in F12 came from the observation that treatment of F12 at pH 8 (37°C, 2h) converted this species to the main peak on HP-IEC (Fig. 6). Addition of a reducing agent to prevent disulfide-linked multimerization at high pH had no effect. The above conversion was accompanied by minimal deblocking of sequencing through aspartate[15], suggesting that the vast majority of the conversion products were the iso-asp derivative and/or D-isomers. Direct determination of the iso-asp content of the pH 8-treated F12 using the iso-aspartyl methyltransferase assay showed 38% iso-asp.

Identification and quantitation of iso-aspartate sites: The total iso-asp content of the unfractionated aged bFGF (25°C, 13 weeks) was measured to be 31±4 %, whereas the iso-asp content of untreated control was only 1%. (The same level of methylation was achieved using 0.5 μM instead of 1 μM PIMT, indicating that methylation was stoichiometric.) Examination of the peptide maps at 214 nm did not show any obvious difference between the aged and the control samples (Fig. 7, left). However, [^{3}H]-methylation showed predominant labeling of the N-terminal cluster of fragments that spans residues 2 through 50 and contains asp[15]-gly sequence (Fig. 7, right). Due to the presence of 3 lysines, 1 arginine, 1 asp-pro in bFGF(2-50) as well as N-terminal des-ala form, there are at least 8 species within this cluster that co-elute as a broad peak. Only 2% of label appeared in a fragment that spans residues 107–116 and contains three asn residues; this was found in control sample and did not increase with age (Fig. 7, right). Post-column methylation analysis confirmed the above observations, particularly that T17 (which contains asp[99]) was not labeled (Fig. 8).

Bioactivity of degraded bFGF: The bioactivities of F5, F12 (succinimidyl-bFGF), and F23 (asp[28]-pro cleaved bFGF) were similar relative to a fresh reference standard (80%, 112%, 81%, respectively) within the ±20% variation of the method; the activity was not affected by ammonium sulfate which was present at 130 mM in the isolated fractions.

DISCUSSION

We have shown that bFGF solutions aged at pH 5 produce a number of soluble degradation products. The major product was a succinimide at aspartate[15]. Other products included monomers cleaved at asp[28]-pro and asp[15]-gly, as well as disulfide-linked dimers and trimers of intact and asp-pro cleaved species. Fig. 9 shows the placement of the modified sites on the folded structure of bFGF.

Formation of succinimide is limited in proteins due to the required conformational constraints (ϕ and ψ angles of

