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A MASS SPECTROMETRIC METHOD FOR THE IDENTIFICATION OF NOVEL PEPTIDES IN *XENOPUS LAEVIS* SKIN SECRETIONS[1]

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ABSTRACT.—The peptides secreted by the South African frog *Xenopus laevis* were screened systematically using a strategy based on fabms. Hplc of crude and Sephadex G-10 chromatographed secretions showed that many more peptides were present in these secretions than those previously identified, i.e., xenopsin, caerulein, TRH, and PGL[a]. Fractions from the hplc were analyzed directly by fabms to determine the molecular weights of these novel peptides. Subsequent analyses, using a combination of fabms, manual Edman degradation, enzymatic digestions, and amino acid analyses identified the partial and sometimes complete sequences of these peptides which had molecular weights ranging from 700-2700. Many peptides with structural features that are often indicative of biological activity, e.g., C-terminal amides and pyroglutamic acid, were readily identified by fabms. In some cases, molecular weight data combined with partial sequence data was sufficient to identify peptides as originating from spacer regions in the precursors to xenopsin, caerulein, and PGL[a].

For the structure elucidation of unknowns, ms is, in general, a technique of much less information content than nmr. Some of the most impressive applications of ms are therefore in cases where much structural information is already available, e.g., in studies of metabolism or in the sequence determination of peptides. In the latter area, the structures of the twenty basic building blocks are already defined, and it is only the sequence of amino acids that requires determination. It is fortunate that in these problems ms is powerful, because the quantities of compounds available (frequently only a few micrograms or less) precludes the application of nmr.

In the case of peptides, fabms is suitable for the determination of molecular weight on samples as small as 10 pg in favorable cases. Depending on the sample, quantities of ca 1 μg or greater permit the determination of total sequence, partial sequence, or (rarely) no sequence at all. In view of this variable performance of the technique in providing sequence information, fabms is often a powerful adjunct to DNA sequencing. Sometimes when a gene has been sequenced, much has still to be learned about the processing (e.g., into small peptides) of the initial gene product. Molecular weight determination and partial sequence determination of these peptides by fabms permits, in conjunction with the gene sequence, complete structure determination of the peptides.

To utilize the above principles, we have recently been engaged in the characterization of peptides in the skin secretion of the frog *Xenopus laevis*. This peptide secretion occurs when the frog is handled but can be promoted by an injection of epinephrine or nor-epinephrine into a dorsal sac. The constituents of the secretion are of great interest because two of the peptides, xenopsin and caerulein, already identified in the secretion are closely related in sequence to the mammalian peptides neurotensin and gastrin, respectively.

Bioassays and other screening methods used for identifying new peptides in neural and gastrointestinal tissue are generally limited by the low levels of most peptides in these tissues and the difficulty in choosing an appropriate assay. If a specific function can be identified, such as gut contraction or hormone release, then an appropriate and

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sensitive bioassay can often be devised. However, when the function(s) or biological activities are not known, the detection and isolation of these peptides become considerably more difficult.

In *X. laevis*, as in many amphibians, the peptide content of its dorsal skin is much greater than that of mammalian neural and gastrointestinal tissue (1). Xenopsin, an amphibian analogue of neurotensin, was first isolated from *Xenopus* skin extracts by using a bioassay based on the contraction of rat stomach and guinea pig ileum (2). Caerulein and TRH have also been identified in *Xenopus* skin extracts (3,4) although they were first discovered in other sources (5,6). However, an examination of the hplc chromatograms obtained in this laboratory of *Xenopus* skin secretions showed a multitude of compounds, many more than one would expect, considering the extensive screening these skin extracts and secretions have undergone (2-4). Conceivably, a number of additional bioactive compounds, perhaps peptides, are contained in these secretions but have so far escaped detection due to the limited nature or sensitivity of the original bioassays. Recently, Kreil and co-workers predicted the existence of a new peptide from *Xenopus* (PYL^a) on the basis of a cDNA sequence encoding it and not from any chemical or biological assay (7). A shortened version of this peptide (PGL^a) was subsequently isolated, although no biological function has yet been identified despite a series of tests (P. Emson, University of Cambridge, and G. Kreil, Institute for Molecular Biology, Salzburg, Austria, personal communication, 1985).

Clearly, techniques are needed to screen biological extracts for novel peptides without relying solely on bioassays. In an attempt to address this problem, Tatemoto and Mutt (8) have devised a chemical assay for identifying peptides containing C-terminal amides. While this procedure has had notable success in the identification of PYY and PHI, it is obviously limited to peptides containing this structural feature.

