Validation of a Peptide Map for Recombinant Porcine Growth Hormone and Application to Stability Assessment[1]

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A reverse-phase HPLC method for the analysis of tryptic digests of recombinant porcine growth hormone (pGH) has been developed and validated. Digestion was performed at 4°C for a 20-hr period with TPCK-treated trypsin at a 1:20 (w/w) trypsin:pGH ratio. Gradient elution HPLC, using an Aquapore RP300 C8 column, was incorporated for separation of the digestion products and peak identification was carried out by mass spectrometry (MS). The digestion procedure and subsequent chromatography were linear in the initial concentration range of 4.55–45.46 μ*M* (100 to 1000 μg/mL) pGH. The variability in the fragment retention times was low and the normalized peak area variability was less than 5% for all but three of the fragments. The utility of the trypsin digestion and chromatography procedures has been demonstrated by assessing chemical changes in pGH induced by incubation at elevated pH. Upon incubation of pGH in 0.2 *M* Tris buffer at pH 9 (ionic strength adjusted to 0.5 with NaCl) and 37°C over a period of 400 hr, significant degradation in the regions corresponding to the digestion fragments T23–T25 (residues 181–182 linked by a disulfide bond to residues 184–191), T9 (residues 96–108), and T5–T18 (residues 43–64 linked by a disulfide bond to residues 158–166) was observed. The disappearance of the peaks corresponding to fragments T23–T25 and T9 both displayed apparent first-order degradation kinetics over the time period investigated with half-lives of 131 and 154 hr, respectively. The disappearance of the peak corresponding to fragment T5–T18 was complicated by poor resolution of the parent peak and the degradation products. These results demonstrate the utility and limitations of the mapping procedure for the determination of the reaction kinetics for pGH.

KEY WORDS: porcine growth hormone; peptide map; protein stability.

INTRODUCTION

Peptide mapping is a technique commonly used in protein chemistry to identify alterations in the primary sequence of a protein which are difficult to detect by standard chromatographic or electrophoretic methods applied to "intact" protein structures. Peptide mapping involves cleavage of a protein by either chemical or enzymatic methods, to produce characteristic peptide fragments which can then be separated and analyzed. Peptide maps are frequently referred to as protein "fingerprints" and have been used extensively to determine the purity of recombinant biologicals (1).

Peptide maps are known to be inherently variable with technique reproducibility being strongly dependent on conditions used for digestion and chromatography. Even under strictly controlled conditions, peak "artefacts" and variable peak responses are commonly encountered (1). For that reason, the utility of peptide maps in evaluating chemical stability profiles for proteins relies heavily on proper validation and often requires the utilization of complimentary analytical techniques.

The goal of the present study was to validate a peptide map for recombinant porcine growth hormone (pGH) and to assess the applicability of the method for analyzing chemical degradation profiles. pGH is a 22-kD protein which displays significant sequence homology to bovine growth hormone (bGH) (2). Two peptide maps for the analysis of bGH have been previously reported in the literature, however, both were associated with significant between-day variability and, consequently, were utilized primarily in a qualitative manner (3,4). An improved and validated procedure for bGH was recently described by Dougherty *et al.;* however, attempts in our laboratory to apply the digestion method to pGH demonstrated the need for adaptations and subsequent assessment of variability (5). Although investigations which addressed chemical degradation of pGH and bGH utilizing qualitative peptide mapping procedures have been reported, the mapping techniques were not fully described and failed to include all peak identifications and an appropriate description of assay variability (6,7).

The peptide map which has been developed and validated for pGH involves trypsin digestion with subsequent separation of the peptide fragments by reverse-phase HPLC. Samples of pGH which were exposed to elevated pH were assessed to determine the suitability of the method for the detection of chemical modifications in the protein sequence and for the analysis of degradation kinetics.

MATERIALS AND METHODS

Chemicals

Lyophilized recombinant DNA-derived methionyl-porcine growth hormone (pGH) was prepared and supplied by Bresatec Ltd. (Adelaide, South Australia). N-terminal sequence analysis (Applied Biosystems Inc., Foster City, CA, Model 470A Protein Sequencer) confirmed a homogeneous product with an N-terminal sequence of Met-Phe-Pro-Ala- (S. Bastiras, personal communication). Trypsin, which had been treated with tosylamido-2-phenylethyl-chloromethyl ketone (TPCK), and Tris–HCl were from Sigma Chemicals (St Louis, MO). HPLC-grade trifluoroacetic acid (TFA), dithiothreitol (DTT), and Tween 20 (Surfact-Amp 20) were obtained from Pierce (Rockford, IL). All other chemicals and reagents were of at least analytical grade quality. Deionized water (Milli-Q, Millipore Inc., Milford, MA) was used in all experiments.

