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Approach to studying proteinase specificity by continuous-flow fast atom bombardment mass spectrometry and high-performance liquid chromatography combined with photodiode-array ultraviolet detection

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ABSTRACT

Fast atom bombardment mass spectrometry (FAB-MS) and high-performance liquid chromatography using a photodiode-array ultraviolet detector were applied to study a dynorphin-converting endopeptidase from the human pituitary gland. The specificity of the enzyme was tested towards various opioid peptides derived from the prodynorphin precursor, *i.e.* dynorphin A, dynorphin B and α-neoendorphin. Peptide fragments were analysed directly by continuous-flow FAB-MS and those containing aromatic amino acids were detected independently by the photodiode-array ultraviolet detector. The results obtained suggest a similar processing of these structure-related substrates and it appears that the enzyme recognizes the dibasic stretch in their sequence. It is also clear from this study that the combination of the above techniques provides a powerful tool for studies of enzymatic conversion among the prodynorphin-derived peptides and it should be applicable to studies of similar mechanisms in other peptide systems.

INTRODUCTION

It is well known that many of the biological processes occurring in living systems are regulated by a variety of proteinases present in various tissues or body fluids [1]. In recent years a particular interest has been focused on proteolytic regulation in peptidergic pathways in the central nervous system (CNS). One important pathway concerns the conversion and degradation of the prodynorphin-derived neuropeptides (Table I) as they are reported to be involved in pain control [2]. Specific cleavage of these peptides may alter their receptor activation profile from κ- to δ-specific, *i.e.* the parent peptides exhibit an affinity for the so-called κ opioid receptors, whereas their products (Leu-enkephalin or Leu-enkephalin-Arg^6) preferentially bind to δ receptors [3].

The detailed and quantitative determination of the converted fragments by radioimmunoassay (RIA) is difficult because of the lack of all necessary antibodies and in such cases fast atom bombardment mass spectrometry (FAB-MS) and high performance liquid chromatography (HPLC) techniques are the methods of choice. The simultaneous analysis of several fragments by direct probe is limited, however,

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TABLE I

STRUCTURE OF PRODYNORPHIN-DERIVED PEPTIDES

Peptide	Structure	[M + H]$^+$
Dynorphin A	Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro–Lys–Leu–Lys–Trp–Asp–Asn–Gln 1 5 10 15	2147
Dynorphin B	Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Gln–Phe–Lys–Val–Val–Thr 1 5 10	1570
α-Neoendorphin	Tyr–Gly–Gly–Phe–Leu–Arg–Lys–Tyr–Pro–Lys 1 5 10	1229

due to the signal suppression of the more hydrophilic peptides in a matrix with a high glycerol concentration [4]. Continuous-flow (CF) FAB-MS analysis overcomes this problem and also allows the quantitation of the molar ratios of the released peptides [5]. The analysis can be further extended to a multispectral search for fragments containing aromatic amino acids. This latter technique allows the rapid, on-line verification of the separated products and can also serve to monitor the progress of the reaction.

The aim of this work was to apply CF-FAB-MS and HPLC combined with a photodiode-array UV detector for the identification of the cleavage sites of various prodynorphin-derived peptides converted by the same enzyme purified from the human pituitary gland.

EXPERIMENTAL

The peptides used in this study were purchased from Bachem (Bubendorf, Switzerland). They were analysed by FAB-MS and by reversed-phase HPLC for impurities that could interfere with the described techniques. All other chemicals were of analytical-reagent grade and were purchased from various commercial sources.

Frozen human pituitary glands were collected at autopsy and generously supplied by Kabi (Stockholm, Sweden). The dynorphin-converting enzyme (DCE) was purified to apparent homogeneity, essentially following the procedure outlined elsewhere [6]; the detailed isolation protocol will be published in a separate paper.

