Isolation and characterization of porcine somatotropin containing a succinimide residue in place of aspartate¹²⁹

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Abstract

Aspartate¹²⁹ in porcine somatotropin was converted into a cyclic imide residue (succinimide) under acidic solution conditions. Reversed-phase high performance liquid chromatography was utilized to isolate and quantitate this altered species, which accounted for approximately 30% of the total protein. The molecular mass of this modified species was determined by electrospray mass spectrometry to be 18 Da less than normal porcine somatotropin, indicative of a loss of 1 H₂O molecule. Tryptic peptide mapping demonstrated that the peptide composed of residues 126–133 was altered in this modified protein. Amino acid analysis, amino acid sequencing, mass spectrometry, and capillary zone electrophoresis were used to demonstrate that aspartate¹²⁹ in this peptide had been converted into a succinimide residue. Further confirmation that this peptide contained a succinimide was obtained by hydrolyzing the modified peptide at pH 9.0, which yielded both the aspartate and isoaspartate peptides.

Keywords: capillary electrophoresis; mass spectrometry; peptide mapping; porcine somatotropin, reversed-phase high performance liquid chromatography; succinimide

The deamidation of asparagine at neutral to basic pH and the isomerization of aspartate to isoaspartate residues is generally accepted to occur through a cyclic imide (succinimide) intermediate. The proposed mechanism for deamidation of asparagine is shown in Figure 1. The isomerization of aspartate to isoaspartate is also proposed to occur by the same mechanism with the elimination of H₂O from aspartate yielding the intermediate succinimide. This is a two-step process with the initial rate-limiting step occurring when the α -amino nitrogen of the $n + 1$ residue attacks the β -carbonyl carbon of asparagine or aspartate to form a succinimide. This succinimide is subsequently hydrolyzed to form both aspartate and isoaspartate (Bornstein & Balian, 1977; Clarke, 1987). The succinimide intermediate has been shown to exist in numerous small peptides, but evidence for its existence in proteins has only been indirect except for one report on

its presence in lyophilized human growth hormone (Tchima et al., 1991). The human growth hormone was thermally stressed to produce this succinimide by incubating the lyophilized protein at 45 °C for 4 months. The presence of a succinimide in a protein solution has never been confirmed perhaps because of the instability of this residue in solution.

Many studies have been performed to measure the rates of formation of succinimide and isoaspartyl residues in model peptides containing either asparagine or aspartic acid residues (Geiger & Clarke, 1987; Stephenson & Clarke, 1989; Patel & Borchardt, 1990; Tyler-Cross & Schirch, 1991; Wright, 1991). These studies have demonstrated that isoaspartate formation occurs most rapidly when the residue on the carboxyl-side of the asparagine or aspartate is small, such as glycine or serine. This is presumably because a large side chain would sterically interfere with the formation of the intermediate succinimide.

Another factor that has been shown to be important for isoaspartate formation in proteins is the local conformation surrounding the asparaginyl or aspartyl resi-

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Fig. 1. Mechanism of formation of isoaspartate and aspartate from asparagine. The α -amino group from the carboxyl-side residue attacks the carbon of the side-chain carbonyl of asparagine releasing NH_3 and forming a succinimide residue. Hydrolysis of the succinimide leads to the formation of both isoaspartate and aspartate.

due (Kossiakoff, 1988; Lura & Schirch, 1988; Wearne & Creighton, 1989). Proteins, because of their secondary and tertiary structures, have a more limited conformational flexibility compared to small peptides. Therefore the conformation of the protein must permit the correct stereochemistry to be present in order for the intermediate succinimide to form. From examining bond angles and distances in proteins of known structures it has been concluded that the most stable conformation for asparagine in proteins does not allow for facile succinimide formation (Clarke, 1987). Therefore succinimide formation may not occur in proteins unless the local conformation is very flexible. A study comparing the rate of deamidation in unfolded vs. native RNase (Wearne & Creighton, 1989) demonstrated that the unfolded protein deamidated at a 30-fold higher rate than the native protein, apparently because of the greater flexibility in the unfolded protein.