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Methods

Trypsin Digestion Procedure. The digestion procedure for pGH was an adaptation of a previously described method for bGH (5). Lyophilized pGH was dissolved in a buffer consisting of 0.1 *M* Tris–HCl, 1 m*M* $CaCl_2$, and 0.02% (v/v) Tween 20 and the pH adjusted to 8.3. The protein concentration was determined by UV absorbance at 278 nm using a molar extinction coefficient of 15,714 M^{-1} cm^{-1}. Trypsin, prepared fresh in cold 1 m*M* HCl, was added to give an enzyme/substrate ratio of 1:20 (w/w) and digestion was carried out at 4°C for 20 hr. The reaction was quenched by acidification with TFA to give a final pH of 2–3 (approximately 0.5%, v/v). Samples were stored at −20°C (for a maximum of 2 weeks) until analysis.

Chromatographic Separation of Peptide Fragments. Chromatographic separation of digested fragments was conducted using a Beckman System Gold HPLC consisting of a Model 126 pump and a Model 166 variable wavelength UV detector. Samples were centrifuged (3000 rpm) for 5 min prior to injection of a 200-μL aliquot. Separation was achieved using an Aquapore RP300 (220 × 2.1-mm i.d.) C8 column (Applied Biosystems Inc., Foster City, CA) and a flow rate of 0.4 mL/min. A series of linear gradients, utilizing 0.1% aqueous TFA (solvent A) and 0.1% TFA in 70% ACN (solvent B), was used to elute the peptide fragments. The percentage of solvent B was varied from 0 to 24% B over 62 min (0.39% B/min) and then from 24 to 60% B over 68 min (0.53% B/min). Detection was by UV absorbance at 214 nm.

Assay Validation. The linearity of the digestion and chromatographic procedures was determined over an initial pGH concentration range of 4.55–45.56 μ*M* (100–1000 μg/mL). Linear regression, calculated using the least-squares estimation method, was performed using a standard statistical package (MINITAB, Minitab Inc., State College, PA). Confidence limits (95%) were calculated about the intercept values by application of a two-sided Students' *t* test. The lower limit of detection of the chromatographic method, arbitrarily defined as the sample concentration giving rise to a signal which was five times the background signal, was determined by analyzing serial dilutions of a digested pGH sample (initial concentration of 11.36 μ*M* for the digestion). Within-day and between-day reproducibilities were determined by analyzing at least four independent digestion samples and comparing the retention times and peak areas of the resulting fragments.

Mass Spectral Analysis of Peptide Fragments. Fragments eluting from the HPLC were manually collected immediately after passage through the detector. Fragments were analyzed by liquid secondary ion mass spectrometry using a Finnigan MAT95 mass spectrometer equipped with a cesium ion gun at 20 kV and 5-μA emission. The sample matrix was 20% (v/v) glycerol and 20% (v/v) thioglycerol in water. Fragments with molecular weights greater than 2000 were analyzed using a Perkin Elmer Sciex API3 triple quadrapole mass spectrometer equipped with an ion spray interface. The orifice potential was 90 V and the samples (in 0.1% TFA/ACN) were directly infused into the instrument.

Amino Acid Analysis of Peptide Fragments. Selected fragments were collected following chromatographic separation and analyzed for amino acid composition using the Millipore-Waters Pico-Tag system as previously described (8).

Preparation of Degradation Samples

Samples of pGH were degraded by incubating at 37°C for 400 hr in 0.2 *M* Tris buffer, pH 9.0, containing 0.02% (w/v) sodium azide and adjusted to an ionic strength of 0.5 using NaCl. Aliquots from the reaction mixture were removed as a function of time and diluted to give a final Tris concentration of 0.1 *M* (pH 8.3) and a total pGH concentration of 13.64 μ*M* (300 μg/mL). Trypsin was added and digestion and chromatography were carried out as described above. Aliquots of the degradation sample were removed periodically and the optical density at 450 nm determined to ensure that there was no evidence of precipitation.