The enzyme assay was performed as described by Silberring and Nyberg [6], with minor modifications. Briefly, 0.5 μg of enzyme was incubated with the particular peptide (15 μg) and the reaction volume was adjusted to 60 μl with 0.1 *M* disodium hydrogenphosphate–hydrochloric acid buffer (pH 6.0). The reaction was terminated with 500 μl of 0.04% trifluoroacetic acid (TFA) and the samples were desalted on Sep-Pak C_{18} cartridges (Waters, Milford, MA, USA) and evaporated before analysis by FAB-MS. The instrument used was a Finnigan MAT 90 double-focusing mass spectrometer with reverse geometry (*B*, *E* where *B* = magnetic field and *E* = electric field). Xenon was used as a source beam operating at 7 kV and 2 mA. Positive mode magnetic scans were acquired at a full accelerating voltage of 5 kV. The samples were analysed by the CF-FAB-MS technique with the interface supplied by Finnigan MAT, as described by Caprioli *et al.* [7]. After desalting, the incubation mixture was

evaporated and then redissolved to the appropriate concentration in the mobile phase which consisted of 5% glycerol, 5% acetonitrile and 0.1% TFA. Elution was performed through a 0.075-mm fused-silica capillary column at a flow-rate of 5 μl/min, as described elsewhere [8].

An identical degradation experiment was performed in parallel where the samples were separated directly on the reversed-phase HPLC column (TSK ODS-120T; 250 × 4.6 mm; particle size 5 μm) and analysed on-line with a Model 2140 photodiode-array UV detector (Pharmacia, Uppsala, Sweden) as described previously [9]. The column was developed by a linear gradient of acetonitrile (15–45%) containing 0.04% TFA, maintaining a flow-rate of 0.5 ml/min.

The RIA for dynorphin A_{1-6} (DYN A_{1-6}) (or Leu-enkephalin-Arg^6) was based on the charcoal adsorption technique and conducted as described elsewhere [6].

RESULTS AND DISCUSSION

Fig. 1a shows the conversion pattern detected by CF-FAB-MS when DYN A was used as a substrate. The most abundant peak at *m/z* 713 was identified as DYN A_{1-6} (YGGFLR), suggesting that the heptadecapeptide is initially split into two fragments: DYN A_{1-6} and DYN A_{7-17} (RIRPKLKWDNQ, *m/z* 1455). The secondary cleavage was observed between Lys^{11}-Leu^{12}, which leads to the formation of two fragments: DYN A_{7-11} (RIRPK, *m/z* 669) and DYN A_{12-17} (LKWDNQ, *m/z* 804). The question remains open as to whether DCE recognized the Arg^6–Arg^7 stretch as a primary target or whether both events occur simultaneously. Detailed studies require time-dependent experiments which were not the aim of this work. DYN A itself was not observed in the mass spectrum as it was completely converted, nor was any signal recorded at *m/z* 555 belonging to DYN A_{1-5} (YGGFL), *i.e.* Leu-enkephalin, which is probably formed by the sequential action of DCE and a carboxypeptidase B-like enzyme [10]. The pituitary enzyme recognizes the C-terminal side of the basic amino acids, but its specificity seems to be limited to certain recognition sites as it does not process the bond between Arg^9-Pro^{10} and Lys^{13}-Trp^{14}. The signal at *m/z* 713 is ambiguous, as it can also be formed by the fragment DYN A_{5-9} (LRRIR), but the HPLC data and the specific RIA confirm that DYN A_{1-6} is, in fact, present in the incubation mixture.

The fragments obtained after degradation were also analysed on the reversed-phase HPLC combined with a photodiode-array UV detector. Using this technique, three fragments were resolved (data not shown) and the spectra of the two most intense peaks (eluting at retention times of 14 and 20 min) recorded in the range 190–320 nm (Fig. 1b). The peak, eluted at a retention time of 20 min, contains tyrosine, which gives a characteristic maximum around 276 nm, returning to the baseline at 290 nm [11]. Thus, this peak can be identified as the fragment. DYN A_{1-6}, and RIA measurement revealed that the peak eluting at 20 min belongs to Leu-enkephalin-Arg^6. The second component eluting at 14 min is probably identical to the fragment DYN A_{7-17}, as its second maximum returns to baseline at 310 nm (Fig. 1b), which is characteristic for tryptophan [11].