During the examination of numerous acidic buffers for formulation studies of porcine somatotropin (PST), a new species comprising a significant portion of the total protein was generated. This report describes the isolation and characterization of this new PST species, which contains a stable succinimide¹²⁹ residue.

Results

Figure 2 shows reversed-phase high performance liquid (RP-HPLC) chromatograms of recombinant PST before

Fig. 2. Reversed-phase high performance liquid chromatographic (RP-HPLC) analyses of porcine somatotropin (PST) before (A) and after (B) formulation in a low pH buffer. The new component labeled, PST-X, is generated in significant amounts during this step.

and after incubation at pH 5.0, 37 °C, for 72 h. Generation of the new later-eluting component in Figure 2, PST-X, was also observed when PST was incubated at pHs from 3.0 to 7.0. This new component accounted for approximately 30% of the total protein from the pH 5.0 incubation.

This new component was purified by RP-HPLC for determination of its chemical composition. A previously published peptide-mapping procedure for PST (Violand et al., 1990) was used to analyze the purified protein (Fig. 3). This analysis showed that in PST-X the normal peptide of residues 126–133 was decreased in intensity compared to a standard PST, and a new modified pep-

Fig. 3. RP-HPLC analyses of tryptic digests (pH 9.0) of normal PST (A) and PST-X (B). The peptide labeled 1 contains residues 126–133 with residue 129 being an isoaspartate. Peptide 2 contains the normal sequence for residues 126–133.

tide eluting before the normal 126–133 peptide was observed. The normal and modified peptides were isolated and analyzed by amino acid analysis, amino acid sequencing, and fast-atom bombardment mass spectrometry (FAB-MS). The normal sequence for residues 126–133 is Glu-Leu-Glu-Asp-Gly-Ser-Pro-Arg. The amino acid composition and mass of the modified peptide (protonated masses of 903 Da) were determined to be identical to that of the normal peptide. However, amino acid sequencing of the modified peptide yielded only the first three residues even though amino acid analysis had shown that the entire sequence was present. These data strongly indicated that the modification present in this peptide was the conversion of Asp¹²⁹ to an isoaspartyl residue, because the Edman degradation chemistry cannot proceed through an isoaspartyl residue (Smyth et al., 1962).

To demonstrate that this modified peptide contained an isoaspartyl residue a peptide was chemically synthesized with the sequence Glu-Leu-Glu-isoAsp-Gly-Ser-Pro-Arg. The chemically synthesized peptide and the peptide from PST-X coeluted when analyzed under the identical RP-HPLC conditions used for the peptide mapping in Figure 3. These data showing that PST-X contained an isoaspartate in place of Asp did not, however, agree with other analyses of this protein. Isoelectric focusing (IEF) of the isolated PST-X showed that this protein had a significantly higher pI (8.3) than standard PST (7.4) (Fig. 4). The conversion of an Asp to an isoAsp would not be expected to increase the pI of a protein almost 1 pH unit, assuming no large conformational changes in the protein. The molecular mass of PST-X was determined by electrospray mass spectrometry (Fig. 5) to be 21,778 Da, 18 Da

less than normal PST and indicative of the loss of 1 H₂O molecule.

Another puzzling fact was that intact PST-X eluted after standard PST on RP-HPLC yet the modified peptide from the RP-HPLC tryptic map of PST-X eluted earlier than the normal peptide. For all other RP-HPLC variants of PST analyzed there has always been a correlation between the relative elution position of the intact modified protein and the altered peptide from the peptide map (i.e., a later-eluting protein yields a later-eluting peptide). From the above data it was postulated that PST-X contained a succinimide instead of isoAsp and the succinimide was not observed from the peptide map because of its instability during this procedure. If PST-X contained a succinimide then it might be hydrolyzed during the trypsin digestion procedure, which was performed at pH 9.0.

