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Purification and characterization of the central segment of prothymosin-α: Methodology for handling highly acidic peptides

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Abstract: Prothymosin-α is a highly acidic protein consisting of 110 amino acids. The central segment of this protein, residues 51–89, is thought to be involved in metal binding which may be necessary for its physiological function. To carry out studies of this peptide, this central segment was synthesized in a linear fashion using Fmoc-based methods on rink amide MBHA resin. However, this peptide could not be purified with the typical straightforward approach of RP HPLC followed by negative mode electrospray ionization mass spectrometry (ESI-MS). This was attributed to the high proportion of acidic residues: 26 out of the 39 residues are aspartic and glutamic acids. The acidity of the peptide prevented retention on the RP HPLC column. Additionally, the ability of the highly negatively charged peptide to retain sodium ions prevented molecular weight determination with ESI-MS. A systematic approach to the purification of this highly acidic peptide was undertaken. Ultimately, strong anion exchange chromatography was used to purify the peptide. Extensive desalting using dialysis was required prior to ESI-MS, and the choice of the buffer proved to be critical. In the end, a purification method was devised that yielded a highly purified peptide and is readily compatible with analysis by ESI-MS. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: prothymosin-α; acidic peptides; strong anion exchange; negative ion mode ESI-MS; dialysis

INTRODUCTION

Prothymosin-α (ProTα) is a highly acidic protein consisting of 110 residues found in the nucleus of cells [1]. Although its function is unclear, there are strong correlations between the expression level of this protein and cell proliferation [2]. The level of prothymosin-α was found to be higher in cancerous tissues than in healthy ones, and therefore it can serve as a biomarker for the presence of malignant cells [3,4]. The central region of ProTα, spanning residues 51–89, is particularly rich in aspartic acid (Asp) and glutamic acid (Glu) and is a putative zinc-binding domain [5]. In fact, 67% of the residues in this 39 amino acid segment are Asp and Glu, and therefore it serves as an excellent general model for moderately sized, highly acidic peptides.

There are numerous other examples of highly acidic proteins and protein domains. For example, parathymosin-α is a homolog of prothymosin-α, each with approximately 50% of their total residues being acidic [6]. A collection of small acidic peptides ranging from 6 to 10 residues was discovered associated with chromatin [7,8]. These peptides are representatives of phosphorylation sites characterized by acidic clusters found in many transcription factors [9]. The protein RNA polymerase II also contains a similar acidic cluster [10]. When dealing with highly acidic peptides, two common problems encountered are purification and characterization. In particular, retention on many chromatographic media is poor and good resolution is difficult to achieve. Further, given the high negative charge borne by ProTα, the presence of salts and trifluoroacetic acid (TFA) lead to significant interference in characterization by electrospray ionization mass spectrometry (ESI-MS). For the 39 amino acid peptide spanning residues 51–89, denoted ProTα(51–89), purification using a strong anion exchange (SAX) column followed by dialysis yielded purified material. Analysis by ESI-MS was made possible by dialyzing the purified peptide sample against an ammonium acetate buffer followed by pure water. Here we discuss the significance of this methodology in overcoming the problems often associated with the characterization of highly acidic sequences.

MATERIALS AND METHODS

Rink amide MBHA resin, HBTU, HOBt and Fmoc-amino acids were purchased from NovaBiochem. *N*,*N*-Diisopropylethylamine (DIEA) and triisopropylsilane were obtained from Aldrich. *N*,*N*-Dimethylformamide and trifluoroacetic acid were purchased from Fisher Scientific. ProTα(51–89) and ProTα(50–89)N50W were purified on a Phamacia Biotech mono-Q 5/5 column using a BioRad HPLC system. Slide-A-Lyzer cassettes with 3.5 kD MWCO with 0.5–3.0 ml and 3–12 ml volumes were obtained from Pierce Biotechnology. ESI-MS was performed on a Bruker Esquire 3000plus quadrupole ion trap mass spectrometer.