When a reaction mixture of DYN B incubated with the same proteinase was analysed by CF-FAB-MS, only two fragments, at *m/z* 712 and 876, respectively, were observed (Fig. 2a), supporting the indication that in this case the major conversion

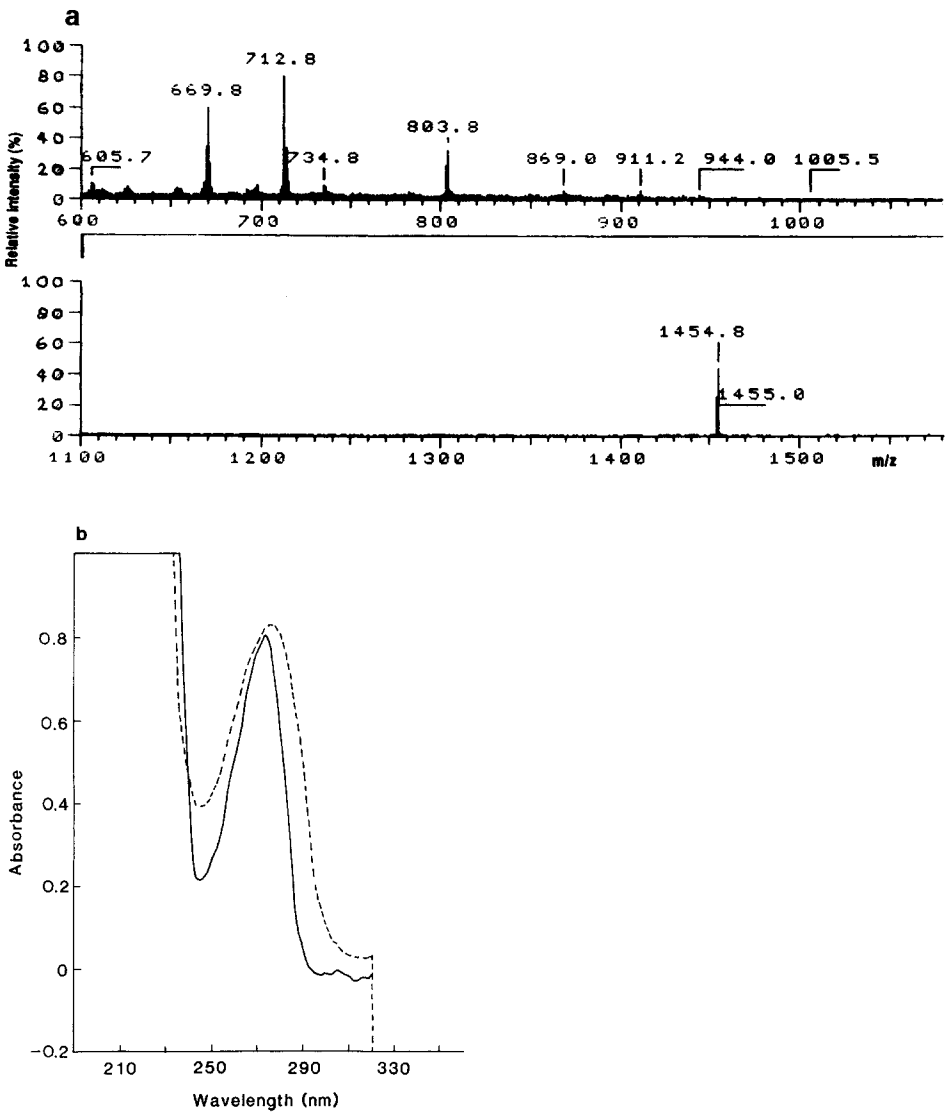

Fig. 1. Analysis of the dynorphin A fragments. (a) CF-FAB mass spectrum of the peptides obtained during conversion by pituitary DCE. DYN A itself was completely processed by the enzyme. (b) Enhanced spectra of the particular fragments containing tyrosine or tryptophan taken within the range 190–320 nm. The spectra were recorded during reversed-phase HPLC separation of the reaction mixture of DYN A and pituitary DCE. The solid line represents the peak containing tyrosine, eluting at a retention time of 20 min, whereas the broken line corresponds to the peak (containing tryptophan) eluting at 14 min. Separation conditions are given in the text.

