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MASS SPECTROMETRIC CHARACTERIZATION OF MICROSCALE ENZYME CATALYZED REACTIONS OF SURFACE-BOUND PEPTIDES AND PROTEINS.

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Many important biomolecules occur in nature only in very low abundance. Ever more sensitive analytical tools are thus required for the elucidation of their structure and chemical behavior. We have recently demonstrated (1,2) a new mass spectrometric method for investigating microscale chemical reactions of both gas and condensed phase reagents with the surface of organic solids. Here we report the results of an investigation of the utility of this new method for measuring enzyme catalyzed reactions of surface-bound peptides and proteins.

Details of the method have been described previously (1,2). Briefly, the technique involves practically non-destructive ^{252}Cf fission fragment ionization time-of-flight mass spectrometric analysis (3) of monolayer to submonolayer amounts of molecules noncovalently bound to solid surfaces (4,5) prior to and after chemical modification. The corresponding differences in the mass spectra yield information concerning the products resulting from and the rate of the reaction under investigation. Thus, for example, inspection of the mass spectra of the nonapeptide bradykinin taken prior to and after incubation with carboxypeptidase Y (CPY) (Fig.1) provides a clear indication of the masses and relative amounts of the residual peptide products formed from the successive carboxyl-terminal amino acid hydrolyses. Prior to any reaction, the mass spectrum (Fig.1A) of a monolayer (approximate) of bradykinin (10^{-9} mol) adsorbed onto a thin film of nitro-

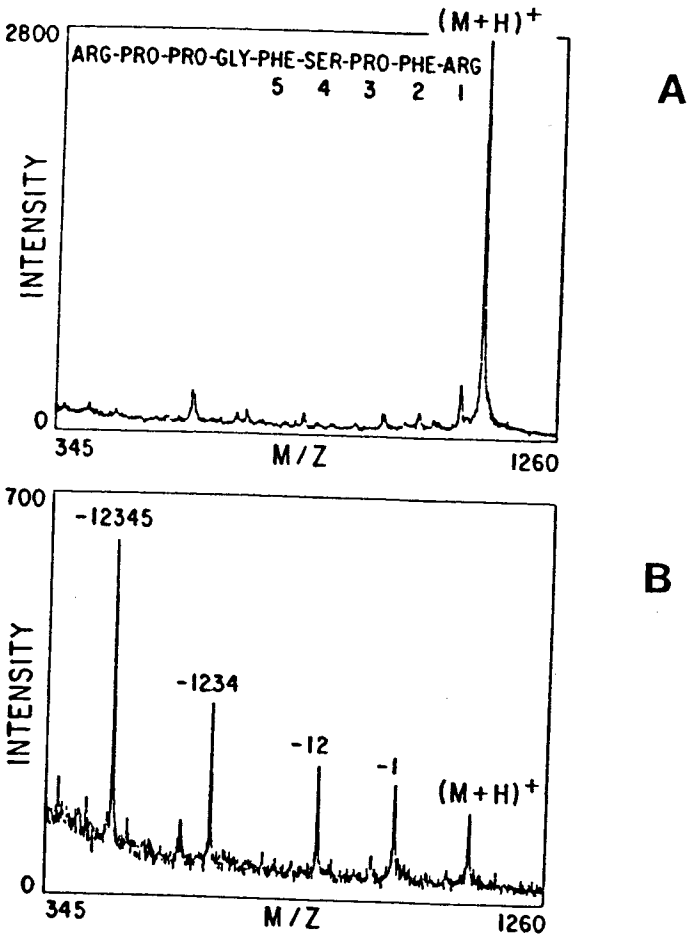

Fig. 1. ^{252}Cf fission fragment ionization time-of-flight mass spectra of bradykinin, bound to a nitrocellulose surface, taken prior to (A) and after (B) incubation with carboxypeptidase Y. (A) Partial mass spectrum of unreacted bradykinin. The solid peptide sample film was prepared by applying 10^{-9} mol of bradykinin to a thin film of nitrocellulose (6). (B) Partial mass spectrum of bradykinin after a 5 min incubation with carboxypeptidase Y at 41°C. The peaks labeled -1, -12, -1234, and -12345 correspond to protonated peptides resulting from the loss of respectively 1, 2, 4 and 5 carboxyl-terminal residues from bradykinin.