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General Procedure for the Solid-phase Synthesis of ProTα(51–89) and ProTα(50–90)N50W

The synthesis was performed manually using a silanized column with a porous glass frit. The peptide was assembled in a linear manner on the 0.75 mmol scale using Rink amide MBHA resin with a 0.69 mmol/g substitution. Upon cleavage, the peptide produced from this resin was *C*-terminally amidated. Coupling reactions were performed in DMF by *in situ* activation of a 4 equiv. excess of Fmoc-amino acid/HBTU/HOBt (3 mmol/3 mmol/3 mmol) and 4 equiv. (3.0 mmol) of DIEA. Coupling times were usually 1.5 h unless the reaction was deemed incomplete, according to the Kaiser test, in which case the coupling was performed again for 2.5 h. The Fmoc protecting group was removed using 20% (v/v) solution of piperidine in DMF and the reaction time was 45 min. After each coupling or deprotection step the resin was rinsed extensively with DMF and the success of these steps were often monitored using the Kaiser test. The final peptide was acetylated using acetic anhydride in DMF with 3.0 mmol DIEA. Upon completion of the synthetic steps, the resin was washed with DMF, dichloromethane, and finally methanol. It was then dried in a vacuum desiccator overnight. The peptide was cleaved from the dried resin in a TIS/H_2O/TFA (3/3/94) solution for 2 h. The reaction mixture was filtered and the peptide precipitated with cold ethyl ether. The precipitate was obtained as a pellet after centrifugation and decanting of the supernatant. The crude peptide was dissolved in water and then freeze-dried. [ProTα(51–89): calculated mass = 4399.61, found = 4402; ProTα(50–89)N50W: calculated mass = 4585.69, found = 4586].

Purification by Strong Anion Exchange Chromatography

The crude peptide was purified on a mono-Q 5/5 strong anion exchange column using 20 mM glycine at pH 5.3 as buffer A and 20 mM glycine with 1 M NaCl at pH 5.3 as buffer B. A gradient elution from 100% A to 0% A in 35 min was used, and ProTα(51–89) had a retention time of 17 min whereas ProTα(50–89)N50W had a retention time of 20 min.

Dialysis

The peaks collected from SAX chromatography were dialyzed extensively to rid the solutions of any sodium. Combined fractions were loaded into 3.5 kD MWCO dialysis cassettes (0.5–3.0 ml or 3–12 ml capacities) and dialyzed against 1 l of 50 mM ammonium acetate 4 times for 2 h each and/or against 1 l of deionized water (18 MΩ cm, pH 5.1–6.0) 4–6 times for 2 h each.

Characterization

All samples were characterized on a Bruker Esquire 3000plus quadrupole ion trap mass spectrometer in negative ESI mode. Purified and dialyzed peptides were diluted in 10/90 methanol/water and buffered to pH ~7 with 2 mM *N*-ethylmorpholine. Sample was delivered to the ESI source with a KD Scientific syringe pump at 250 µl/h, and the electric potential used to initiate ESI was 4000 V. The source conditions were optimized to provide the best S/N with the lowest amount of fragmentation (facile losses of water). The source temperature was set at 50°C. The nebulizer gas pressure was 25 psi and the dry gas flow rate was 5 l/min. The capillary skimmer voltage difference was 88.5 V.

RESULTS AND DISCUSSION

The solid-phase synthesis of highly acidic peptides or proteins poses no unusual challenge, and both linear and convergent methods can be used. In fact, the power of a convergent approach has been demonstrated by the synthesis of full prothymosin-α [11]. Our synthetic strategy for ProTα(51–89) and ProTα(50–90)N50W (see Table 1) involved linear construction of the peptides and included the use of a pseudoproline dipeptide, specifically Fmoc-Glu-(OtBu)-Ser(ψ^{MeMe}pro)-OH, for the addition of Glu-Ser at positions 83 and 84, respectively. This was included in order to facilitate couplings at two subsequent positions identified as difficult coupling sites. In particular, the coupling of Gly69 to Asp70 and coupling of Gly76 to Asp77 proved problematic. At these two positions double couplings were employed.

Although the synthesis may be straightforward, difficulty is usually encountered in the purification of highly acidic peptides. Before detailing the purification of these peptides, it is useful to have a better sense of what is meant by the term *highly acidic*. In the case of ProTα, 54 out of the 110 amino acids are Asp and Glu, thus 49% of the residues are acidic. In the central segment, ProTα(51–89), 67% of the residues are acidic. It appears that not only the percentage of acidic residues is a factor in the chromatographic properties of these peptides, but so are the length and amino acid composition. Henin *et al.* reported success in purification of peptides containing only Leu and Asp, ranging from 5 to 25 residues and containing 52–60% Asp, using standard RP HPLC (C18 column) [12]. With similar peptides ranging from 24 to 33 residues and containing approximately 50% Asp residues, they found that purification employing an SAX column yielded good results. Attempts to purify ProTα(51–89) by RP HPLC were unsuccessful because the peptide was not retained on the C18 column. Analysis of the solvent front peak by ESI-MS revealed the presence of the target peptide.

