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The degradation pathways of glucagon in acidic solutions

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Abstract

Objective: Glucagon is a 29 amino acid peptide hormone that exhibits degradation via both chemical and physical pathways. The objective of the studies reported herein was to identify the degradation products and scheme for glucagon hydrolysis in acidic solutions. *Methods*: Solutions of glucagon in 0.01 N HCl (pH 2.5) were degraded at 60°C for 70 h. One isocratic and two gradient RP-HPLC methods were developed to separate the degradation products. Structure elucidation of the separated peaks was achieved using amino acid sequencing, amino acid analysis, and mass spectrometry. Degradation was carried out in the pH range 1.5–5 to check for changes in degradation scheme with pH. Authentic samples of degradation products were degraded under similar acidic conditions to confirm precursor successor relationships in the degradation scheme. *Results*: Sixteen major degradation products were isolated and identified. The major pathways of degradation were found to be aspartic acid cleavage at positions 9, 15, and 21 and glutaminyl deamidation at positions 3, 20, and 24. Cleavage occurred on both sides of Asp-15 but only on the C-terminal side of Asp-9 and Asp-21. Deamidation of the Asn residue at position 28 was not detected.

Keywords: Degradation pathways; Deamidation; Glucagon; Glutaminyl; Hydrolysis; Peptide cleavage

1. Introduction

Glucagon is a polypeptide hormone that is used for the emergency treatment of insulin induced, sulphonylurea induced, and spontaneous hypoglycemia. It has the ability to raise blood glucose concentrations by increasing hepatic glycogenolysis through activation of liver phosphorylase and to stimulate insulin secretion by direct action on pancreatic β-cells. Recovery of consciousness is usually achieved within 15–30 min of the injection whereupon oral glucose may be used to continue treatment (Marks, 1983). Glucagon also has the ability to reduce smooth muscle tone and motility. It is used as a diagnostic aid in radiologic procedures of the gastrointestinal tract that require a diminished tone and motility of the organ under study (Diamant and Picazo, 1983).

Glucagon for injection is a mixture of glucagon hydrochloride with one or more suitable, dry diluents supplied in single-dose or multiple-dose containers. The pH of the reconstituted solution is between 2.5 and 3.0 (US Pharmacopeia 24, 1999).

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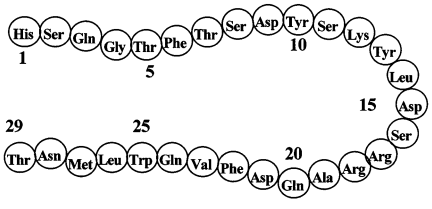
Thus the stability of glucagon in acidic aqueous solutions is relevant to drug product quality.

Structurally, glucagon contains 29 amino acids (Fig. 1). In the crystalline state, a completely helical conformation was proposed for glucagon (Sasaki et al., 1975). However, optical rotatory dispersion (Gratzer et al., 1968) and circular dichroism studies (Srere and Brooks, 1969) of glucagon in dilute aqueous solutions revealed a predominantly random coil conformation with at most 15% α-helix at the C-terminal end. The molecule has an isoelectric point of ~ 7 and exhibits very low solubility (< 0.1 mg/ml) in the approximate pH range of 4–8. It is readily soluble (> 10 mg/ml) at pH values less than 3 or greater than 9 (Bromer, 1983). It is known to self-associate at high concentrations and forms aggregates and gels at mild temperatures in acidic and basic solutions. The aggregation is promoted by salt, increased pH (within the acid range of solubility), agitation, and increased temperature up to 30°C (Beaven et al., 1969). Aggregation is accompanied by an increase in secondary structure in the form of anti-parallel β-sheets (Gratzer et al., 1968).



Separation method (see text)	Arbitrary Peak #	Fragment sequence
A	I	FVQWLMNT 22-29
	II	FVEWLMNT (22-29)d_{24}
	III	SRRAQDFVQWLMNT 16-29
	IV	SRRAQDFVEWLMNT (16-29)d_{24} YSKYL 10-14
	V	TSKYLDSRRAQDFVQWLMNT 10-29
	VI	TSKYLDSRRAQDFVEWLMNT (10-29)d_{24}
	VII	HSQGTFTSDYSKYLDSRRAQDFVEWLMNT (1-29)d_{24}
B	VIII	HSQGTFTSD 1-9
	IX	HSEGTFTSD (1-9)d_3
	1X	YSKYLDSRRAQD 10-21
	XI	YSKYLDSRRAED (10-21)d_{20}
	XII	YSKYLD 10-15
C	XIII	HSQGTFTSDYSKYLDSRRAQD 1-21
	XIV	HSQGTFTSDYSKYLD 1-15
	XV	HSQGTFTSDYSKYL 1-14

Fig. 1. Sequences of glucagon and its major degradation products in acidic aqueous solutions. The sequence of each degradation fragment is denoted using the one-letter abbreviations for the amino acids and by the numbers of the first and last amino acid residues. Deamidated fragments are denoted by parentheses followed by 'd_n' where 'n' is the number of the glutamine residue which deamidates.