RESULTS AND DISCUSSION

Trypsin Digestion

Trypsin has been widely used as a digestion enzyme in peptide mapping due to its high specificity for cleavage at the C-terminal side of lysine and arginine residues (9). Freshly prepared solutions of trypsin were used for all digestion procedures as trypsin solutions which had been previously frozen (as a storage measure) resulted in nonreproducible cleavage patterns. For pGH digestion, a 20-hr incubation time was required to digest the samples adequately, as shorter digestion periods (12–16 hr) resulted in incomplete cleavage patterns and significant variability in peak response. A digestion temperature of 4°C was found to eliminate the formation of a visible precipitate (as indicated by the optical density at 450 nm) which was present following digestion at either 25 or 37°C. This trend in precipitation patterns is consistent with previous observations during the digestion of bGH (5).

During the initial stages of assay development, the digestion buffer did not contain Tween 20 and samples were routinely filtered through 0.22-μm cellulose acetate filters prior to HPLC injection to avoid injection of any particulate material which could be present. Following peak identification, it was discovered that fragment T8 (Fig. 1) was completely lost on the filter, presumably because of adsorption. When the digested sample was centrifuged rather than filtered prior to HPLC injection, an additional peak (later identified to be T8) eluted at 120 min, but with a response significantly lower than expected. The digestion was repeated including 0.02% (v/v) Tween 20 in the digestion buffer (to reduce nonspecific adsorption to the tube during the digestion), and the response for the peak corresponding to T8 increased by a fourfold factor relative to a sample digested in the absence of Tween 20. All subsequent digestions with trypsin were performed with the addition of Tween 20 (0.02%, v/v) to the digestion buffer.

Chromatographic Separation of Peptide Fragments

Figure 2 displays a representative chromatogram for the elution of pGH peptide fragments resulting from the trypsin digestion. The majority of the peaks in the chromatogram were well resolved, with the exception of some of the early-eluting peaks (fragments T6/T22, T20, T10, T14/T13). Peak resolution was found to be strongly influenced by the flow rate and gradient rate, with the respective optimized condi-

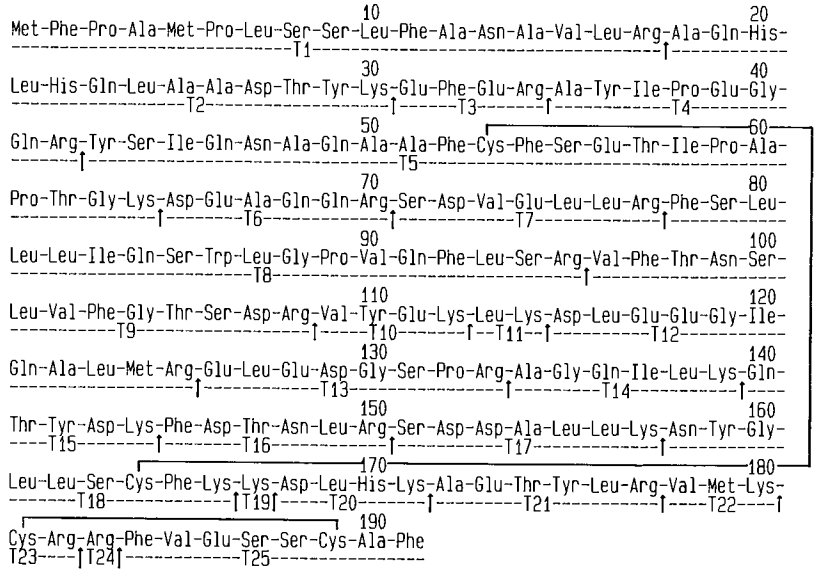

Fig. 1. The amino acid sequence of methionyl-pGH. Arrows indicate predicted tryptic cleavage sites and the disulfide bonds which bridge positions 53–164 and 181–189 are represented by the solid lines.

tions being 0.4 mL/min and 0.39% solvent B/min over 62 min followed by 0.53% solvent B/min over 68 min. Peaks eluting in the early portion of the chromatogram (<30 min) were completely lost if the digestion sample was not acidified with TFA (to a final pH 2–3) prior to injection. Slight variations in mobile phase composition, which invariably occurred with each new solvent preparation, were found to affect significantly the resolution of the early eluting peaks. As a routine practice, large volumes of mobile phase were prepared and filtered in order to minimize variability in peak resolution.