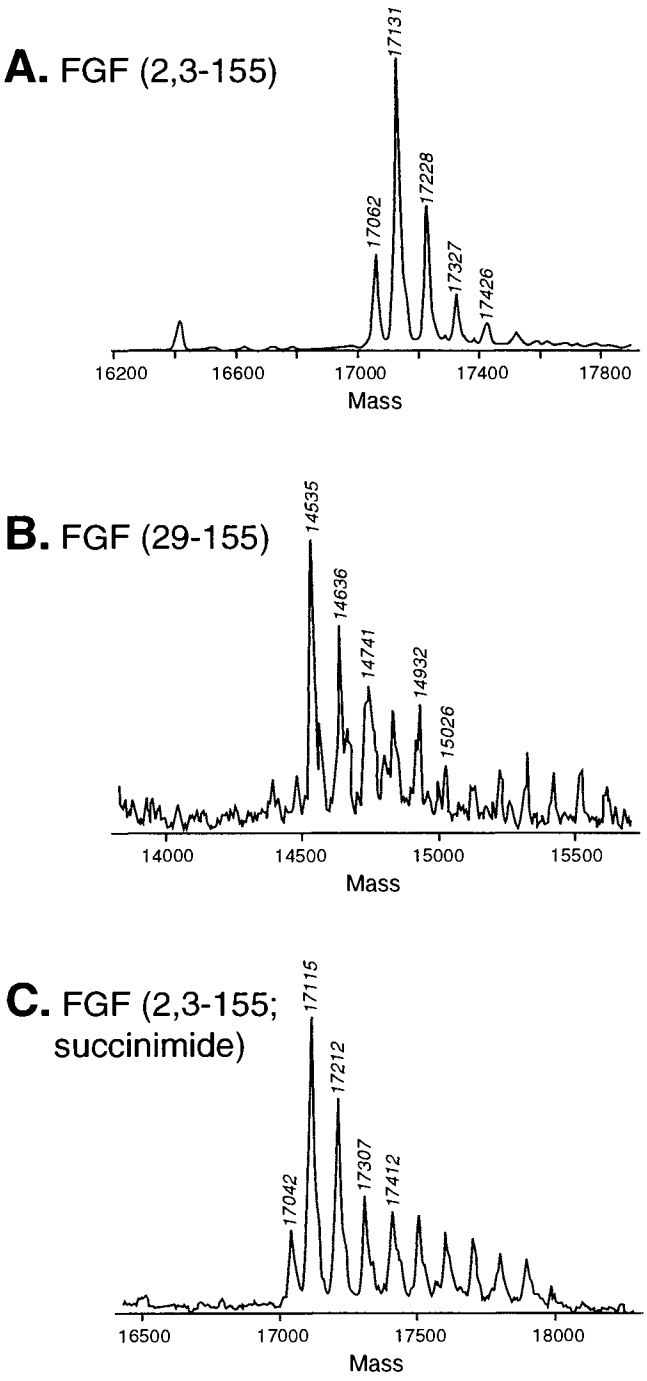

Figure 4: LC/Mass spectrometry of bFGF degradation products. Mass spectra of a fraction within the main HP-IEC peak (F5, A), and the two post main peak components (F23, B and F12, C). The des-ala component (missing 79±3 amu) and the phosphate and/or sulfate adducts (multiples of 100±5 amu) are seen. Mass was corrected using cytochrome C as an external standard which showed a mass of 12,364 amu instead of the theoretical mass of 12,361 amu.

+120° and −120°) around the asx residues which are excluded in the native state of most proteins (4). Moreover, even in small flexible peptides, a bulky C-flanking amino acid would hinder succinimide formation, hence asx-gly is the best candidate (1–5). bFGF represents the third reported protein to date in which a stable succinimide intermediate

Table I: N-terminal sequencing of F12. ~1 nmol F12 and fresh bFGF were subjected to Edman degradation and sequence analysis. The ratio of recovery of each amino acid in F12 versus fresh bFGF (not corrected for carry-over) remained near unity up to aspartate[15] were sequencing stopped. Data for cycles 8–18 are shown here. Note that cycle 1 begins with ala not met in the product.

cycle number	amino acid	recovery ratio (F12 to fresh)
8	leu	1.0
9	pro	1.0
10	ala	0.9
11	leu	0.8
12	pro	0.8
13	glu	0.8
14	asp	0.1
15	gly	0.2
16	gly	0.3
17	ser	0.2
18	gly	0.4

has been identified (8–10). Aspartate[15] is one of three asx-gly pairs predicted by the method of Regano et al (20) to fall in a region of above-average backbone flexibility (Fig. 10). Corroborating these calculations, X-ray crystallography (21) has shown that the segment spanning the 20 N-terminal amino acids is unstructured (Fig. 9). Thus, asp[15] favors succinimide formation since the C-flanking residue is gly, and it is also in a highly flexible segment of the protein. Consistent with this observation was the finding that the calculated rate of appearance of succinimidyl-bFGF (4% per day at 25°C using an exponential fit to the time-course data (not shown)) was comparable to that found in a model hexapeptide at the same pH (6.5% per day at 37°C (5)). We should emphasize that a pH 5 solution was ideal for cumulation of the succinimidyl-bFGF, because the optimal pH for the formation of succinimide is 4–5 (5) and for minimal degradation rate is at or below pH ~5 (9,10); this intermediate was not observed in a pH 6.5 solution, although it may be formed at a similar rate.

The by-products of the succinimide degradation are the corresponding aspartate or iso-aspartate forms (Fig. 5). The major iso-aspartate site in bFGF was identified by tryptic mapping of the methylated peptides. Taking into consideration the slight shift to longer retention times due to methylation (15,22), the major iso-asp site in bFGF was shown to be within a cluster of N-terminal peptides that contained asp[15] (see Fig. 7). Post-column methylation analysis verified that T16 cluster was solely responsible for methylation in this region of the peptide map (Fig. 8). This observation was partly predictable since peptide mapping conditions (pH 8) would convert any succinimidyl-bFGF to its iso-asp derivative. The only other [^{3}H]-labeled peak was a minor component containing asn[110], asn[111], and asn[113] which was present in both control and aged samples. Since the [^{3}H]-methylation method detects as low as 0.5% iso-asp, lack of appearance of other iso-asp sites suggests that the rate of isomerization of other asp or asn residues in bFGF were at least an order of magnitude lower than that of asp[15].