The degradation pathways of glucagon have not been reported. In one study, glucagon degradation in 0.03 N HCl at 105°C was found to release three aspartic acid residues in 13 h (Schultz, 1967). The possibility of iso-aspartyl formation at the position of Asp-9 at neutral pH has also been suggested (Ota et al., 1987). The objective of the studies presented herein was to identify the degradation products and scheme for glucagon hydrolysis in acidic solutions.

2. Materials and methods

2.1. Materials

Purified porcine glucagon was obtained from Lilly Research Laboratories (Indianapolis, IN). Sodium dihydrogen phosphate, *o*-phosphoric acid, acetic acid, formic acid, hydrochloric acid, trifluoroacetic acid, potassium chloride, sodium formate, and sodium acetate were from Fisher (Springfield, NJ). All chemicals were of reagent grade and used as received. Solvents used for chromatography were HPLC grade. Cellulose ester dialysis membranes were from Spectrum (Houston, TX). Degradation product fragments were synthesized manually by SynPep Corporation (Dublin, CA).

2.2. Degradation of glucagon and glucagon fragments in acidic pH range

Aqueous glucagon solutions (0.1–1 mg/ml) were degraded at 60°C in the pH range 1.5–5 using dilute HCl, phosphate, formate, or acetate buffers. Degradation was allowed to proceed to two to three half-lives. Aliquots were removed from reaction mixtures at various time intervals and stored at 4°C until analyzed. Authentic degradation products were subject to hydrolysis at pH 1.5 and 2.5 under conditions identical to those used for glucagon.

2.3. Separation of degradation products

High performance liquid chromatography (HPLC) analyses were performed using a Shimadzu RP-HPLC system consisting of an SCL-10AVP system controller, LC-10ATVP pumps, SIL-10ADVP auto-injector, SPD-10AVP UV-VIS detector, and a CTO-10ASVP column oven. Chromatograms were integrated and data stored using Class VP Chromatography Data System software (Version 4.2).

Separation of degradation products was achieved on a Lichrospher RP-18, 4.6 × 250 mm, 5μ-column using isocratic elution (Method A). The mobile phase contained 29:71 acetonitrile: buffer (buffer = 0.1 M sodium phosphate monobasic and 0.002 M cysteine free base, adjusted to pH 2.6 with 85% phosphoric acid). Flow rate was 1.0 ml/min, detection was at 214 nm, and column temperature was 35°C. Degradation products that were unresolved with the isocratic method were separated by gradient elution using a Zorbax 300SB C8, 4.6 × 250 mm, 5μ column. The mobile phase contained methanol and a 0.024% solution of trifluoroacetic acid in water. The gradient used was 5–35% methanol in 30 min (Method B). Flow rate was 1.0 ml/min, detection was at 214 nm, and column temperature was 25°C. Another gradient method (Method C) was developed in an attempt to separate additional degradation peaks using the same column and mobile phase as the previous gradient method. The gradient was 5–50% methanol in 90 min. Flow rate was 1.0 ml/min, detection was at 214 nm, and column temperature was 25°C.

Degradation product peaks were collected from repeated 200 μl reaction mixture samples using a FRC 10A fraction collector (Shimadzu). Seven peaks were collected with the isocratic method (Method A), five more peaks with the first gradient method (Method B), and an additional three peaks with the second gradient method (Method C). The collection procedure was validated by re-injecting peak fractions back on the column to check peak integrity. Individual peaks were collected in flat top 1.5 ml polypropylene microcentrifuge tubes (Fisher Scientific) and stored in the refrigerator at 4°C until analysis. Since analysis of the fractions was typically done 4–7 days after fraction collection, the stability of the fractions at 4°C was evaluated. No significant degradation of peptide fractions was observed up to 18 days at 4°C.

2.4. Degradation product identification

Structure elucidation of degradation products was done using either amino acid sequencing, amino acid analysis, or mass spectrometry. Most of the fractions were identified using more than one method.

Amino acid analysis was performed by hydrolyzing an aliquot of each fraction using 100 μl of 6 N HCl at 110°C for 24 h. When hydrolysis was completed, the vials were placed in a SpeedVac® (Model SC210A) where all the residual liquid was removed by evaporation. Sample hydrolysates were then re-dissolved in sodium citrate buffer diluent. A Beckman 6300 high-performance ion-exchange analyzer was used to analyze each sample. Separation was done using a 12 cm hydrolysate column and a three-step temperature program in combination with three sodium citrate buffers to separate amino acids in various charged states. Standards consisting of a mixture of all amino acids plus an internal standard were also hydrolyzed. The system used ninhydrin to react with the amino acid giving a color reaction. The intensity of the color was proportional to the concentration of amino acid.