Table 1 Amino acid sequence of synthetic peptides representing the central region of prothymosin-α. (A) ProTα(51–89) and (B) ProTα(50–90)N50W

(A)	Ac-EVDEEEEEGGEEEEEEEEGDGEEEDGDEDEEAESATGKR-NH_2
(B)	Ac-WEVDEEEEEGGEEEEEEEEGDGEEEDGDEDEEAESATGKR-NH_2

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Calderan *et al.* used RP ion-pair chromatography to separate very small peptides, ranging from 5 to 7 residues, which contained 33–100% Glu residues [13]. Their method was capable of separating peptides differing by a single Glu residue with excellent resolution. Using the tetrabutylammonium ion as a counterion, the elution order is dependent on the hydrophobicity of the tetrabutylammonium–peptide complex, which increases as a function of the number of acidic residues present. Following the method of Calderan *et al.* for purification of ProTα(51–89), it was found that the peptide was not retained by the chromatographic media and so no separation was achieved.

Peptides are usually not purified using normal phase chromatography because the interaction with the polar media is too strong. We reasoned that a hydroxyapaptite column ($Ca_5(OH)(PO_4)_3$) may have the intermediate polarity necessary to retain ProTα(51–90) sufficiently well. Although this approach led to a longer retention time than observed for the reversed-phase column, sufficient resolution was not achieved.

Ultimately, SAX chromatography proved capable of retaining ProTα(51–89) (Figure 1). The peak eluting at 17 min was collected in two fractions. Analysis by ESI-MS showed that the second fraction contained very pure ProTα(51–89). The peptide ProTα(50–89)N50W, which contains a *N*-terminal Trp to facilitate concentration determination, was purified in an identical manner and again the second half of the main peak contained the desired material. The first half of the peak was found to contain a deletion peptide – in this case a single Asp deletion. In a single SAX run 34% of the target peptide was recovered with 99% purity.

Negative ion mode ESI was selected as the characterization technique because the ProTα-derived peptides have very low isoelectric points (~3) and few protonatable residues. ESI produces multiply charged ions, so the m/z values of larger analytes fall within the limited mass range (m/z 50–3000) of quadrupole ion trap instruments. Characterization by ESI-MS requires the collected fractions to be desalted using dialysis. This offline sample preparation step is a major drawback to using ion exchange as a separation method before ESI-MS. The nature of the interference due to sodium ions is multifaceted. Given the high net negative charge of the peptide, it attracts and retains ubiquitous sodium ions very effectively. The sensitivity of the mass spectral analysis is reduced because the ion intensity is spread across a wide distribution of peptide–sodium complexes. Any synthetic work-up steps or chromatography performed using deionized water having a resistivity less than 18 MΩ cm will contain enough sodium to be problematic. Additionally, ion-pairing agents like TFA also easily interfere in negative mode ESI-MS. TFA is widely used in peptide synthesis and purification steps as a cleavage reagent for most Fmoc-based syntheses and as an ion-pairing agent in chromatography. In negative mode ESI-MS, pre-ionized sodium/TFA adducts appear with very high intensities. The adducts are identified easily in a mass spectrum by the spacing of 136 Da, the mass of an added sodium/TFA pair to the neighboring lower m/z adduct. In all cases, these ion pairs completely masked the presence of analyte in the mass spectra. In our experience, even after three days of dialysis against deionized water, with six 1 l buffer switches per day, the peptide retained sodium; so it could not be unambiguously detected by ESI-MS (Figure 2). The ineffectiveness of even a lengthy dialysis time can be explained by the high negative charge borne by the peptide, as discussed above, plus the low concentration of cations (H^+) in the deionized water (pH 6.0). Cations are needed to balance the flow of Na^+ out of the dialysis cassette in order to prohibit the buildup of an electric potential. The H^+ concentration could have been increased by lowering the pH, but that could have resulted in precipitation and/or the introduction of more salts incompatible with ESI. Dialysis of the peptide against 50 mM ammonium acetate rapidly rids the sample of all detectable sodium because charge balance is quickly achieved on both sides of the dialysis cassette. This volatile salt is compatible with ESI-MS, and as little as two, 30 min dialyses against this buffer are sufficient to make SAX purified ProTα(50–89) ready for direct analysis by MS (Figure 3).