Because of the acidity of the ring methylene hydrogen, racemization also occurs during degradation of succinimide

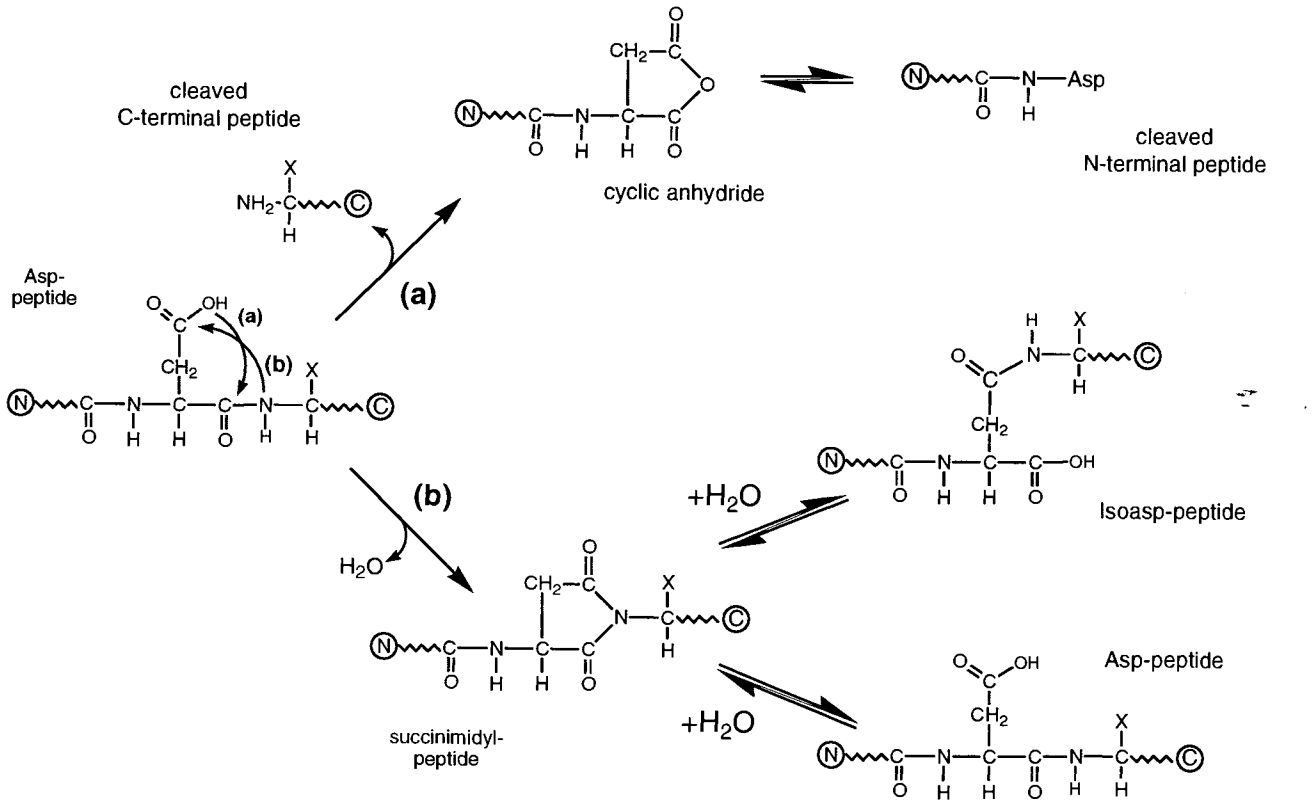

Figure 5: Reactions at aspartate residues in proteins and peptides: Nucleophilic attack of n+1 nitrogen onto the side chain carbonyl of aspartate residues results in the formation of a 5-membered cyclic imide (succinimide) concomitant with loss of water. This reaction is favored in the pH 4–5 range (5,9,10). At neutral to basic pH, the succinimide opens up to form either an aspartate or an iso-aspartate (2). Under highly acidic conditions, cleavage after aspartate is favored which is via an anhydride intermediate (5).

(1,2,5). Racemization would lead to formation of both L- and D- forms of both asp and iso-asp derivatives.