For sequencing, fraction aliquots were concentrated in a SpeedVac® and passed through a Prosorb sample preparation cartridge (Perkin Elmer). The adsorbent filter in these cartridges draws sample solution through a membrane by capillary action. The membrane immobilizes proteins and peptides while buffer components that could potentially interfere with sequencing pass through. The process not only desalts but also concentrates the sample. The membrane holding the peptide was directly loaded on the sequencer. Sequencing was done using an Applied Biosystems 492 automatic sequencer with an on-line PTH analyzer. A standard consisting of a mixture of all amino acids was first injected to obtain reference retention times for each amino

acid. One fraction was assigned more than one sequence (one major and one minor) due to the presence of a mixture of peptides.

Degradation product identity was also verified using Fast Atom Bombardment Mass Spectrometry (FABMS). The instrument used was a Hewlett Packard 1100 LCMSD. Organic solvents present in peak fractions collected with the isocratic method (Method A) were removed by evaporation under nitrogen. Sample peak fractions were then desalted using cellulose ester dialysis membranes (MWCO = 500). De-ionized water was used as the dialysis medium and was changed twice daily. The extent of dialysis was monitored by measuring the conductivity of the dialysate on an Orion Model 160 conductivity meter. Dialysis was stopped when the conductivity measurement was close to that of pure de-ionized water. The dialyzed fractions were then freeze dried (Virtis Advantage lyophilizer) and analyzed by FABMS. Peak fractions collected using the gradient methods (Methods B and C) were simply concentrated in a SpeedVac® before FABMS analysis since these fractions contained only water and volatile solvents.

To verify the sequencing results, two peaks obtained by isocratic elution (Method A) were analyzed by FABMS. The molecular ion masses obtained corresponded to the fragments obtained by sequencing. Hence, additional FABMS analyses were not performed on fractions that were sequenced. However, FABMS was used to confirm every sequence obtained with amino acid analysis alone.

3. Results

3.1. Chromatographic separation of degradation products

Chromatographic separation of degraded glucagon using isocratic elution (Method A) gave seven major degradation peaks (Peaks I-VII, Fig. 2). Sequencing of these products revealed that they were peptide fragments containing the C-terminus of glucagon. A gradient elution method (Method B) was developed to separate the corresponding fragments containing the more polar amino acids of the N-terminus end. An additional five peaks were obtained (Peaks VIII–XII, Fig. 3). A second gradient method (Method C) was developed to check for additional degradation products. This method involved a slow gradient (90 min) from 5 to 50% methanol and yielded several peaks. The peak at retention time of 5.5 min was found to be the same as peak XII obtained with Method B (Fig. 3). The peaks eluting at 35.3, 59.3 min, and 69.3 min were found to be the same as peak I, peak III, and peak V respectively, obtained by Method A (Fig. 2). These peak assignments were made by co-injecting peak fractions or authentic samples of peaks XII, I, III, and V and comparing retention times. The peak eluting at 72.1 min was glucagon. Thus, three new peaks, at 15.3, 20.2, and 26.6 min were obtained by Method C (peaks XIII, XIV, and XV in Fig. 4).

An overlay of sample chromatograms obtained from degradation reactions conducted over the pH range 2.5–5 (Fig. 5) demonstrated that the retention times of the major degradation product peaks were similar at pH 2.5 and 3.5. At higher pH values (4.5 and 5), the chromatograms were somewhat similar but separation quality was compromised by glucagon aggregation.

3.2. Degradation product identities

Of the seven peaks obtained by Method A (Fig. 2), peaks I, III, and V were composed of peptide fragments 22–29, 16–29, and 10–29 respectively, indicating that peptide cleavage had occurred on the C-terminal side of Asp-21, Asp-15, and Asp-9. Peaks II, IV, and VI eluted immediately after peaks I, III, and V (Fig. 2) and were found to be the deamidated forms of fragments 22–29 (peak I), 16–29 (peak III), and 10–29 (peak V) wherein Gln-24 converted to Glu-24. Peak VII was the deamidated form of glucagon with Glu at position 24. A small peptide fragment starting at Tyr-10 co-eluted with deamidated 16–29 in peak IV. Both these peptides could be detected through separate PTH-AA signals. However, both fragments have Asp as their sixth residue, hence the chromatogram for the sixth cycle of sequencing