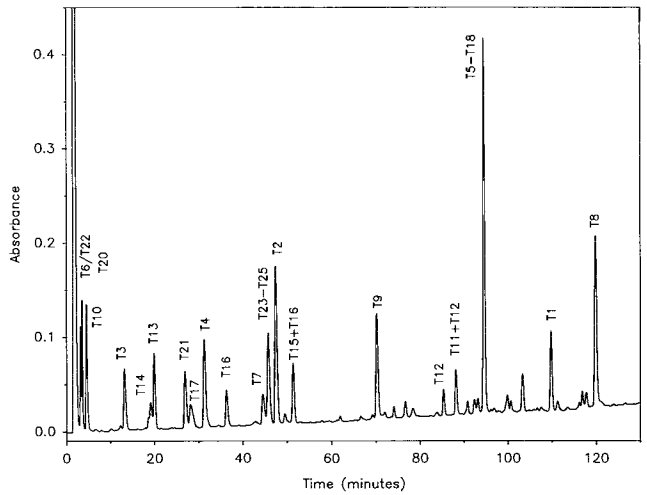

Fig. 2. Chromatogram of a pGH tryptic digest (initial concentration, 11.36 μ*M*). Digestion was carried out at 4°C for 20 hr using an enzyme to substrate ratio of 1:20 (w/w). Chromatography was conducted using an Aquapore C8 column, a flow rate of 0.4 mL/min, and mobile phases consisting of 0.1% TFA (solvent A) and 0.1% TFA in 70% ACN (solvent B) using a series of linear gradients. A hyphen between fragments represents a disulfide bond, a plus sign represents incomplete cleavage, and a slash represents coeluting fragments.

Assay Validation

The linearity of the trypsin digestion and chromatographic procedures was examined over an initial pGH concentration range of 4.55–45.56 μ*M* (100–1000 μg/mL). Table I displays the regression parameters for the peak area–concentration relationships for each of the peptide fragments and illustrates that the majority of the fragments exhibited a high degree of linearity over the concentration range evaluated. Fragments which displayed intercepts which were statistically different from zero at the 95% confidence limit (fragments T16, T7, T23–25, T12, and T5–T18) were those which were either incompletely resolved from neighboring peaks or fragments which were only partially cleaved.

The lower limit of detection of the chromatographic method was determined by serial dilution of a pGH sample which had been digested at an initial concentration of 11.36 μ*M* (250 μg/mL). The limit of detection was arbitrarily defined as the minimum concentration which gave rise to a signal which was at least five times the background signal. All except six peaks (T7, T12, T11 + T12, T14, T16, T17) could be reliably quantitated at a concentration of 1.44 μ*M* and several peaks (T2, T9, T5–T18, T8) could be detected at a concentration of 0.72 μ*M*.

Reproducibility of the digestion and chromatography was assessed using an initial pGH concentration of either 9.09 μ*M* (200 μg/mL) or 11.36 μ*M* (250 μg/mL). The within-day reproducibility of fragment retention times and peak areas was examined and the results are presented in Table II. The coefficient of variation (CV) in the peak area data was calculated either from the raw integrated data or by normalizing the area of fragment peaks relative to peak T3 in the chromatogram. This normalization procedure standardized the peak area data for potential differences in sample concentration or injection volume. The peak corresponding to fragment T3 was used for normalization as it was well resolved and reproducible, exhibited appropriate linearity

Table I. Regression Statistics Describing the Relationship Between Peak Area (Nominal Units) of Peptide Fragments and the Initial pGH Concentration (4.55–45.56 μ*M* or 100–1000 μg/mL) Subjected to Tryptic Digestion