An iso-asp to asp ratio of ~3 to 1 has commonly been reported in peptides (1,7,23), but we found a ~1:3 ratio when the purified succinimide was opened at pH 8. Lower iso-asp to asp ratios (e.g., 0.6:1 in insulin suspensions (24)) have been attributed to a more rigid state of the protein in amorphous or crystalline phases. One possible explanation for low apparent iso-asp content in the pH 8-treated succinimidyl-bFGF might be the formation of D-iso-asp form which would probably not be detected by the iso-aspartyl methyltransferase assay. Because sequencing of the succinimidyl-bFGF treated at pH 8 showed >80% blockage at asp[15] whereas only 38% iso-asp was determined, the remaining ~40% modified asp might be accounted for as the D-iso-asp form which would have blocked sequencing but escaped the methyltransferase assay. Direct determination of D-forms of asp and iso-asp needs to be made in order to explain this discrepancy. Without knowing the contribution of D-forms, the iso-asp content of aged bFGF was measured at ~30%[4] at 25°C after 13 weeks or ~0.3% per day. At 4°C, <2% iso-asp was detected after storage for 24 weeks. Reported rates at pH 7.4 and 37°C for human growth hormone and tissue plasminogen activator ranged from 2 to 9% per day (15,22).

Besides cyclization, aspartate residues are labile to cleavage, particularly under acidic conditions. The probability of cleavage is enhanced when the C-terminal flanking residue is proline, possibly due to the greater basicity of the

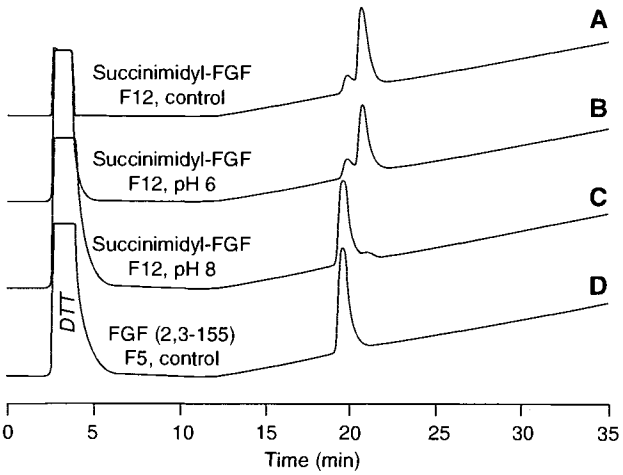

Figure 6: Effect of pH on the succinimidyl-bFGF. The HP-IEC profiles of A) succinimidyl-bFGF control (F12, pH 6), B) F12 incubated (37°C, 2h) at pH 6 in the presence of 20 mM DTT to dissociate any disulfide-linked dimers, C) F12 incubated (37°C, 2h) at pH 8 (using Tris buffer) and 20 mM DTT, and D) F5 control incubated (37°C, 2h) at pH 6 in the presence of 20 mM DTT.

[4] This includes a maximal contribution of 4% from the succinimide under the conditions of sample preparation prior to the assay.