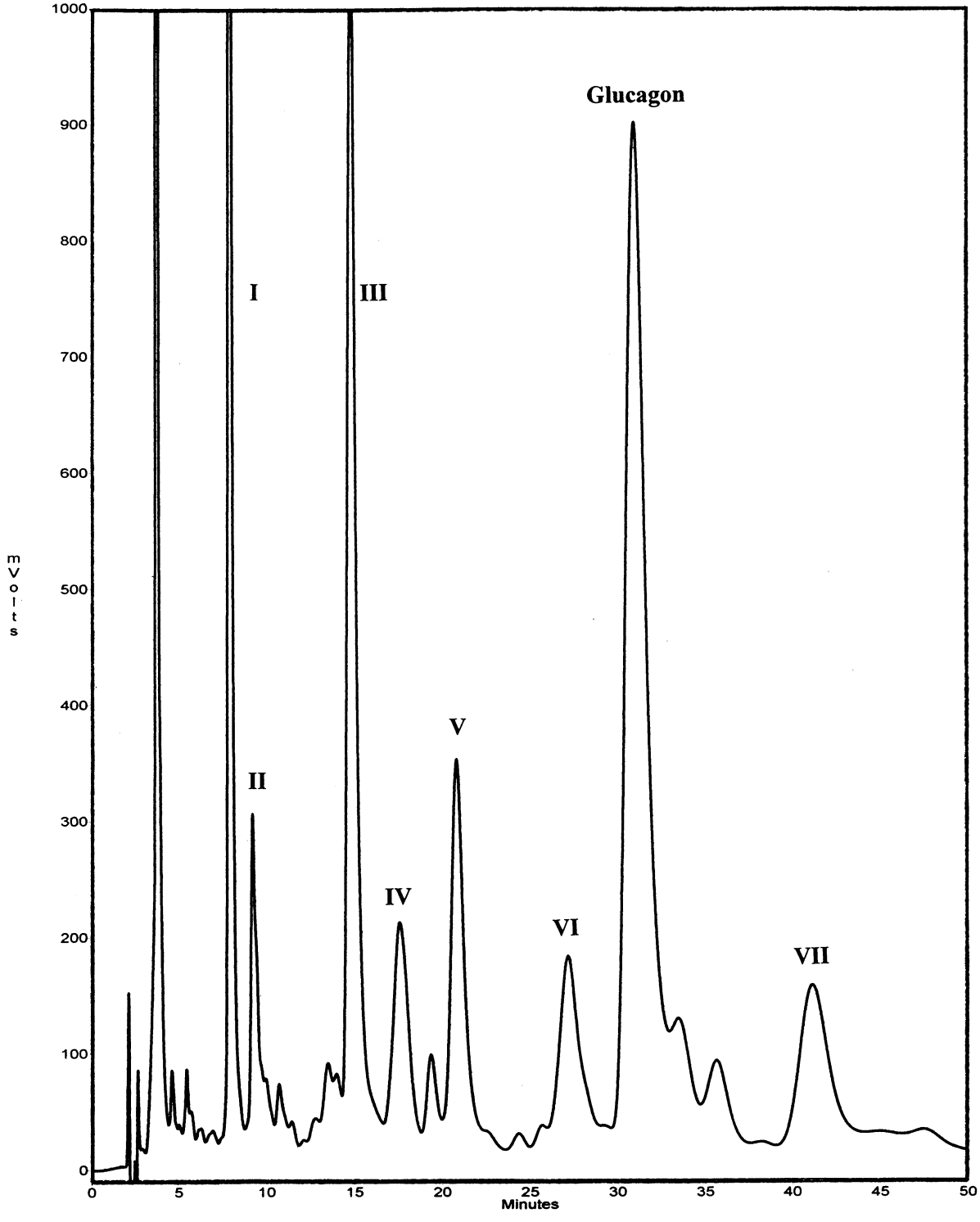


Fig. 2. Separation of degradation products of glucagon by isocratic elution using RP-HPLC Method A (column was a Lichrospher RP-18, mobile phase was 29:71 acetonitrile:buffer, buffer = 0.1 sodium phosphate monobasic and 0.002 M cysteine free base, adjusted to the pH 2.6 with 85% phosphoric acid).