Fragment[a]	r^2	Intercept[b]	Slope (μM^{-1})
T6/T22	0.993	−2.49 ± 10.92	3.74
T20	0.993	−7.13 ± 13.38	4.51
T10	0.994	−20.89 ± 21.54	7.55
T3	0.994	−13.12 ± 15.23	5.57
T14/T13	0.993	−15.85 ± 22.65	7.74
T21	0.997	−8.73 ± 10.03	5.35
T17	0.992	−8.00 ± 10.31	3.21
T4	0.996	−13.04 ± 19.10	8.18
T16	0.986	−15.74 ± 12.85	3.06
T7	0.950	9.17 ± 8.44	1.03
T23–T25	0.998	−21.97 ± 16.30	10.05
T2	0.997	−23.40 ± 23.95	14.45
T15 + T16	0.990	14.80 ± 17.28	4.86
T9	0.995	−17.34 ± 27.65	10.67
T12	0.990	−6.44 ± 6.06	3.51
T11 + T12	0.995	0.82 ± 8.08	3.19
T5–T18	0.996	−54.75 ± 53.16	24.86
T1	0.995	−4.41 ± 20.57	8.47
T8	0.989	−2.62 ± 46.30	12.54

[a] Refer to Fig. 1 for amino acid sequences of fragments. A slash (/) indicates coeluting peaks, a hyphen (-) represents fragments linked by a disulfide bridge, and a plus sign (+) indicates uncleaved fragments.

[b] One hundred peak area units is approximately equal to a peak height corresponding to 0.2 absorbance unit. The data are reported as the extrapolated mean intercept value with 95% confidence limits.

Table II. Within-Day Variability of the Retention Time (Mean ± SD) and Peak Area Values for Fragments from Tryptic Digestion of pGH (n = 5): The Initial pGH Concentration was 9.09 μ*M* (200 μg/mL)

		CV (%)	
Fragment[a]	Retention time (min)	Raw peak area data[b]	Normalized peak area data[b]
T6/T22	3.35 ± 0.20	3.43	2.01
T20	3.94 ± 0.23	2.31	0.90
T10	4.86 ± 0.35	3.45	2.24
T3	13.85 ± 0.42	2.19	—
T14/T13	20.62 ± 0.27	1.90	0.46
T21	28.38 ± 0.27	2.56	1.87
T17	30.15 ± 0.27	7.10	6.41
T4	32.20 ± 0.17	2.14	0.64
T16	37.83 ± 0.17	2.76	2.03
T7	46.29 ± 0.20	1.30	1.49
T23–T25	47.58 ± 0.20	2.25	1.48
T2	48.68 ± 0.16	1.60	0.66
T15 + T16	52.36 ± 0.15	2.30	2.09
T9	70.95 ± 0.11	1.80	0.93
T12	85.69 ± 0.07	4.05	2.93
T11 + T12	88.45 ± 0.06	2.13	0.89
T5–T18	94.57 ± 0.06	2.33	1.06
T1	109.92 ± 0.07	2.27	2.15
T8	119.90 ± 0.11	22.37	23.11

[a] Notation as described in Table I, footnote *a*.

[b] Peak area coefficient of variation data were calculated from either raw peak area values or when individual fragment peak areas were normalized relative to the area of fragment T3.

characteristics, and did not contain residues which were particularly susceptible to degradation. The retention times for all peaks were highly reproducible, and with the exception of fragment T8, the CVs for all fragment peak areas were less than 8%. In all but two cases, the CVs determined from the normalized peak area data were lower than those from the nonnormalized data. The variability in the peak area of fragment T8 cannot be fully explained but is most likely a consequence of its hydrophobicity and tendency to adsorb to surfaces or to form a fine precipitate. Between-day variability (Table III) for retention times was less than 2% for all fragments. Between-day variability in peak area was slightly higher than that observed for within-day variability, with the CVs for the normalized peak area data generally being less than 7% except for fragment T8 as described above.

Fragment Identification by Mass Spectral Analysis

Table IV displays the results for the MS analysis of the peptide fragments resulting from trypsin digestion and chromatographic separation. Based upon the amino acid sequence for pGH (Fig. 1), 23 fragments were expected upon digestion with trypsin, provided that each of the two disulfide bonds remained intact. Two of the expected fragments (T19 and T24) were only single amino acid residues (lysine and arginine, respectively) and would, therefore, not be detected by the chromatographic method. Two fragments (T13 and T14) periodically coeluted, however, their individual identities were confirmed by altering the initial gradient so as to resolve the two components which were then subjected to MS analysis. MS analysis indicated that fragments T6 and T22 were coeluting in the first eluting peak. This was confirmed by free solution capillary electrophoresis (CE) (data not shown) in which the single HPLC peak gave rise to two main peaks in the electropherogram.