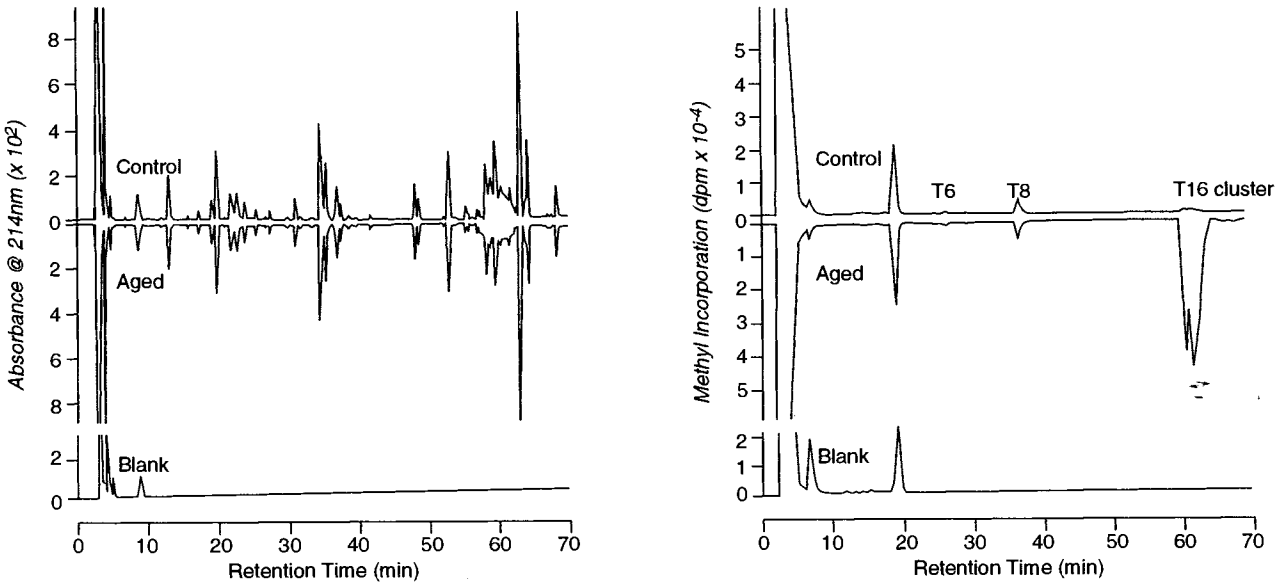

Figure 7: Tryptic map of bFGF. (A) The 214 nm absorbance profile of control (top) and aged (mirror image) samples. (B) The radioactivity profile of [^{3}H]-methylation sites of control (top) and aged (mirror image) samples. T4, ile^{43} thru arg^{48}. T6A, gln^{87} thru arg^{90} and other components without asn or asp. T6C, val^{49} thru arg^{53} and other components. T7, gly^{76} thru arg^{80}. T8, leu^{107} thru arg^{106}. T13, asn^{36} thru arg^{42}. T16, ala^2 thru lys^{27} and a number of fragments encompassing residues 2 thru 50. T17, cys^{96} thru arg^{106}. The two sharp peaks co-eluting with the broad T16 cluster are fragments that span 119–129 and its des-arg derivatives and do not contain asp or asn (Wallace, unpublished results).

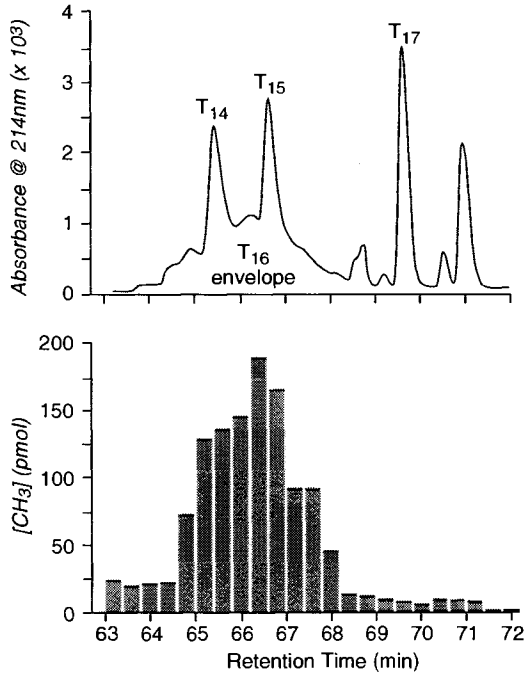

Figure 8: Post-column evaluation of methyl incorporation into tryptic fragments of aged bFGF. 4 nmol of a tryptic digest of aged bFGF (see Methods) were analyzed by RP-HPLC using 0.5 ml/min flow rate between 46 and 76 min. 0.2 ml fractions were collected in the 63–72 min region. The fractions were dried down and tested for methyl incorporation as described in Methods (bottom panel). The absorbance profile at 214 nm under identical conditions is shown in the top panel. Methyl incorporation occurred in the broad peak containing T16, but not in T17 (see legend to Fig. 7).

prolyl nitrogen causing enhanced protonation of the leaving group during hydrolysis (6). We observed as much as 3% conversion of bFGF to a cleavage product as asp^{28}-pro within 13 weeks at 25°C and pH 5 (~0.03% per day) which is slower than that reported for macrophage colony stimulating factor (1% per day at pH 5 and 40°C, (25)). There is another asp-pro site in bFGF at position 57 which is also in an exposed loop (Fig. 9). Interestingly however, asp^{57}-pro cleavage product was not detected in the soluble protein fraction,