Incubation of a sample containing about 20% PST-X at a pH of 9 or 11 resulted in the disappearance of PST-X (Fig. 6). This indicated that PST-X was not stable to alkaline treatment and supported the supposition that this protein contained a succinimide and not an isoaspartate. Because of this possibility, the normal peptide mapping procedure was modified by adding fivefold more trypsin and performing it at an acidic pH (6.5) instead of the normal pH 9.0. Analysis of the pH 6.5 trypsin digest demonstrated that a later-eluting peptide of residues 126–133 (Fig. 7B) was obtained for PST-X. In contrast, the modified peptide eluted earlier when the digest was done at pH 9.0 (Fig. 3B). This result indicated that a different modified peptide was obtained from PST-X depending on the pH used for the trypsin digestion. Both the Asp- and isoAsp-containing synthetic peptides were shown to be completely stable to the trypsin digestion conditions thus eliminating the possibility that either of these residues was modified during the digestion at either pH 9.0 or 6.5 (unpubl.).

The modified peptide from the pH 6.5 digest was purified and extensively characterized. Amino acid analyses yielded the same composition as normal residues 126–133. Amino acid sequencing only yielded the first three amino acids for this peptide. These two analyses cannot differentiate between a peptide containing an isoAsp or a succinimide residue. Discrimination between these two species was obtained through the use of mass spectrometry and capillary zone electrophoresis (CZE). The mass difference between a succinimide and either Asp or isoAsp is 18 Da because the succinimide is generated by the loss of one water molecule. The protonated mass of the modified peptide from the pH 6.5 digest was determined by FAB-MS to be 885 Da, which was 18 Da less than either the normal aspartate or the isoaspartate containing peptides that had a protonated mass of 903 Da. These results agreed with the postulation that the modified residue was a succinimide.

Because the electrophoretic mobility of a peptide with a succinimide should be significantly different than the

Fig. 4. Analytical isoelectric focusing (IEF) of purified PST-X. Lane 1, IEF standards; lane 2, normal PST; lanes 3–5, PST incubated at pH 5.0, 50 μ g, 100 μ g, and 20 μ g, respectively; lane 6, purified PST-X.

Fig. 5. Comparison of the positive ion electrospray mass spectra of (A) PST and (B) PST-X. Deconvolution of the multiple charge states of the two proteins yields molecular weights of 21,796 amu and 21,778 amu, respectively, which differ by 18 amu and indicates loss of one H₂O molecule. The calculated molecular weight of PST is 21,798 amu.

same peptide with an Asp or isoAsp residue, CZE was used to analyze these peptides. A synthetic Asp, synthetic isoAsp, and the isolated modified peptides of residues 126–133 were analyzed at pH 6.2 using CZE (Fig. 8). At this pH the Asp and isoAsp peptides would have an overall charge of approximately -2 since they contain four negative charges (2 Glu, 1 Asp, and C-terminal COO⁻) and two positive charges (NH₃⁺-terminal α -amino and Arg). The succinimide form of this peptide would have an overall charge of approximately -1 since it has one less

negative charge (Asp to succinimide) and it should have less mobility when analyzed as an anion by CZE. The modified peptide from the pH 6.5 digest did elute significantly later than the other two peptides at this pH. The high resolution of this technique was evident because even the Asp and isoAsp peptides were almost baseline resolved even though they should have nearly identical charges. Analysis of these same three peptides as cations was performed at pH 2.5 where they should all contain approximately two net positive charges (Fig. 9). CZE was able to

Fig. 6. RP-HPLC analyses of PST containing approximately 20% PST-X. The solid line shows the chromatogram before incubation at pH 9.0 and the dashed line shows the same sample after incubation at pH 9.0, 22 °C, for 4 h.

Fig. 7. RP-HPLC analyses of tryptic digests (pH 6.5) of normal PST (A) and PST-X (B). The peptide labeled 1 in A is residues 126–133 with the normal Asp¹²⁹. The peptide labeled 2 in B is modified and contains residues 126–133.