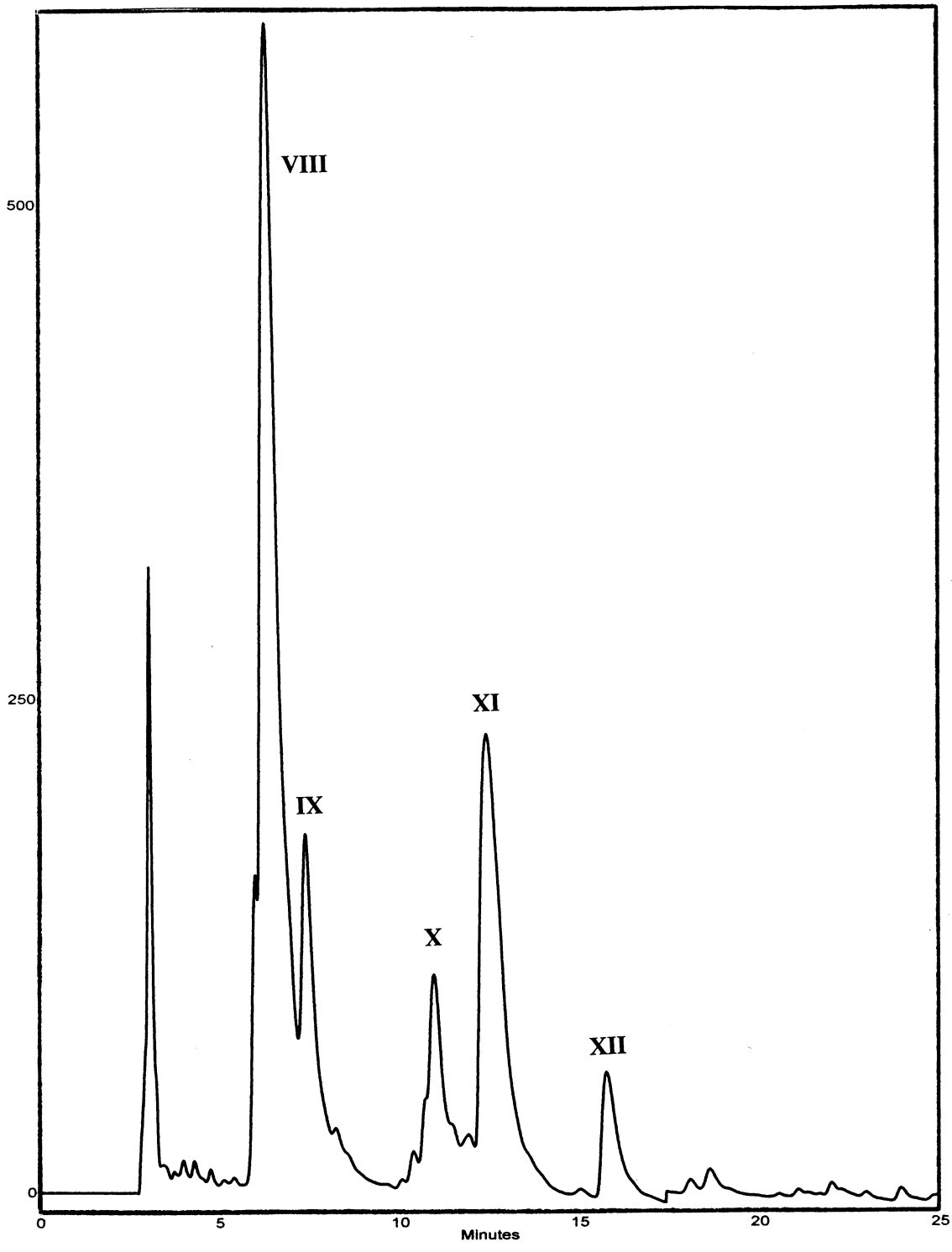


Fig. 3. Separation of degradation products containing the more polar amino acid residues of the N-terminal end using RP-HPLC Method B (column was a Zorbax SB300 C8, mobile phase was methanol and 0.024% TFA in water, gradient was 5–35% methanol in 30 min).