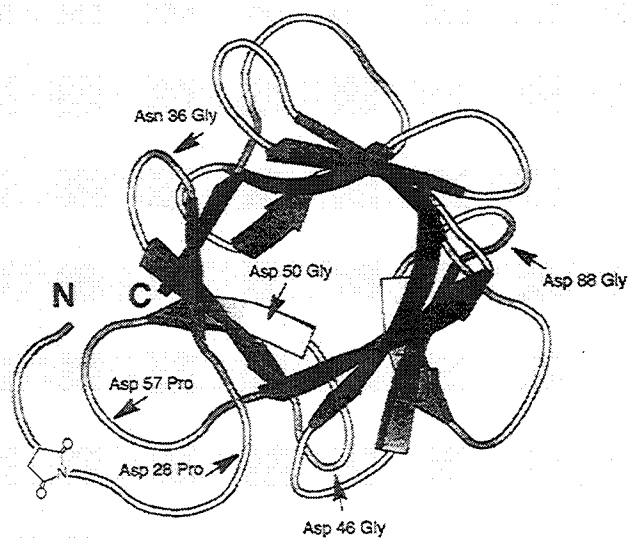

Figure 9: Location of degradation sites in the folded structure of bFGF. There are 12 antiparallel β-strands that form a β-barrel structure with a hydrophobic core (modified drawing from ref. 12). The positions of succinimide, asp-pro, asp-gly, asn-gly sites are shown.

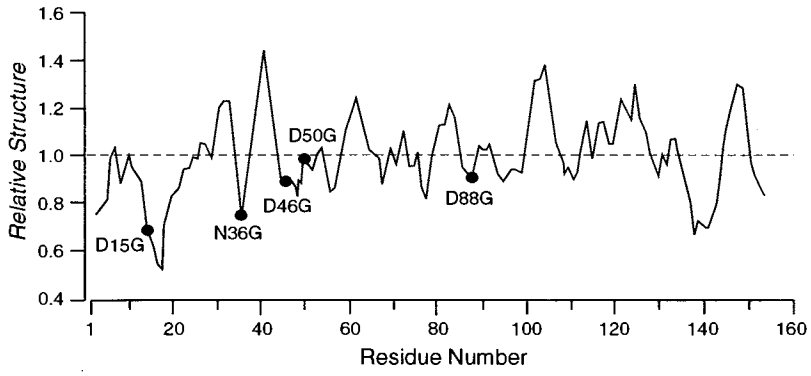

Figure 10: Flexibility plot of bFGF. The product of hydrodynamic radius and hydrophobility for each amino acid is plotted as a function of the residue number. Lower index of relative structure indicates greater flexibility of the peptide segment. The positions of asp-gly and asn-gly residues are shown. This plot was generated by the method of Ragone et al. (20).

possibly due to major disruption of the protein's folded structure.

Cleavage at aspartate residues may also occur when C-flanking residues other than proline are present, but this occurs at very low pHs. We have detected a minor (<1% of total soluble protein) cleavage product involving asp[15]-gly. Because acid cleavage at aspartates involves formation of an anhydride (5) rather than a succinimide (Fig. 5), the succinimide form at asp[15] would compete with the anhydride form; at pH 5, the former was the predominant product.

Analysis of the flexibility plot (15,22) of bFGF (Fig. 10) suggests that asn[36]-gly would be prone to deamidation. However, deamidation was not a major degradation product at pH 5, partly because deamidation is favored at neutral to alkaline pH's. Approximately 0.6% of total protein at pH 5 eluted ~2 min earlier than main peak by HP-IEC (Fig. 2A). A component with this retention time was the major degradation product at pH 6.5 (not shown). The more acidic deamidated product is predicted to elute earlier, but this possibility needs to be directly investigated.

Interestingly, despite the presence of two methionines at the surface of the protein, methionine oxidation was not found as a major (>1%) degradation product of bFGF when HP-IEC fractions were analyzed by reverse phase HPLC and MS (not shown). Based on the rate of appearance of oxidized methionine in IL2 at 25°C and pH 5 (0.08% day^{-1} (26)), appreciable amounts (5–10%) would be expected in the current study. It is possible that oxidative stress was initially absorbed by the exposed cysteines which readily form disulfide linkages.

In conclusion, we have identified the major soluble degradation products of bFGF at pH 5. The evidences for succinimidyl-bFGF at the position of asp[15] were: (1) late elution on HP-IEC consistent with one less negative charge, (2) stop in Edman degradation, (3) conversion to original bFGF at pH 8, (4) detectable by [^{3}H]-methylation after such conversion, (5) C-flanking glycine residue and a region of high backbone flexibility. None of the modifications found here were near the receptor or heparin binding domains of bFGF (residues 115 to 124 and 128 to 144, respectively, (21)) and bioactivity was predictively not compromised.

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