Fig. 8. Capillary zone electrophoresis at pH 6.2 of purified PST residues 126–133. Peptide 1 contains Asp¹²⁹, peptide 2 contains isoAsp¹²⁹, and peptide 3 contains a succinimide¹²⁹.

Fig. 9. Capillary zone electrophoresis at pH 2.5 of purified PST residues 126–133. Peptide 1 contains succinimide¹²⁹, peptide 2 contains Asp¹²⁹, and peptide 3 contains isoAsp¹²⁹.

effectively separate all three peptides at pH 2.5 even though their overall charges are very similar. The degree of separation of the succinimide¹²⁹ peptide was much less at pH 2.5 than 6.5 as would be expected.

Further confirmation of the identity of the succinimide¹²⁹-containing peptide was performed by hydrolysis at alkaline pH. A succinimide peptide will yield both the Asp and isoAsp peptides after hydrolysis (Fig. 1). Therefore if the modified peptide does contain a succinimide residue, then its hydrolysis products should confirm its identity. It has been demonstrated in the literature that the ratio of the isoAsp to Asp peptides after hydrolysis of a succinimide peptide is usually about 3:1 (Geiger & Clarke, 1987). Figure 10 shows that after incubation at pH 9.0 the isolated peptide from the pH 6.5 digest of PST-X yielded both the isoAsp- and Asp-containing peptides in a ratio of 3.7:1. The Asp or isoAsp peptides of residues 126–133 were completely stable when incubated at these identical conditions. Incubation of the isolated PST-X peptide under identical conditions used for the pH 9.0 peptide mapping digestion procedure also yielded the same result, demonstrating that during this procedure the succinimide peptide would be converted into its Asp and isoAsp peptides.

Discussion

This report describes the isolation and characterization of a PST-succinimide¹²⁹ species, which is generated during incubation of this protein at acidic solution conditions. This is the first reported characterization of a stable succinimide from a protein solution. The PST-succinimide¹²⁹ and isoAsp¹²⁹ species do not appear to be formed at alkaline pH. This was determined by analyzing the protein incubated at alkaline pH (9.0 or 10.0, 37 °C, 96 h) by both RP-HPLC and by peptide mapping. The instability of

Asp¹²⁹ at acidic pH is most probably because the unionized but not the ionized form of the side-chain carboxyl is the reactive species for succinimide formation. This is because the OH group is a much better leaving group than O⁻ (Geiger & Clarke, 1987). This would also imply that at more acidic pHs the rate of succinimide formation would be faster with decreasing pH because more of the Asp would be in the unionized form (pK_a of Asp is 4.7). Initial results on pH vs. the percentage of succinimide¹²⁹ are shown in Figure 11. These results demonstrate that the optimal pH for succinimide¹²⁹ formation is approximately 5, and at pH <5 less of this modified molecule is formed. Although decreasing the pH below 5 increases the protonation of the side-chain carboxyl group and ac-

Fig. 10. RP-HPLC analyses of the purified modified tryptic peptide from PST-X. The modified peptide was analyzed before (A) and after (B) incubation at pH 9.0, 22 °C, for 15 h. This incubation converted the peptide into two new peptides that contained either an Asp¹²⁹ (B2) or an Isoasp¹²⁹ (B1).

Fig. 11. The effect of pH (3–9) on the % succinimide¹²⁹ formed in PST incubated at 37 °C for 72 h. The percentage of succinimide¹²⁹ was measured by RP-HPLC.

celerates succinimide formation, the lower pH would also be expected to increase the protonation of the backbone peptide nitrogen involved in the nucleophilic attack on the Asp¹²⁹ side chain and this would have the opposite effect of diminishing the rate of succinimide formation.

One reason that succinimide forms of proteins may not be isolated and characterized as such on a routine basis may be because of the conditions used for the analyses of these modified proteins. This report shows that utilizing an alkaline pH for the trypsin digestion procedure for peptide mapping will result in the modification being identified as an isoaspartate instead of the succinimide. Because most peptide mapping procedures utilize trypsin at alkaline pH, this conversion of a succinimide during analysis may be a common event. This anomaly was overcome by performing the digestion at pH 6.5 with an excess of trypsin.