product is DYN B_{1-6} (YGGFLR) without any secondary cleavage. This is in contrast to the findings when DYN A was served as a substrate. The other ion is formed by DYN B_{7-13} (RQFKVVT). These results were confirmed by HPLC studies (Fig. 2b), where three major peaks were found when the chromatogram was taken at 214

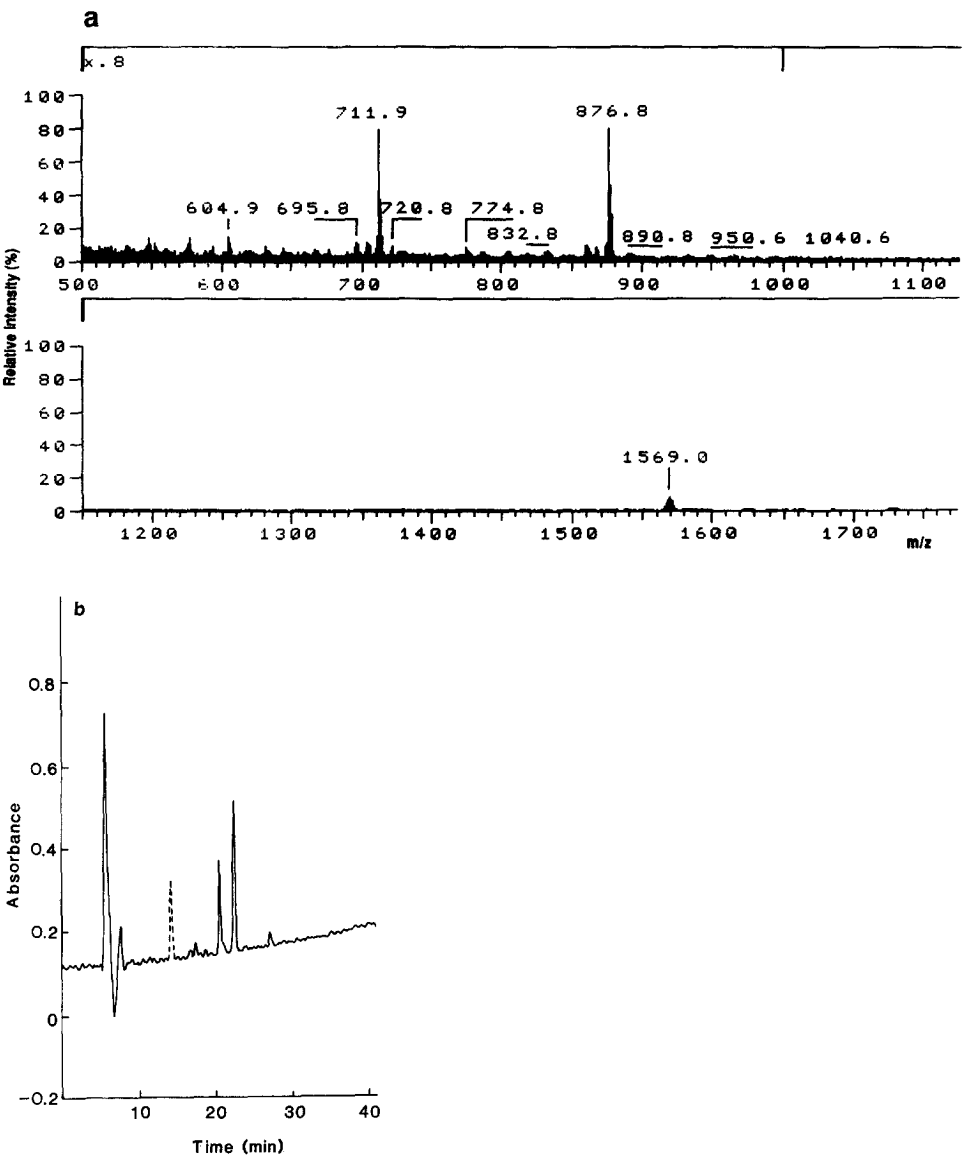

Fig. 2. Analysis of the dynorphin B fragments following conversion of the tridecapeptide by pituitary DCE. (a) CF-FAB mass spectrum of the converted fragments. (b) Reversed-phase HPLC chromatograms, taken at 214 (broken line) and 276 (solid line) nm. (Note that the peak eluted at 14 min belongs to DYN B_{7-13}, which does not contain tyrosine). For further details, see text.

nm. However, the same separation recorded at 276 nm (which is the wavelength characteristic for tyrosine) shows only two peaks, eluted at 20 and 22 min, respectively. The DYN B structure contains only one tyrosine located at the N-terminus and the peak with the retention time of 14 min thus belongs to DYN B_{7-13}, which also correlates with the FAB-MS data.