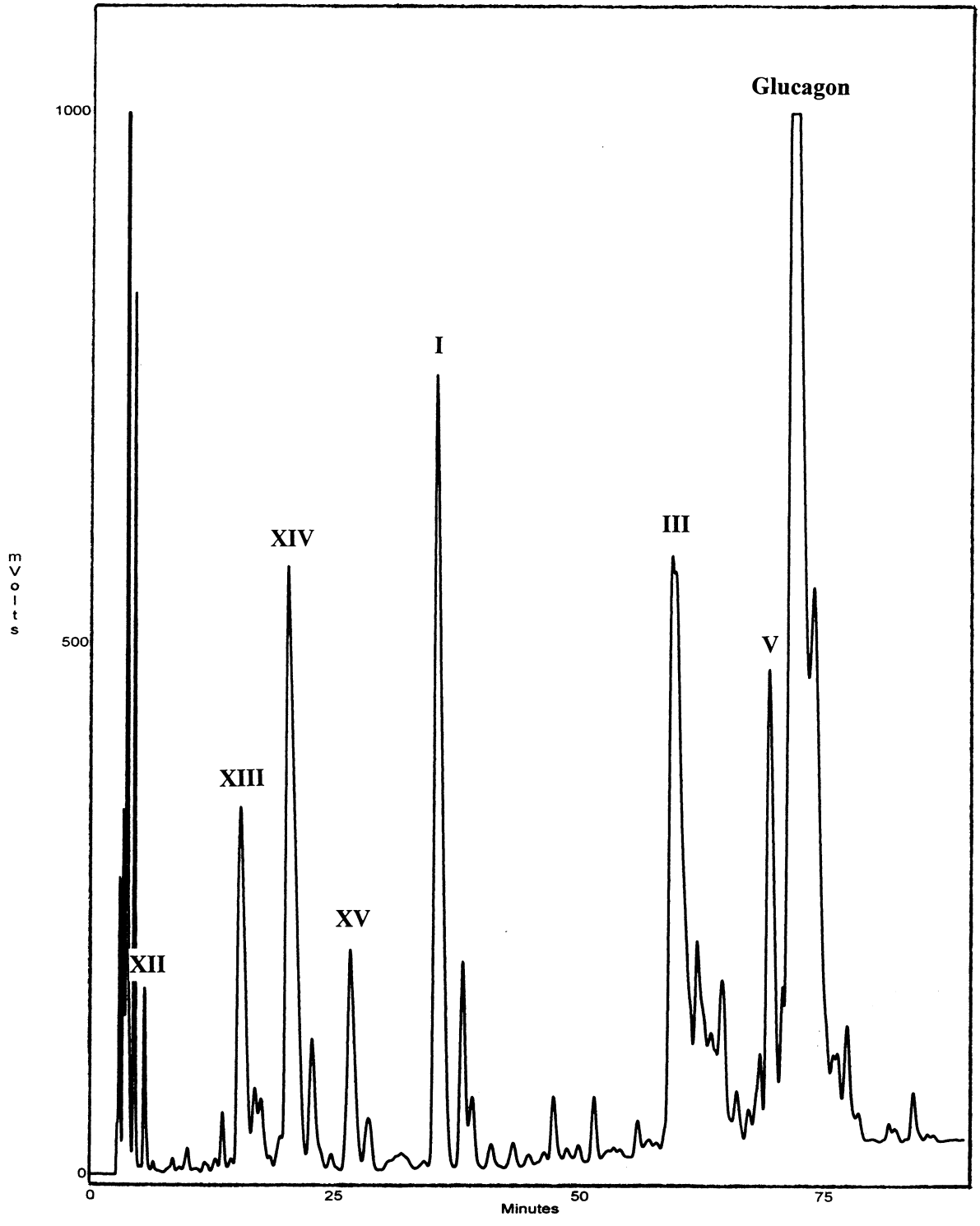


Fig. 4. Separation of additional degradation products containing the more polar amino acid residues of the N-terminal end using RP-HPLC Method C (column was a Zorbax SB300 C8, mobile phase was methanol and 0.024% TFA in water, gradient was 5–50% methanol in 90 min).