In examination of the skin secretions of *X. laevis*, we have attempted to identify novel peptides of potential biological importance. A ms strategy was used to screen rapidly for peptides in these secretions, even when present in complex mixtures, to determine the molecular weights of their constituents. Fabms (9) produced abundant protonated molecular ions (MH^+) and, in some cases, fragment ions that were used to determine the nature of the compounds present in the secretions. In conjunction with manual Edman degradation, enzymatic digestions, and amino acid analyses, fabms identified most of these skin compounds as novel peptides. In addition, the partial or complete amino acid sequences of some peptides showed the presence of C-terminal amides and N-terminal pyroglutamic acid. Other peptides were found to be additional proteolytic fragments of the precursors to xenopsin (10), caerulein (11), and PGL^a (7).

METHODS

PREPARATION AND CHROMATOGRAPHY OF *XENOPUS* SKIN SECRETIONS.—Secretions from *X. laevis* were collected in 50 mM ammonium acetate (pH 8) after injection of 50 nmol of norepinephrine into the dorsal sac (3). Skin secretions appeared after 5 min and were frozen in liquid nitrogen within 15 min after initial injection. The material was lyophilized and then dissolved in 1% HOAc, filtered, and again lyophilized. Alternatively, the material was passed through Sephadex G-10 prior to the second drying to remove low molecular weight compounds. The dried material (ca. 5 mg/frog) was taken up in 2% HOAc and injected onto a Spherisorb ODS2 hplc column (25×0.4 cm or 25×0.8 cm). A linear gradient starting with 0.1% trifluoroacetic acid/H_2O was taken to 60% 0.1% trifluoroacetic acid/MeCN over a period of 60 to 120 min. The eluent was monitored by uv absorbance at 210-215 nm, and fractions were collected for subsequent analyses.

MASS SPECTROMETRIC ANALYSIS.—Samples for fabms analysis were dissolved in 1-5% HOAc and dried on the mass spectrometer probe tip under vacuum. Approximately 1-2 μl of glycerol or a 1:1 mixture of thioglycerol-diglycerol was added to the probe tip and mixed thoroughly with the sample. The probe was inserted into the ion source of a Kratos MS 50 mass spectrometer equipped with an Ion Tech FAB gun operating at 30 μA and 8 kV with xenon (12). Mass spectra were taken at a scan speed of 100-300 sec/decade with a Kratos high field magnet capable of analyzing *m/z* 1-3,200 at an accelerating voltage of 8 kV.

EDMAN DEGRADATION AND ENZYMATIC DIGESTION.—Samples collected from hplc were subjected to one or more cycles of manual Edman degradation after analysis by fabms using the procedure of Tarr (11). The truncated peptides were subsequently reanalyzed by fabms, as pure peptides or in mixtures, to determine the N-terminal residue(s) by the corresponding change in their molecular weights (14).

Enzymatic digests of peptides using trypsin, chymotrypsin, and carboxypeptidase Y were analyzed directly by fabms without prior purification. The molecular weights of the resulting peptide fragments were determined from the observed MH^+ ions. Enzyme/substrate ratios of 1:100 to 1:50 (w/w) were generally used and mixtures were incubated at 37° for 1-2 h (trypsin and chymotrypsin) or up to 12 h for carboxypeptidase Y digests. A 50 mM ammonium acetate buffer (pH 8.3) was used for the tryptic and chymotryptic digests while distilled H_2O was adequate for carboxypeptidase Y digestions.

RESULTS

The chromatographic separation by reversed phase hplc of *Xenopus* skin secretions shown in Figure 1 gives a clear indication of the complexity of this material. Judging by