cellulose (NC) (6), is dominated by a single peak with a measured mass-to-charge ratio (m/z) of 1060.7 corresponding to the protonated peptide molecule. After reaction of the peptide layer with 10^{-11} mol of CPY, the mass spectrum (Fig.1B) exhibits a series of additional peaks which correspond to the protonated reaction products arising from the loss of respectively 1,2,4, and 5 C-terminal amino acid residues from bradykinin (7). The observed relative intensities of the product peaks reflect the mass spectrometric response for each product as well as the rate constants for removal of the different residues. Since the mass spectrometric responses for peptides of roughly equal size are much the same, the absence of the peak corresponding to the loss of three residues from bradykinin reflects the higher rate constant for the hydrolysis of the third (proline) residue compared with the fourth (serine) residue, and the absence of the peak corresponding to bradykinin minus six residues indicates that the glycine residue is not significantly hydrolyzed during the 5 minute incubation. This example illustrates the potential utility of the present method for C-terminal sequence determinations of peptides (8). We have performed similar analyses utilizing CPY with angiotensin II, β-endorphin, insulin B-chain, and insulin, and we find that, in all cases, products resulting from the removal of C-terminal residues are observed and can be identified. The presently described technique for C-terminal sequencing is complementary to that previously described by Caprioli et al (9) wherein enzyme catalyzed reactions are carried out in solution on the sample probe of a fast atom bombardment mass spectrometer.

The sensitivity limits of the microscale chemical reaction probe were investigated by performing carboxypeptidase B (CPB)-catalyzed hydrolysis of the C-terminal arginyl residue of bradykinin. Figs.2A and 2B show the mass spectra obtained from 1.0×10^{-12} mol of bradykinin taken prior to and after reaction with CPB. The observed signal-to-noise ratio for the peak corresponding to protonated bradykinin is 26:1 (Fig.2A) and for that corresponding to the protonated reaction product is 18:1 (Fig.2B). The spectrum accumulation times were 8.7 minutes and 28.4 minutes, respectively. Since less than 10 ppm of the sample is consumed during these mass spectrometric analysis periods (3), it is feasible further to improve the signal-to-noise ratio by simply accumulating data

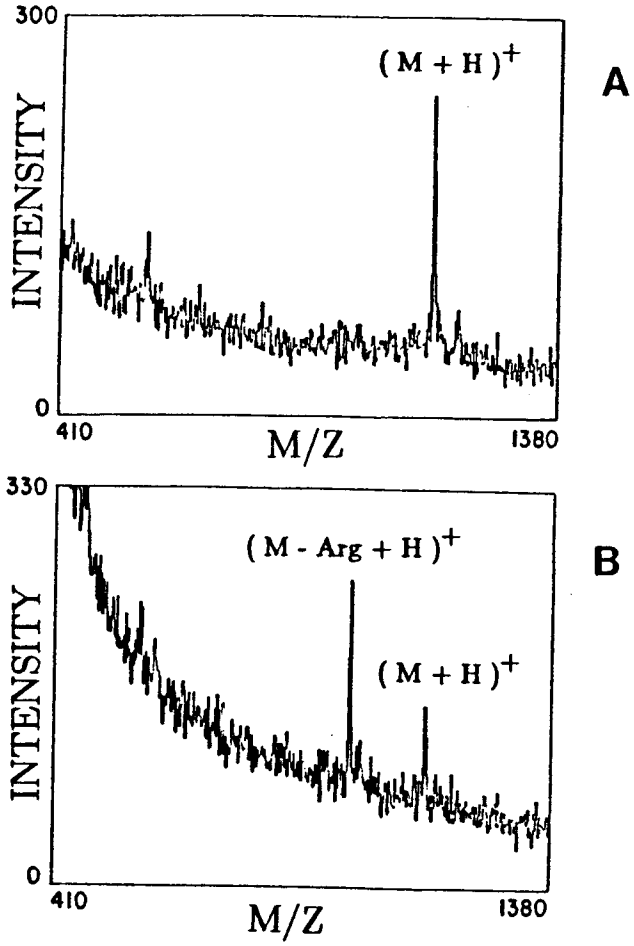

Fig. 2. ^{252}Cf ionization mass spectra of 10^{-12} mol bradykinin bound to nitrocellulose taken prior to (A) and after (B) incubation with carboxypeptidase B. (A) Partial mass spectrum of unreacted bradykinin. (B) Partial mass spectrum of bradykinin after incubation in a solution containing 4 x 10^{-8}M carboxypeptidase B and 0.05M CH_3COONH_4 (pH 7.82) for 12 minutes at 36°C. The reaction was carried out by dipping the peptide coated nitrocellulose surface into a large volume (16 ml) of the enzyme solution.

for a longer time.