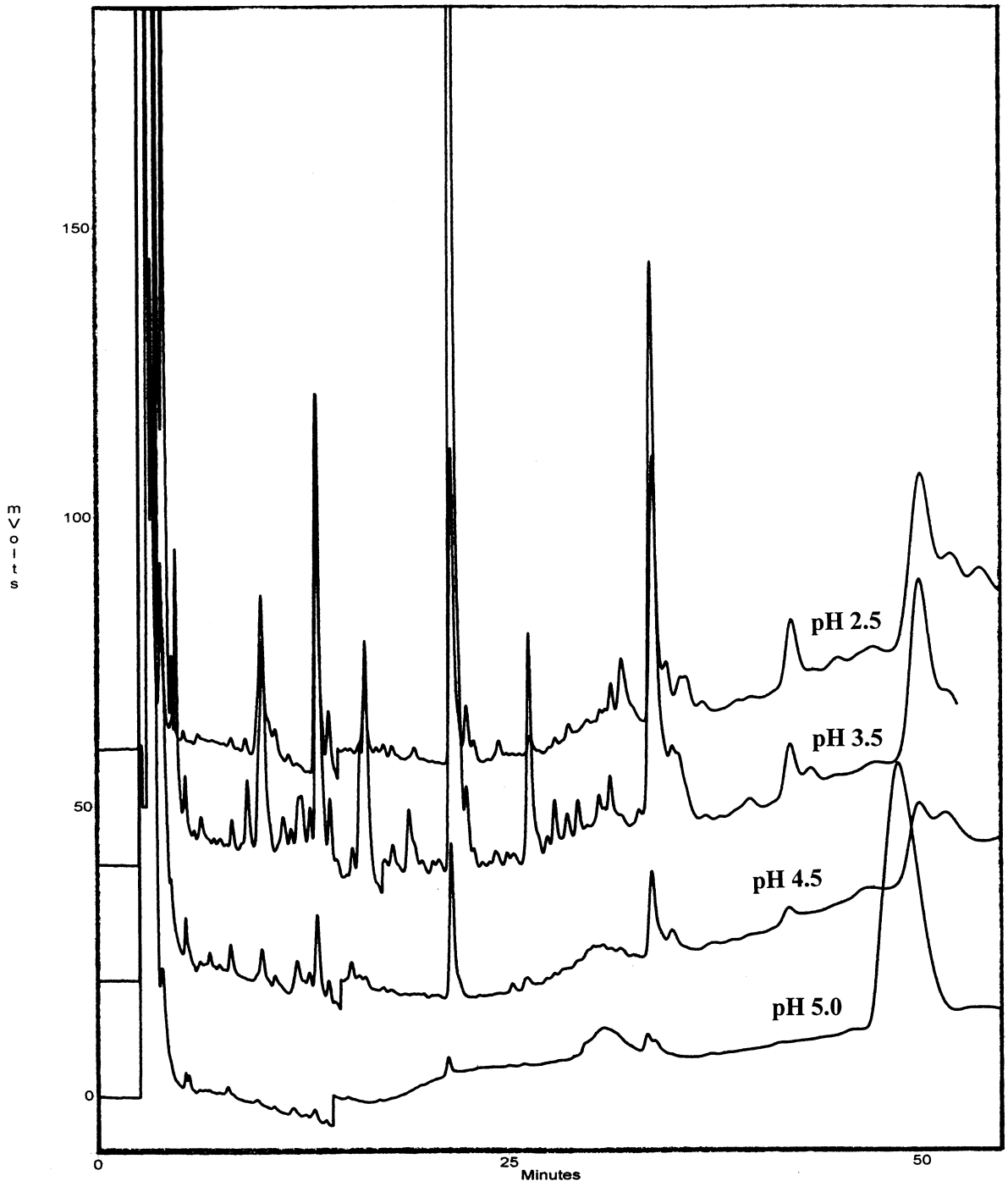


Fig. 5. Overlaid chromatograms of glucagon degraded in the pH range 2.5–5.0 using Method C.

gave only one peak for Asp. This made it difficult to determine whether the signal came just from the major fragment (deamidated 16–29) or from both fragments. A comparison of the combined picomole yields for the two fragments (as the sequencing progressed) indicated that the Asp picomole yield was not large enough to include two signals. Hence the sequence for the co-eluting peak was assigned as 10–14. This suggested the possibility that the Asp-15 residue cleaved on both its C-terminal and N-terminal sides.

Peaks VIII through XII (Fig. 3) were obtained by gradient elution (Method B) and contained the peptide fragments containing the N-terminal residues that were not recovered by isocratic elution. Peaks VIII and X were fragments 1–9 and 10–21, respectively. This confirmed the result that Asp-9 and Asp-21 did not cleave on the N-terminal side. Peak XII was fragment 10–15, which further confirmed cleavage on both sides of the Asp-15 residue. Peaks IX and XI were the deamidated products of peaks VIII and X respectively, which indicated that the glutamine residues in positions 3 and 21 were susceptible to deamidation as well as Gln-24 (peaks II, IV, VI, and VII).

The three peaks obtained with Method C (Fig. 4) were analyzed using amino acid analysis and confirmed by FABMS. Peak XIII was found to be fragment 1–21, peak XIV was fragment 1–15, and peak XV was fragment 1–14. This further confirmed that peptide cleavage occurred on both sides of Asp-15 and only on the C-terminal side of Asp-21. The peaks that immediately followed peaks XIII, XIV, and XV in Fig. 4 were not analyzed. These are expected to be the deamidated forms of the fragments 1–21 (peak XIII), 1–15 (peak XIV), and 1–14 (peak XV), respectively. The results of structure elucidation of the degradation products are summarized in Fig. 1.

3.3. Verification of precursor-successor relationships

The synthesized peptide fragments 22–29 and 16–29 corresponding to peaks I and III in Fig. 2 were subjected to degradation under acidic conditions. The resultant degradation chromatograms indicated that these fragments were subject to the expected peptide cleavage and deamidation pathways. Degradation of fragment 22–29 resulted in the appearance of a peak with retention time corresponding to deamidated 22–29. Degradation of the peptide fragment 16–29 gave rise to HPLC peaks corresponding to 22–29 and the deamidated forms of 16–29 and 22–29. The peaks corresponding to fragment 10–29 (peak V, Fig. 2) and fragment 1–15 (peak XIV, Fig. 3) were collected and also degraded under acidic conditions. Fragment 10–29 gave peaks corresponding to 22–29 and 16–29, and the deamidated forms of 10–29. Fragment 1–15 gave peaks corresponding to 1–14 and 1–9.

4. Discussion

Sixteen degradation products from thermally stressed, acidic solutions of glucagon were isolated and identified. The major pathways of glucagon degradation were glutaminyl deamidation and aspartyl peptide cleavage. The former was demonstrated to occur at residues 3, 20, and 24 and the latter at residues 9, 15, and 21. Cleavage at Asp-9 and Asp-21 occurred on the C-terminal side of the amino acid whereas cleavage at Asp-15 occurred on both N- and C-terminal sides. The proposed degradation scheme is illustrated in Fig. 6.