During storage of PST at acidic pH, the formation of isoAsp¹²⁹ was also observed. This species was found by peptide mapping of the normal RP-HPLC peak from Figure 2B. This molecule is most likely generated by slow hydrolysis of succinimide¹²⁹ at the acidic conditions. Analysis of the rate of formation of both the succinimide¹²⁹ and isoAsp¹²⁹ forms of PST is currently being examined. This can most readily be performed by peptide mapping at pH 6.5 and subsequent determination of the amount of each peptide of residues 126–133. The presence of isoAsp¹²⁹ in a purified bovine somatotropin preparation has previously been reported by Wood et al. (1989). The succinimide¹²⁹ species would not have been present in this isolated protein because alkaline conditions were used during the purification and analytical procedures.

Several methods besides RP-HPLC were used in an effort to purify PST-succinimide¹²⁹. Ion-exchange chromatography and hydrophobic interaction chromatography did not result in resolution of this modified species from the standard PST. Preparative IEF using an LKB 110-mL column yielded samples containing 50% PST-succinimide¹²⁹ from a sample that initially contained 20% of this species. IEF may not be able to yield pure PST-suc-

cinimide¹²⁹, however, because of the instability of this protein at its pI, which is 8.5. Therefore as the protein focuses at its pI it slowly hydrolyzes to the Asp and isoAsp forms. RP-HPLC has been the only successful method for yielding pure PST-succinimide¹²⁹.

Only Asp¹²⁹ was identified as forming a succinimide under the conditions examined in this report even though there are 10 other aspartyl residues in PST. This may have been, however, because this was the only succinimide that was resolved by the RP-HPLC method used to separate out succinimide¹²⁹. However, the IEF gel in Figure 4 demonstrated that PST-succinimide¹²⁹ was the only significantly higher pI species present in this sample, and any succinimide formed from aspartate would be expected to have a higher pI than normal PST. The X-ray structure of PST shows that this region of PST contains very low density and indicates that this portion of the protein is probably very flexible (Abdel-Meguid et al., 1987). This flexibility may allow the correct orientation to be obtained for the atoms involved in this reaction. Aspartate¹²⁹ is also the only Asp in PST that has a glycine as the $n + 1$ residue. A small residue in the $n + 1$ residue has been shown to be important for allowing rapid succinimide formation in model peptides. Glycine does not sterically interfere and also allows for more flexibility in the peptide backbone in this region of PST. A combination of these factors and the local conformation around Asp¹²⁹ probably make it a preferred site for this modification to occur in PST. Preliminary results using a variant with Val substituted for Gly¹³⁰ did not show the presence of any PST-X when it was incubated under identical conditions as those in Figure 2B showing that placing a larger amino acid after Asp¹²⁹ eliminated formation of this modified species.

Interestingly, a previous report (Violand et al., 1990) has demonstrated that at alkaline pH isoAsp⁹⁹ readily forms from Asn⁹⁹. At the acidic conditions used in this report to generate succinimide¹²⁹ no isoAsp⁹⁹ was present. These data indicate that under alkaline conditions Asn⁹⁹ is very labile, whereas at acidic conditions Asp¹²⁹ is susceptible to forming the succinimide and subsequent isoAsp molecule. These results also showed that isoAsp⁹⁹-PST was separable from normal PST (Asn⁹⁹) by RP-HPLC; however, this report showed that isoAsp¹²⁹-PST was not separable from normal PST (Asp¹²⁹) using similar RP-HPLC conditions. Future studies will be required to understand this difference in separation of these molecules.

Materials and methods

Proteins

Porcine somatotropin was purified as previously described (Violand et al., 1990).

*Incubation of PST at various pHs to generate succinimide*¹²⁹

Porcine somatotropin was dissolved at 2 mg/mL in 20 mM acetate, 20 mM MES, and adjusted to the desired pH with HCl or NaOH. It was then incubated for 72 h at 37 °C. The sample was then analyzed by RP-HPLC to determine the percentage of succinimide¹²⁹.