The mass spectrometric technique also allowed us to probe fine details of the interaction between the enzymes in solution and the polypeptides adsorbed on the NC film. Thus, for example, we addressed the important question of whether the polypeptide remained attached to the NC throughout the CPB-catalyzed removal of the C-terminal arginyl residue from bradykinin. The determination was made by dipping the bradykinin-coated (10^{-12} mol) NC film into a large enough reservoir of reagent solution (16 ml, 4 x 10^{-8}M CPB) so that, if detachment occurred at any stage during the reaction, the reactant and product would be irreversibly lost from the NC surface (10) and thus would not be observed. Since significant amounts of both product and reactant are present on the NC after reaction (Fig.2B), we conclude that at least part of the reaction occurs with molecules which are and remain bound to the NC surface.

Trypsin is an endopeptidase which hydrolyses peptides on the C-terminal side of the basic amino acid residues lysine and arginine. We have investigated the use of the new technique to identify the resulting tryptic fragments from NC surface-bound vasoactive intestinal peptide, dynorphin, bovine insulin B-chain, and porcine proinsulin. In each case products resulting from the tryptic digestion were readily observed and determined mass spectrometrically. Thus, for example, Fig.3 shows the mass spectra obtained from 1.7 x 10^{-10} mol of porcine proinsulin adsorbed on NC prior to and after reaction with trypsin. Prior to reaction (Fig.3A) the high mass portion of the mass spectrum is dominated by ion peaks corresponding to the addition of 1,2,3 and 4 protons to proinsulin. After reaction of the same sample foil with trypsin these four peaks largely disappear from the spectrum and are replaced by a series of lower molecular weight protonated tryptic fragment product peaks. The measured mass-to-charge ratios of these tryptic fragment ions are given in Table 1 together with the masses calculated for the postulated ions given in column 2 of the table. Peaks A,B,D and F correspond to readily rationalized tryptic fragment ions. We do not know the identity of peak G. Peaks C and E are respectively 71±1 daltons below peaks B and D and arise from a previously unobserved genetic variant of porcine proinsulin (11). This example illustra-