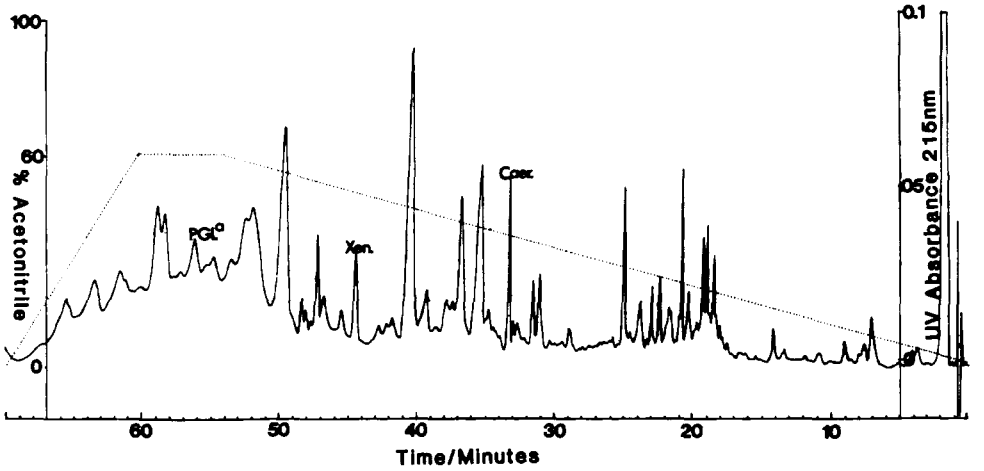

FIGURE 1. An hplc chromatogram of *Xenopus laevis* skin secretions. The elution positions of xenopsin, caerulein, and PGL^a are indicated. TRH is not present because the crude secretions in this case were first chromatographed by Sephadex G-10 and only the breakthrough was collected (M_r>700).

the uv absorbance, the levels of xenopsin, caerulein, and PGL^a are similar to those of twenty or more unidentified peaks. The broad elution pattern also indicates considerable chemical diversity amongst these compounds, at least in terms of hydrophobicity. When these unidentified peaks were collected and analyzed by fabms, generally as impure fractions, a large number of MH^+ ions could be identified (see Figure 2). By the simple subtraction of the mass of a proton, the molecular weights were assigned from these fabms spectra, and a list was tabulated (Table 1). Although the natures of these compounds were not yet determined, the molecular weights were clearly in the range one would expect for peptides of between five and thirty amino acids in length.

COMBINED EDMAN DEGRADATION AND FABMS.—A combination of Edman degradation and fabms (14) was carried out on a number of the hplc fractions. These experiments were able to show the peptide nature of these compounds by causing a change in their molecular weights corresponding to the loss of their N-terminal amino acids. One such fraction gave two adundant MH^+ ions at *m/z* 996 and 976, which matched the molecular weights (M_r=995 and 975) predicted for two regions in the caerulein precursor bordered by single basic residues (to be published). Two cycles of Edman degradation followed by fabms analysis after each step showed the MH^+ ions shifting to *m/z* 1074 and 1054 (1st cycle) and *m/z* 927 and 941 (2nd cycle), respectively. The increase in mass of the MH^+ ions after the first cycle is from the addition of