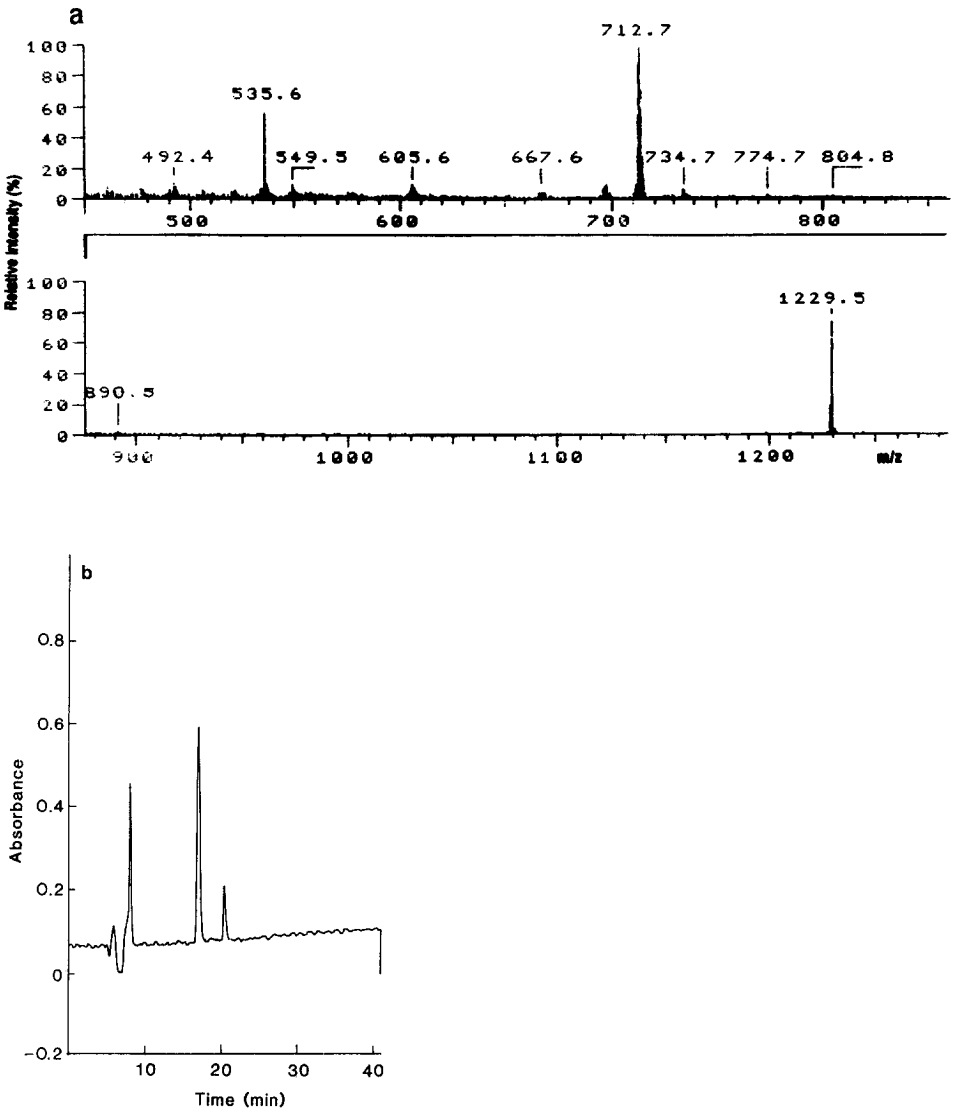

Fig. 3. Analysis of the α-neoendorphin fragments released by the pituitary DCE. (a) CF-FAB mass spectrum of the converted fragments. (b) Chromatogram taken at 276 nm following reversed-phase HPLC. A detailed description of the separation conditions is given in the text.