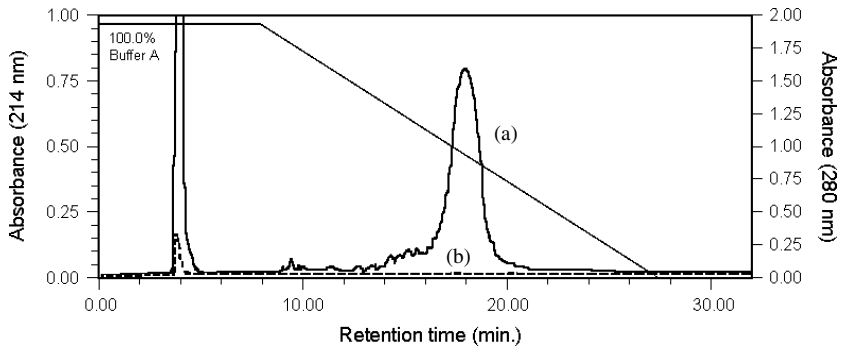

Figure 1 Strong anion exchange HPLC profile of crude ProTα (51–89) monitored at 214 nm (a) and 280 nm (b). For conditions see 'Materials and Methods'.

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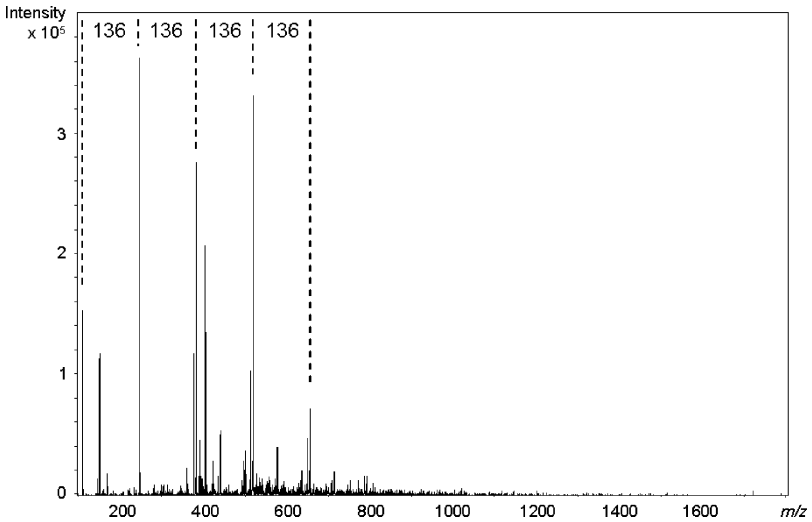

Figure 2 ESI-MS of ProTα sample after strong anion exchange HPLC and dialysis against water. Na/TFA complexes, evidenced by peaks spaced at 136 Da, dominate the signal.

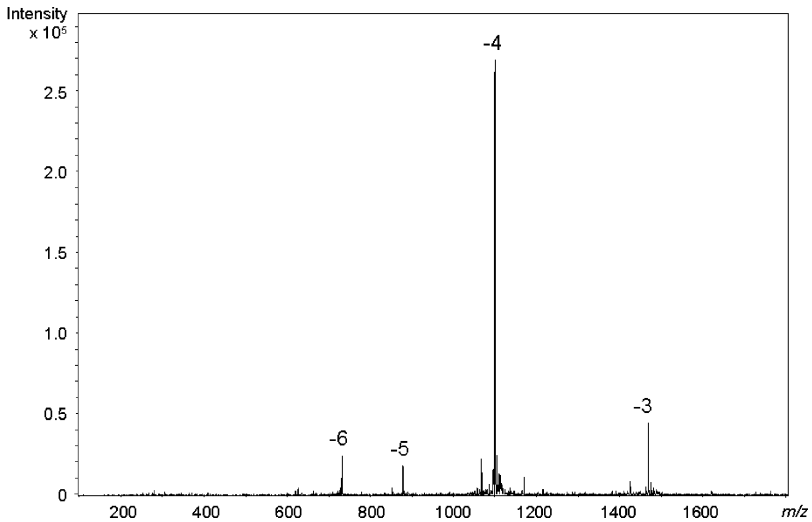

Figure 3 ESI-MS of ProTα sample after SAX HPLC and dialysis against ammonium acetate.

CONCLUSIONS

The methods described above can greatly facilitate the purification and analysis of highly acidic peptide sequences. Problems associated with characterization can easily lead one to the conclusion that a synthesis has failed when in fact it has not. While matrix-assisted laser desorption/ionization (MALDI) can be used to ionize and volatilize analytes in the presence of salts and other interferences, ESI is often the only available ionization method. ESI is popular because of its compatibility with on-line HPLC methods, and because ESI can be used with low m/z range mass analyzers. Thus, it is critical to develop a purification method that is compatible with ESI-MS. The method presented herein can be easily and quickly applied to the characterization of a variety of highly acidic peptides.

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