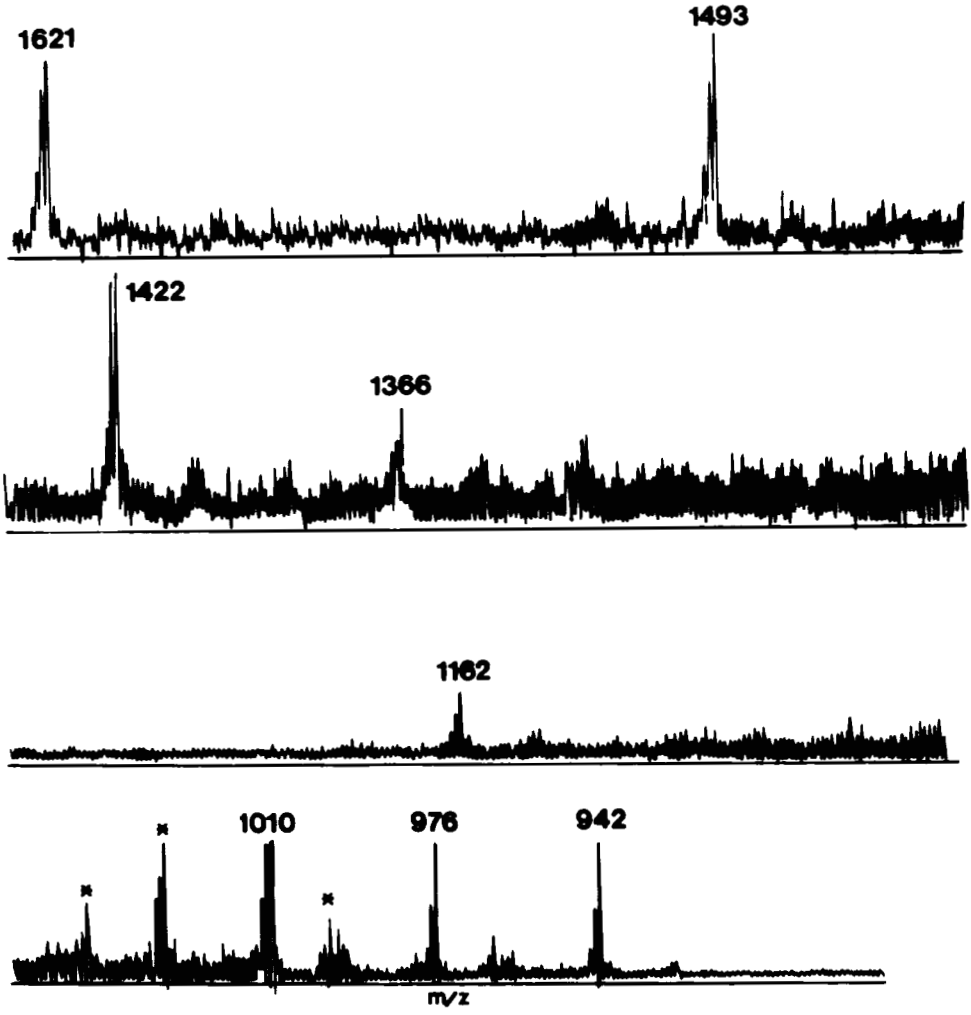

FIGURE 2. A partial fabms spectrum of an hplc fraction from *Xenopus laevis* skin secretions. The protonated molecular ions (MH^+) are labeled for each peptide. The ions of lower abundances identified by asterisks correspond to the sodium and potassium adducts (MNa^+ and MK^+) of peptides already identified from their MH^+ ions.

phenylisothiocyanate to lysine, forming the phenylthiocarbamyl derivative. After subtracting this mass (135 Da), the change in mass for the peptides is a loss of 57 Da (glycine) for both peptides in the first cycle and a further loss of 113 Da (leucine or isoleucine) and 147 Da (phenyalanine) in the second cycle for the peptides with M_r 975 and 995, respectively. These data are consistent with the sequence of the two spacer regions in the precursor to caerulein:

Gly-Phe-Gly-Ser-Phe-Leu-Gly-Lys-Ala-Leu M_r=995
Gly-Leu-Ala-Ser-Phe-Leu-Gly-Lys-Ala-Leu M_r=975

Subsequent separations and purification of these two peptides gave fabms spectra containing sufficient fragmentation to confirm their entire sequences (data not shown).

FABMS PEPTIDE SEQUENCING.—As mentioned earlier, fabms spectra of a single peptide or a simple mixture of peptides can often be used to deduce complete or partial amino acid sequences (12). The major fragmentations observed in the positive ion mode are cleavages on either side of the amide nitrogen, usually accompanied by proton trans-

TABLE 1. Protonated Molecular Ions (MH^+) of Compounds in the Skin Secretions of *Xenopus laevis* Determined by Fabms and their Corresponding Molecular Weights (M_r)

MeCN					
60%[a]	55-60%	50-55%	45-50%	35-40%	30-35%
2662 (2661)[b]	2465 (2464)	1855 (1854)	1564 (1563)	1667 (1666)	1623 (1622)
2646 (2645)	2408 (2407)	1656 (1655)	1510 (1509)	1607 (1606)	933 (932)
2608 (2607)	2287 (2286)	1429 (1428)	1427 (1426)	1443 (1442)	875 (874)
2600 (2599)	2194 (2193)	1318 (1317)	40-45%	1352 (1351)	
2584 (2583)	1983 (1982)	1226 (1225)	1722 (1721)	1252 (1251)	
2475 (2474)	1779 (1778)	1175 (1174)	1658 (1657)	1245 (1244)	
2454 (2453)	1621 (1620)	1141 (1140)	1542 (1541)	742 (741)	
2445 (2444)	1493 (1492)	1010 (1009)	1117 (1116)	741 (740)	
2038 (2037)	1366 (1365)	996 (995)	1057 (1056)		
1967 (1966)[c]		980 (979)[d]	1053 (1052)		
		976 (975)	929 (928)		
		942 (941)			

[a]Approximate hplc elution position expressed as %MeCN (mobile phase)
[b]Molecular weights are in parenthesis
[c]MH^+ and M_r of PGL[a]
[d]MH^+ and M_r of xenopsin

fer (see Figure 3). In general, a series of such ions can be seen corresponding to cleavages at a number of the amino acid linkages. By calculating the mass differences between these ions, the sequence can be determined, although not always completely. Simple as this approach may seem, careful inspection of the fragment ions is important for a correct interpretation.