MS analysis also indicated that fragment pairs T15 + T16 and T11 + T12 were incompletely cleaved consistent with previous results for bGH in which the corresponding fragments were also incompletely cleaved (5). The resistance to cleavage at lysine 114 and lysine 144 (cleavage points for fragments T11 and T15, respectively) may have been due to the surrounding amino acids in the respective regions of the sequence. The presence of neighboring acidic residues (as in lysine 114 and lysine 144; see Fig. 1) has been shown previously to decrease dramatically the rate of trypsin cleavage (9).

The presence of an adjacent Lys or Arg residue has been reported to reduce the rate of trypsin cleavage or to result in only partial cleavage (9). Trypsin digestion of bGH has been shown to yield several different cleavage positions around the C-terminal disulfide bond which was attributed to the −Arg−Arg− sequence present at residues 182–183 (5). The same sequence (−Arg−Arg− at positions 182–183) is also present in pGH, in addition to the presence of the sequence −Lys−Lys− at positions 166–167. MS analysis of the T23–T25 and T5–T18 fragments showed that the respective molecular weights were consistent with cleavage both at Arg 182 and Arg 183 and at Lys 166 and Lys 167. It may be

Table III. Between-Day Variability in the Retention Time (Mean ± SD) and Peak Area Values for Fragments from Tryptic Digestion of pGH (n = 4): The Initial pGH Concentration was 11.36 μM (250 μg/mL)

Fragment[a]	Retention time (min)	CV (%) Raw peak area data[b]	CV (%) Normalized peak area data[b]
T6/T22	3.08 ± 0.02	5.32	0.80
T20	3.45 ± 0.03	7.10	1.71
T10	4.36 ± 0.05	6.79	1.25
T3	12.16 ± 0.20	5.94	—
T14/T13	18.98 ± 0.17	5.94	0.88
T21	26.16 ± 0.17	6.15	0.76
T17	27.62 ± 0.04	7.67	2.07
T4	30.54 ± 0.09	6.74	1.32
T16	35.61 ± 0.13	10.84	6.93
T7	43.90 ± 0.05	9.69	4.58
T23–T25	44.97 ± 0.13	7.67	2.63
T2	46.56 ± 0.10	6.40	1.03
T15 + T16	50.46 ± 0.14	8.66	3.68
T9	69.36 ± 0.08	8.59	2.97
T12	84.47 ± 0.10	3.59	4.36
T11 + T12	87.19 ± 0.07	6.94	2.08
T5–T18	93.71 ± 0.19	8.06	2.51
T1	109.10 ± 0.30	11.78	7.09
T8	119.07 ± 0.33	29.39	33.20

[a] Notation as described in Table I, footnote *a*.

[b] Peak area coefficient of variation data were calculated either from raw peak area values or when individual fragment peak areas were normalized relative to the area of fragment T3.

Table IV. Mass Spectral Analysis of Tryptic Fragments Following Chromatographic Separation and Collection

Fragment[a]	Monoisotopic mass Expected	Monoisotopic mass Observed
T6	746.3	746.2
T22	377.2	377.3
T20	512.3	512.3
T10	538.3	538.3
T3	580.6	579.9
T14	629.4	628.9
T13	902.4	902.4
T21	752.4	752.1
T17	761.4	760.9
T4	933.5	933.4
T16	765.4	765.0
T7	831.5	830.7
T23–T25	1164.5	1164.8
T2	1495.8	1495.3
T15 + T16	1400.7	1400.3
T9	1442.7	1442.9
T12	1274.6	1275.1
T11 + T12	1515.8	1516.0
T5–T18	3386.7	3386.4
T1	1865.0	1866.2
T8	2104.2	2104.0

[a] Notation as described in Table I, footnote *a*.

that the small peak following the T2 peak (eluting at approximately 49 min) represents an additional minor cleavage product with Arg 183 attached either to the C terminus of T23 or to the N terminus of T25. Likewise, one of the small peaks eluting just prior to the peak corresponding to T5–T18 may represent a minor cleavage product with Lys 167 attached to the C terminus of T5–T18.