Tryptic mapping of PST

The tryptic digestion and analyses were performed as previously described (Violand et al., 1990). Digestion of the protein at pH 6.5 was performed as described above, except the level of trypsin was increased fivefold.

*RP-HPLC separation of PST-succinimide*¹²⁹

A Perkin-Elmer Series 4 HPLC system was used with a Perkin-Elmer ISS-100 autosampler and a Kratos 783 detector. Chromatography was performed on a Vydac C-18 column (218TP54) at 2 mL/min, using an acetonitrile gradient of 50–65% over 15 min at constant 40 mM trifluoroacetic acid (TFA). The column eluate was monitored at 278 nm, and the data were collected and stored using Nelson 440 chromatography software. The PST-succinimide¹²⁹ was collected from the HPLC column and solvent was removed using a Savant Speed-Vac.

Isoelectric focusing

This analysis was performed as previously described (Bogosian et al., 1989) except the concentration of urea in the gel was 3 M urea instead of 6 M.

Mass spectrometry of peptides

Mass spectrometry of peptides was performed using FAB-MS on a Finnigan TSQ mass spectrometer equipped with an Iontech FAB source as previously described (Violand et al., 1990).

Electrospray mass spectrometry of proteins

Lyophilized PST samples were dissolved in water:methanol:formic acid (50:50:1, v:v:v) at approximately 30 pmol/ μ L. This solution was introduced into an electrospray mass spectrometer at 4 μ L/min with a syringe pump. A Sciex API III triple-quadrupole mass spectrometer (Sciex, Inc., Toronto, Canada) equipped with an atmospheric pressure ion source was used to sample positive ions produced from a pneumatically assisted electrospray interface (Bruins et al., 1987). Sample ions generated by the electrospray process were mass analyzed by scanning the first quadrupole in 0.1 amu increments from 1,000 to 2,400 amu in approximately 10 s, and passing mass-

selected ions through the second and third quadrupoles operated in the rf-only mode, to the multiplier, which was operated in the pulse-counting mode. Approximately 20 scans were averaged together to obtain the mass spectra obtained in this study. Mass calibration of the mass spectrometer was accomplished just prior to mass analysis of the proteins by matching ions of polypropylene glycol to known reference masses stored in a mass calibration table of the mass spectrometer. The average molecular mass of the protein and modified protein was calculated from the multiply protonated ions in the mass spectrum using software based upon published equations (Bruins et al., 1987). Briefly, the molecular weight, M , is calculated from the equation $m = (M + nX)/(1/n)$ where n = the number of charged species added, m = the mass/charge ratio, and X is the mass of the charged species. For two species with mass/charge ratios, m_1 and m_2 , where n_2 is the number of charges associated with m_2 , then $m_2 = (M + n_2X)/n_2$ and $m_1 = [M + (n_2 + 1)X]/(n_2 + 1)$. Therefore $n_2 = (m_1 - X)/(m_2 - m_1)$ and $M = n_2(m_2 - X)$. Once the mass of the charged species is established, the number of charges and the molecular weight can be calculated from pairs of adjacent peaks.

Amino acid analysis

Amino acid analysis was performed as previously described (Violand et al., 1990).

Amino acid sequencing

Amino acid sequencing was performed as previously described using an Applied Biosystems Sequenator (Bogosian et al., 1989).

Capillary zone electrophoresis

Capillary zone electrophoresis was performed using a Bio-Rad HPE 100 system. The capillary cartridge used was a coated 30-cm by 25- μ m Bio-Rad cartridge. The peptides were dissolved in 0.1 M sodium phosphate buffer, pH 2.5 or 6.2, obtained from Bio-Rad. The capillary was run at 8 kV, and peptide elution was monitored at 200 nm.

Peptide synthesis

Synthetic peptides were prepared using an Applied Biosystems automatic peptide synthesizer as previously described (Violand et al., 1990).

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