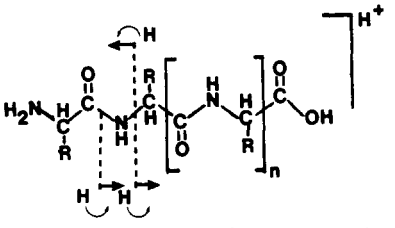

FIGURE 3. The major fragment ions observed in positive-ion mode fabms spectra of peptides. Cleavages on either side of the amide nitrogen with concurrent proton transfer produce fragment ions with charge retention on the C-terminus (↦) and the N-terminus (↤).

One such case involved the observation of a peptide (MH^+=933) in an hplc fraction containing other MH^+ ions. When this fraction was rechromatographed by hplc using a shallower gradient, a single peak was isolated that gave the fabms spectrum shown in Figure 4. An analysis of this spectrum showed the sequence to be identical to the first eleven amino acids in PGL[a], beginning and ending in glycine. However, the ions corresponding to the losses of glycine from both the N-terminus (−57 Da, *m/z* 876) and C-terminus (−58 Da, *m/z* 875) were much larger than the other fragment ions. This indicated that another peptide of M_r=874 (MH^+=875) was most likely co-eluting with PGL[a] (residue 1-10). Since C-terminal glycine is often a recognition site

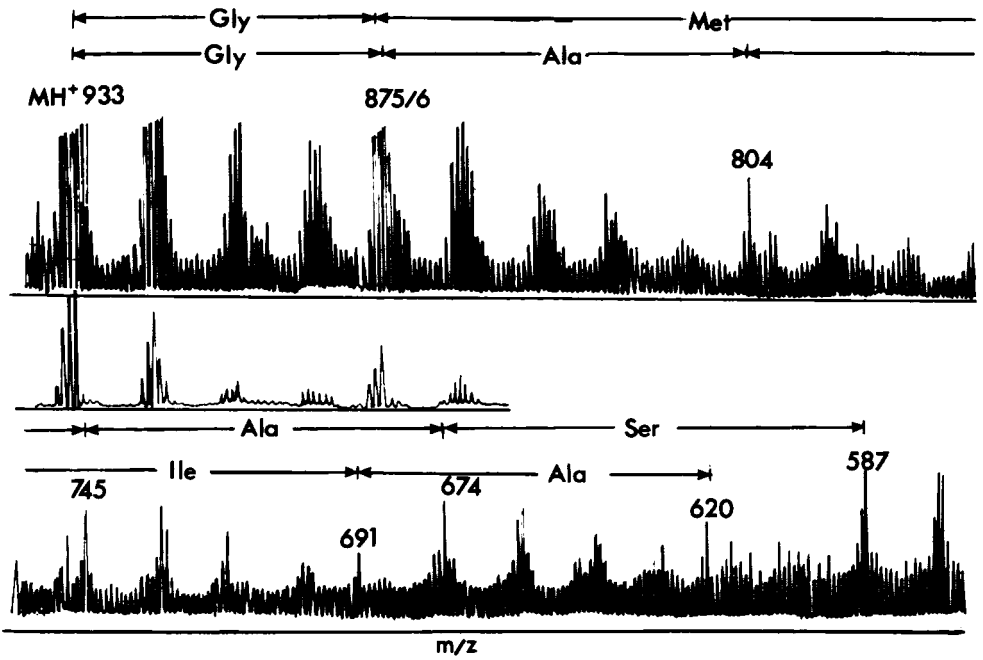

FIGURE 4. Positive-ion fabms spectrum of PGL^a (residue 1-11) showing an abundant $MH^+=933$ and extensive fragmentation. The fragment ions are consistent with the sequence of PGL^a (residue 1-11): Gly-Met-Ala-Ser-Lys-Ala-Gly-Ala-Ile-Ala-Gly. An abnormally abundant fragment ion at *m/z* 875 is partially due to the presence of PGL^a (residue 1-10)-amide (see text).

for amidation (13), it was thought that this additional peptide might be PGL^a (residue 1-10) amide, formed by removal of glycine and amidation of the neighboring alanine. In fact, isocratic hplc separated out a peptide with $MH^+=875$, which was later identified as PGL^a (residue 1-10) amide. It is interesting to note that the purified PGL^a (residue 1-11) gave a fabms spectrum with the fragment ions *m/z* 875-876 at similar abundances to the other fragment ions.