Utility for Evaluation of Sample Degradation

The utility of the peptide map for examining reaction kinetics was evaluated by analyzing pGH samples which had been incubated at pH 9.0 and 37°C for 400 hr. At elevated pH, the prevailing degradation pathways in proteins are expected to include (i) deamidation of asparagine residues to form both the corresponding aspartate and isoaspartate residues (10,11) and (ii) β-elimination of disulfide bonds to form thiocysteinyl and dehydroalanine residues, the latter of which can react further with a lysine amino group to form lysoalanyl linkages (12,13). Deamidation of glutamine residues is also a potential degradation pathway, however, rates of glutamine deamidation at elevated pH have been shown to be significantly slower than those for asparagine (10).

Significant changes in the chromatographic profiles were seen for samples incubated at the elevated pH and subsequently digested. Figure 3 illustrates that the major changes occurred in the regions of pGH corresponding to fragments T23–T25 (residues 181–182 linked by a disulfide bond to residues 184–191), T9 (residues 96–108), and T5–T18 (residues 43–64 linked by a disulfide bond to residues 158–166). The disappearance of the T9 peak was accompanied by the appearance of two new peaks, one immediately preceding the parent T9 peak (peak a) and another small peak following the parent T9 peak (peak b). Amino acid analysis of peak (a) confirmed that it had a sequence identical to that for the parent T9 fragment (containing Asx), suggesting that it represented a deamidated product of asparagine 99.

Deamidation of asparagine 99 present in bGH and pGH has been previously shown to result in the formation of both the iso and the normal aspartate variants in an approximate ratio of 3:1 (7). In the study by Violand *et al.*, trypsin digestion and peptide mapping of deamidated samples showed that the isoaspartate product for bGH eluted just prior to the parent T9 peak while the aspartate product eluted following the parent T9 peak (7). It is likely that the same chromatographic elution patterns were operative in the present study of pGH degradation as the ratio of the normalized peak area for the peak preceding T9 (peak a) relative to that for the peak following T9 (peak b) was 2.9:1. The authors also reported a small degree of cleavage of the peptide bond between asparagine 99 and serine 100 following incubation at pH 10 and 24°C for 96 hr (7). While a small degree of cleavage may have occurred in the present investigation, any peaks corresponding to cleaved products would have most likely been below the limit of detection of the assay when the initial pGH concentration was 13.63 μM.

Further degradation of pGH at elevated pH was reflected in the time dependent disappearance of the peaks corresponding to peptide fragments T23–T25 and T5–T18 (Fig. 3). Two new peaks eluted in the region surrounding the peak corresponding to fragment T5–T18, one being incom-

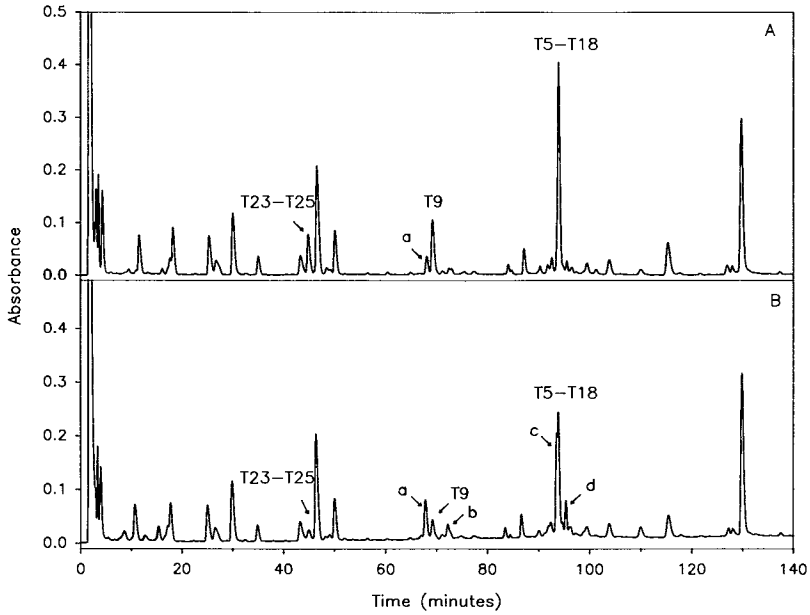

Fig. 3. Comparison of chromatograms obtained for a digest of pGH following incubation in 0.2 *M* Tris buffer, pH 9.0 (μ = 0.5) at 37°C for (A) 68 hr and (B) 300 hr. Peaks a–d are additional peaks that formed during the degradation. Chromatographic conditions and fragment symbols are as described in the legend to Fig. 2.

pletely resolved from the parent peak (peak c) and another eluting just after the parent peak (peak d). The two disulfide bonds in pGH are present in the peptide fragments T23–T25 and T5–T18, with the T5–T18 peptide also containing two asparagine and two glutamine residues.