Deamidation is a common protein degradation pathway that involves the loss of ammonia from the side chain amides of asparagine or glutamine to form the corresponding side chain carboxylic acid residues: aspartic or glutamic acid (Manning et al., 1989). Numerous examples of asparaginyl and, to a lesser extent, glutaminyl deamidation have been reported (Robinson and Rudd, 1974; Geiger and Clarke, 1987; Patel and Borchardt, 1990a; Windisch et al., 1997). Some of the effects of primary, secondary, tertiary, and quaternary structure on deamidation kinetics have been described (Kossiakoff, 1988; Wearne and Creighton, 1989; Patel and Borchardt, 1990b; Wright, 1991; Darrington and Anderson, 1994; Xie and Schowen, 1999). In general, the presence of neighboring glycine (specifically at the $N+1$ residue), serine or threonine facilitates deamidation

(Wright, 1991) whereas the presence of significant secondary structure (Wearne and Creighton, 1989) or the lack of solvent access to the potential deamidating residue due to tertiary structure may tend to slow reaction rates. Moreover, asparaginyl deamidation is reported to be more facile than glutaminyl deamidation (Robinson and Rudd, 1974). It has been suggested that this kinetic difference may be due to the greater length of the glutamine side chain which precludes stabilization of a side chain carbonyl oxyanion transition state by hydrogen bonding with the neighboring peptide nitrogen (Wright and Robinson, 1982).

In glucagon, the lack of asparaginyl deamidation at residue 28 in the presence of glutaminyl deamidation at residues 3, 20 and 24 was a somewhat unexpected observation. Although the sole asparagine residue in glucagon is located in a region of the peptide associated with some helical structure, this structure has been shown to be lost as the temperature is raised in the range 22–50°C (Yi et al., 1992). Since the current study was conducted at 60°C, secondary structure effects were unlikely.

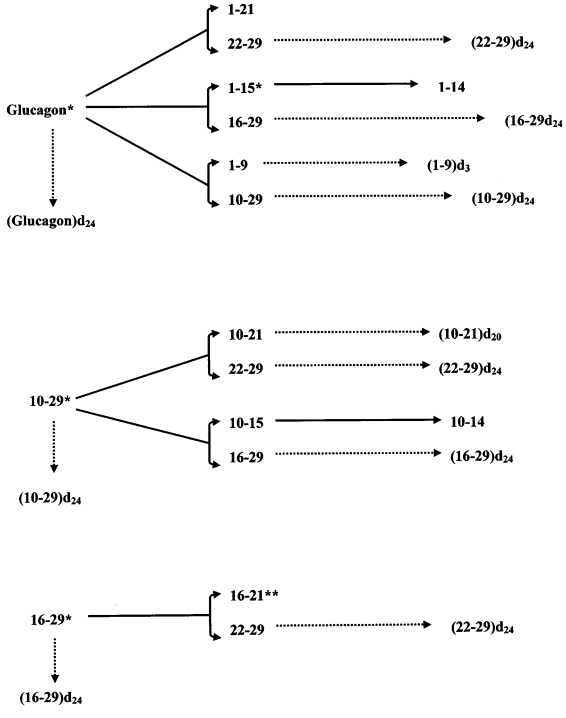


Fig. 6. Proposed degradation scheme of glucagon in acidic aqueous solutions. Deamidation and aspartyl cleavage pathways are represented by dashed and solid arrows respectively. Only observed, but not hypothesized, degradation products are depicted. * Starting materials; ** has not been chromatographically separated.

Most of the studies that support the greater susceptibility of asparaginyl residues to deamidation were conducted at pH conditions approaching neutrality (Robinson and Rudd, 1974; Geiger and Clarke, 1987; Patel and Borchardt, 1990a). The relative reactivity of glutamine and asparagine and the effects of primary, secondary, and tertiary structure on glutaminyl deamidation in acidic conditions have not been reported and are the focus of our ongoing studies.

Preferential peptide cleavage at aspartic acid residues in the presence of acid is a well-known hydrolysis pathway that was historically used to fragment proteins for compositional analysis (Light, 1967). Cleavage can occur between aspartic acid and either the $N-1$ or $N+1$ neighboring residue. The mechanism is believed to involve intramolecular catalysis by the side chain carboxylic acid and formation of a six- or five-membered ring intermediate (Inglis, 1983). Peptide cleavage was observed at all three of the aspartic acid residues in glucagon. Cleavage on both sides of Asp-15 was observed. The relative rates of peptide cleavage at the three residues are expected to vary based on sequence effects. Future work will evaluate the relative reactivity of the aspartic acid residues.

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