ENZYMATIC DIGESTION.—Enzymatic digestion followed by fabms analysis played a crucial part in the structural elucidation of a number of *Xenopus* peptides, particularly with the larger peptides ($M_r>1500$). For example, a peptide with $MH^+=2600$ (M_r 2599) was thought to originate from a spacer region in the caerulein precursor on the basis of its molecular weight and hplc elution position. Initial spectra contained only weak fragment ions, and it was difficult to confirm this assignment. However, a partial tryptic digest on ca. 1-2 nmol of this peptide was analyzed directly by fabms with no prior separation and yielded four abundant MH^+ ions at *m/z* 812, 1112, 1124, and 2600. The first three ions corresponded to those expected for the peptides produced from tryptic cleavage at the three internal lysines and offered the additional data to confirm this preliminary assignment.

For smaller peptides, the strategy was identical. A peptide with $MH^+=1053$ matched that expected for PGL^a (residue 12-21), but more data was needed. A partial tryptic digest produced three MH^+ ions in a single fabms spectrum that matched residues 12-15 ($MH^+=459$), 16-19 ($MH^+=430$), and 16-21 ($MH^+=613$) in the PGL^a sequence:

12 21
Lys-Ile-Ala-Lys-Val-Ala-Leu-Lys-Ala-Leu-amide $M_r=1052$
◄--- 459 ---►◄--- 430 ---►
◄---------- 613 ---------►

C-TERMINAL AMIDES.—A number of peptides were found to contain C-terminal amides and the fabms data were critical in their identification. As mentioned above, a peptide with a $MH^+ = 1053$ was isolated from *Xenopus* skin secretions which was thought to be PGL[a] (residue 12-21). Amino acid analysis yielded a composition $Ile_1Leu_2Val_1Ala_3Lys_3$ which would predict a peptide of $M_r = 1053$, one mass unit larger than determined by fabms. Because the composition did not have an unassigned amide, i.e., Asx or Glx, it could be assumed that this mass discrepancy corresponded to a C-terminal amide. In fact, carboxypeptidase Y digestion of this peptide changed the MH^+ to *m/z* 941, showing a loss of leucine- or isoleucine-amide (112 Da). The actual assignment of leucine-amide was made once it was realized that this peptide corresponded to a fragment of PGL[a].

It should be pointed out that the ion series originating from an amino acid loss at the C-terminus starts with the first cleavage one mass unit larger than an identical loss at the N-terminus (see Figure 3). Confusion can arise when a C-terminal amide is present and this is no longer true, due to identical mass losses from the cleavage of a N-terminal amino acid and the C-terminal amide equivalent. This was the case for a peptide ($MH^+ = 1427$) that was found to contain a C-terminal serine-amide. The fabms spectrum showed two prominent fragment ions involving two consecutive losses of 87 and 115 Da (to *m/z* 1340 and 1225, respectively). These ions could be interpreted as either the N-terminal loss of serine and aspartic acid or the C-terminal loss of serine-amide and aspartic acid. However, Edman degradation determined the N-terminal sequence Lys-Phe, which was supported by the presence of the expected fragment ions at *m/z* 1299 (-Lys) and 1152 (-Lys-Phe). Carboxypeptidase Y digestion failed to remove an amino acid, thus suggesting the presence of an unreactive C-terminal amide. Esterification of this peptide with ethanolic HCl indicated the presence of two carboxyl groups by changing the MH^+ from *m/z* 1427 to 1483. However, a fragment ion 87 mass units lower at *m/z* 1396, the same loss observed in the nonesterified peptide, was also found. This showed conclusively that the C-terminal amino acid was serine-amide because a loss of 116 mass units, corresponding to serine-OEt, would have been the result if it had been a free acid. A partial structure consistent with these data and the amino acid analysis results could now be proposed:

Lys-Phe-(Lys,Ile,Gly_2,Val,Met,Glu,Ala,Phe)-Asp-Ser-amide

N-TERMINAL PYROGLUTAMIC ACID.—A peptide with a $M_r = 1541$ ($MH^+ = 1542$) was found in the skin secretions at levels similar to that of xenopsin. Amino acid analysis gave a composition that at first appeared to be inconsistent with the molecular weight deduced from the fabms data. A composition of $Glx_2Gly_2Met_1Ile_2Leu_1Thr_2Ser_1Lys_2Arg$ would predict a peptide of $M_r = 1559$-1561, depending on the assignments of the two Glx residues. This value was clearly 18-20 mass units larger than the fabms determined $M_r = 1541$. However, this discrepancy could be due to the presence of pyroglutamic acid and the assignment of the remaining Glx as glutamic acid. Inspection of the fabms spectrum showed a fragment ion at *m/z* 1431 that would correspond to the loss of N-terminal pyroglutamic acid (-111 Da), thus providing the necessary confirmation for this assignment.

AMINO ACID ANALYSIS AND FABMS.—An accurate amino acid composition for many peptides was made by comparing their fabms determined molecular weights to the results from the amino acid analyses. As shown for the peptides containing C-terminal amides or N-terminal pyroglutamic acid, mass discrepancies between these results were used to identify these modified amino acids. For other peptides, mass differences between those calculated from the amino acid analyses and their M_r values were used to determine the presence and number of amino acids that are difficult to accurately as-

sign, e.g., tryptophan and cysteine. For example, a peptide with a molecular weight matching a region in the xenopsin precursor gave the expected composition $Gly_3Ala_1Ser_1Lys_1Leu_1Ile_1Gln_1Thr_1$ (M_r calcd.=930) with the exception of a missing Trp. The fabms determined M_r=1116 indicated that one Trp must be present because its mass corresponded to the mass difference between two results of 186 Da.

NEGATIVE-ION FABMS.—In general, peptides that were analyzed by fabms in the negative-ion mode produced data similar to that already obtained in the positive-ion mode. Negative-ion fabms gave primarily the deprotonated molecular ions, $(M-H)^-$, and fragment ions resulting from cleavages similar to those shown in Figure 3, with the obvious difference of concurrent deprotonation (12).

An important exception to this pattern was observed for caerulein, which did not give a MH^+ ion but did give an abundant $(M-H)^-$ ion. In addition to the $(M-H)^-$ ion at *m/z* 1350, an ion of near equal abundance was observed 80 mass units lower at *m/z* 1270, most likely corresponding to the ion $(M-HSO_3)^-$. Presumably, the presence of sulfated tyrosine in caerulein causes the positive and negative molecular ion to be unstable (although less so for the $(M-H)^-$), and fragmentation occurs readily at the sulfate group (16). This effect appears to be limited to sulfated tyrosine and therefore provides an excellent assay for peptides containing this modified amino acid, such as cholecystokinin.

DISCUSSION

As more structural information is obtainable by DNA sequencing and cloning methods, techniques for the rapid and sensitive analysis of peptides and proteins become essential. Fabms represents a particularly useful method, both in the screening of complex biological extracts for novel peptides and in studying peptide biosynthesis. For these applications, the precise determination of the molecular weights of peptides, even in complex mixtures, represents one of the most important uses of fabms. This is especially true if preliminary assignments can be made on the basis of the molecular weight data alone. It is also worth noting that mass spectrometers are now commercially available with extended mass ranges of up to *m/z* 10,000, greatly increasing the size of peptides that can be analyzed by fabms.

In the screening of *X. laevis* skin secretions, a number of peptides were initially identified from their molecular weights as proteolytic fragments of the PGL[a], caerulein, and xenopsin precursors. It appears that they are originating from novel cleavages at single arginine and lysine residues. Subsequent experiments involving combined fabms, Edman degradation, enzymatic digests, and amino acid analyses were carried out to provide the necessary additional structural data. The success of this approach was demonstrated quite effectively for the two caerulein precursor fragments (M_r=995 and 975), whose partial sequences were determined without prior separation by a combination of fabms and Edman degradation. Alternatively, when a relatively pure peptide preparation was obtained, the increased abundances of the fragmentation ions were used to deduce an extensive, if not complete, amino acid sequence.

Although many of the peptides from *Xenopus* could be assigned as additional proteolytic fragments of the precursors to PGL[a], caerulein, and xenopsin, many undoubtably originated from other sources. For these peptides, a sequencing strategy employing both the methods described here as well as DNA sequencing techniques is still in progress. We hope that once this work is completed, it may be possible to consider the purpose and origins of such a large and diverse group of peptides in the skin secretions of *X. laevis*.

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