The degradation pathway for the region corresponding to T23–T25 has not yet been identified but is likely to involve the disulfide bond between Cys 181 and Cys 189. A recent study regarding chemical modification of pGH has implicated the "small loop" disulfide (i.e., the disulfide present in fragment T23–T25) as being involved in the formation of a nonreducible, covalent dimer upon incubation of high pGH concentrations at neutral pH and 40°C (14). These data suggest that the disulfide bond which links residues 181 and 189 may be susceptible to β-elimination and subsequent cross-linking.

The degradation of pGH in the region corresponding to fragment T5–T18 is complex, as the two peptide regions contain both asparagine residues and a disulfide bond. For analysis of degraded samples, the gradient slope in this region of the map was decreased in an attempt to resolve the degraded peak (peak c) from the parent peak (hence the increase in retention times for some of the later-eluting peaks in Fig. 3). Unfortunately, peak c was still poorly resolved from the T5–T18 peak, which precluded evaluation of peak area-versus-time data for the parent peak. Additional studies are underway to fully characterize the degradation of both the T5–T18 and the T23–T25 regions of pGH.

From a kinetic standpoint, the disappearance of the peaks corresponding to fragments T23–T25 and T9 were followed as a function of time over a period of 400 hr. Figure 4 displays a semilog plot of the percentage remaining-versus-time data (calculated using peak areas normalized to fragment T3) for the two peptide fragments illustrating that both were linear over the time period investigated. The apparent first-order rate constants (±SE) were 5.30 (±0.10) × 10^{-3} and 4.49 (±0.07) × 10^{-3} hr^{-1} ($t_{1/2}$ of 130.8 and 154.3 hr) for fragments T23–T25 and T9, respectively. The percentage remaining data for fragment T7, which contains the sequence -Ser-Asp-Val-Glu-Leu-Leu-Arg-, is also shown in Fig. 4 to demonstrate the absence of considerable change in the normalized peak area for this fragment over the time course of

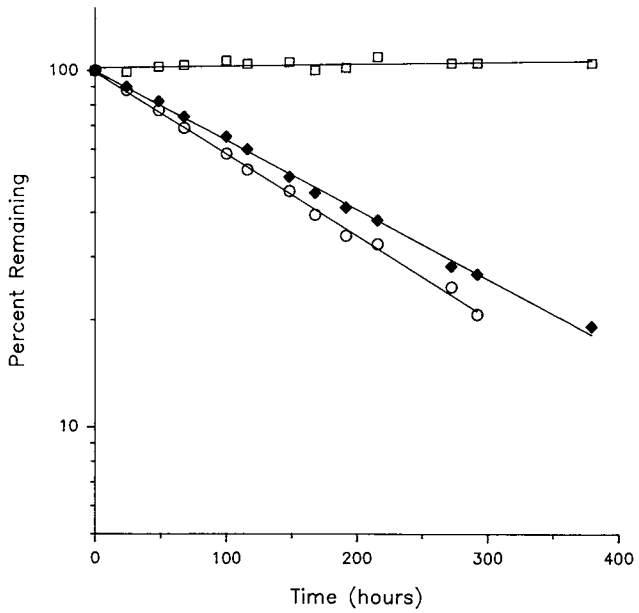

Fig. 4. Percentage remaining-versus-time data for the HPLC peaks corresponding to fragments T7 (□), T23–T25 (○), and T9 (◆) following incubation of pGH in 0.2 *M* Tris buffer, pH 9.0 (μ = 0.5) at 37°C.

the experiment. There was also no evidence of precipitation over the incubation period as shown by the optical density at 450 nm.

In summary, a peptide map for pGH has been developed and validated using trypsin as the digestion enzyme and reverse-phase HPLC with gradient elution as the separation modality. The method was reproducible and could be used to monitor degradation of pGH resulting from incubation at elevated pH. The method was found to be well suited for the determination of reaction kinetics, provided that the degradation products were resolved from the corresponding parent peak. Kinetics and mechanisms of degradation of pGH at elevated pH are currently under investigation.

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