The data given in Fig. 3a indicate that α-neoendorphin (ANEO) is converted directly to YGGFLR (*m/z* 713) and KYPK (*m/z* 535). No other cleavage sites were recorded. It should be noted that in this study all the peptides were incubated with the pituitary DCE for the same time interval. Therefore, ANEO seems to be a poorer substrate than DYN A, as the molecular ion corresponding to unaltered ANEO (*m/z* 1229) is still present in the spectrum. This observation was corfirmed by the analysis of the reaction mixture by reversed-phase HPLC. Thus, the chromatogram, taken at 276 nm (Fig. 3b), also shows three peaks, eluting at 8, 17 and 20 min, respectively.

This result suggests that all fragments contain a tyrosine residue in their structure, which was confirmed by enhancing the spectral range around 276 nm (data not shown). The absorbance profiles were identical, returning to baseline at around 290 nm, thus indicating the same aromatic components in all structures. Retention data as well as the RIA for $ANEO_{1-6}$ (or Leu-enkephalin-Arg^6) revealed that the peak eluting at 8 min belongs to $ANEO_{7-10}$, which has the lowest hydrophobicity index, and the two others to the unconverted decapeptide (17 min) and $ANEO_{1-6}$ (20 min), respectively.

It can be seen from this study that the different techniques applied have been of great use for the identification of the peptide bonds hydrolysed by the pituitary enzyme. A common cleavage site of all the substrates seems to be located between their dibasic stretch, resulting in the release of Leu-enkephalin-Arg^6, which is the common N-terminal sequence of all the prodynorphin-derived opioid peptides. A secondary cleavage sites was only observed for dynorphin A.

Several converting and degrading enzymes from human cereborspinal fluid and spinal cord capable of cleaving prodynorphin-derived peptides to enkephalins have recently been reported [12]. In these studies, however, the completed fragmentation patterns could not be detected as the only quantitative and reliable method was simple HPLC and an RIA towards DYN A_{1-6}. The latter technique allows the measurement of one or two peptides with high precision, whereas no other fragments are detected and detailed studies on, for example, the ratio between various reaction products are difficult or even impossible. Investigations on the specificity of proteinases recovered from the CNS are also difficult as a result of the limited availablity of tissues or body fluids. The HPLC technique utilizes at least a ten-fold higher amount of the enzyme and at least 100 times more of the substrate than the RIA detection, which might influence the final results due to minor contaminants or the "non-physiological" concentrations of the reagents. This work has described the applications of CF-FAB-MS for the rapid and efficient screening of the cleavage sites of several neuropeptides converted by a pituirary proteinase. HPLC combined with photodiode-array UV detection was applied complementary to the FAB-MS technique. The enzyme action seems to be dependent on the applied substrate, regardless of the presence of a dibasic stretch in all structures, as well as the identical N-terminal fragment YGGFLR. This fact might be of physiological importance during maturation of the bioactive fragments. The samples were analysed directly without prior purification, which significantly simplifies the procedure.

An interesting application of FAB-MS to study peptide fragments directly on the probe after enzymatic cleavage was reported by Hafok-Peters *et al.* [13]. In that work the tryptic or chymotryptic fragments of genetically engineered interferon could be identified. The sensitivity of this technique can be comparable with the RIA method in certain cases, thus this procedure requires only minor amounts of all reagents, whereas spectral analysis with the aid of HPLC photodiode-array detectors which require much higher sample amounts, can be complementary to CF-FAB-MS for the determination of ambiguous fragments [8].

CONCLUDING REMARKS

In this work CF-FAB-MS and HPLC with diode-array UV detection were used

to study the cleavage pattern of a pituitary enzyme acting on prodynorphin-derived opioid peptides. In combination these techniques provide a rapid procedure with a high precision for the identification of peptide fragments with aromatic amino acid residues. It is therefore suggested that, when a sufficient amount of sample is available, the possible incorporation of a photodiode-array UV detector into the HPLC–MS system may give a powerful tool for the assessment of definite cleavage sites in substrate peptides containing these particular residues.

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