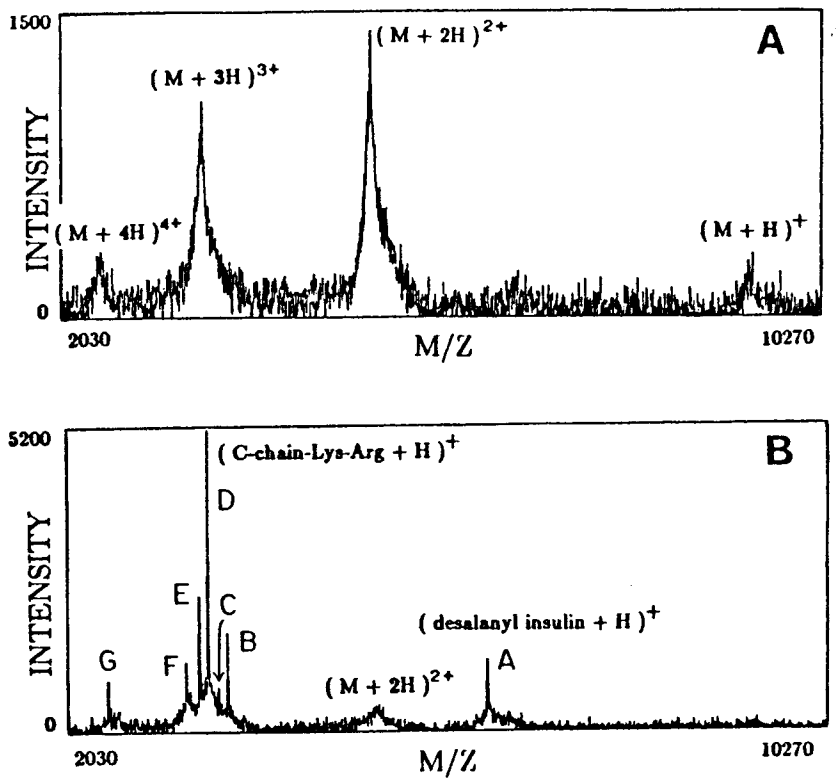

Fig. 3. Mass spectra of porcine proinsulin prior to and after incubation with trypsin. (A) Partial mass spectrum of 1.6 x 10^{-10} mol porcine proinsulin prior to any reaction. (B) Partial mass spectrum of proinsulin after incubation with trypsin for 12 minutes at 38°C. The reaction solution contained 0.05M NH_4HCO_3 and 3.3 x 10^{-6}M trypsin (pH 8.30). 3µl of this solution was dropped onto the peptide covered nitrocellulose surface, spread with a microscope coverslip, and allowed to react. After reaction, the coverslip was removed and the reacted surface was promptly inserted into the mass spectrometer where the volatile reagents evaporated and the spectrum was obtained. The postulated identities of the ions giving rise to the peaks labeled A-G are given in Table 1 together with their measured and calculated m/z values.

Peak #	Postulated ion identity	Calculated mass to charge ratio	Measured mass to charge ratio	Δ
A	(desalanyl insulin + H)$^+$	5707.7	5707.5	-0.2
B	(arg-C-peptide-lys-arg + H)$^+$	3185.4	3186.2	+0.8
C	(arg-C-peptide-lys-arg - 71 + H)$^+$	3114.4	3115.3	+0.9
D	(C-peptide-lys-arg + H)$^+$	3029.3	3029.1	-0.2
E	(C-peptide-lys-arg - 71 + H)$^+$	2958.3	2957.6	-0.7
F	(desalanyl insulin + 2H)$^{2+}$	2854.4	2855.0	+0.6
G	Reaction product not identified		2274.7	

Table 1. Comparison of the measured mass-to-charge ratios (m/z) of the products from the trypsin digestion of porcine proinsulin with the m/z values calculated for a series of postulated tryptic fragment ions. Both the measured and the calculated m/z values are the means of the 5 most abundant isotopic components. C-peptide designates the connecting peptide comprising residues 33-61 of porcine proinsulin (13). Δ designates the difference between the measured and calculated m/z values.

tes the detailed information which can be readily extracted from a small quantity of protein using the present method.

In separate experiments, trypsin-catalyzed hydrolysis reactions of dynorphin carried out in the presence of ^{18}O labelled water allowed the rapid and unambiguous identification of those tryptic fragments containing the C-terminus of the original peptide. The identification was made on the basis of incorporation or nonincorporation of ^{18}O during hydrolysis (12).

The general properties of the mass spectrometric enzyme-catalyzed surface reaction probe are summarized as follows: Sensitivity is in the range 10^{-9}-10^{-12} mol of polypeptide. Low yields (<10%) of reaction product can be detected reliably. The mass spectrometric analyses are practically nondestructive of sample, which allows for reactions to be followed as a function of time and also for sequential reactions to be monitored. These properties make the present technique at least an interesting complementary addition to existing techniques. Beyond that, we believe that it will be a valuable general tool for studying biomolecules.

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6. The method for preparing the solid peptide sample film is based on a previously described technique (5) and is given in detail in Ref. 2. 10^{-9} mol of bradykinin dissolved in 1 ul of 10% aqueous acetic acid was applied to a NC layer. Following adsorption of the peptide to the NC layer the film was thoroughly dried. The NC layer was prepared by electrospraying

50 ug NC (1 ug/ul in acetone) onto an aluminized polyester support with a geometric surface area of 1 cm^2.

7. The measured and calculated (in parentheses) masses of bradykinin and the enzyme hydrolysis products are RPPGFSPFR 1059.7 (1059.6), RPPGFSPF 903.7 (903.5), RPPGFSP 756.6 (756.4), RPPGF 572.4 (572.3), and RPPG 425.2 (425.2). The values given are, in each case, for the most intense single isotopic component.
8. The difficulty of missed amino acid residues engendered by too rapid or too slow hydrolysis can to some extent be reduced by obtaining spectra from the starting material at a series of reaction times. This strategy can be used since the mass analysis is practically nondestructive of sample (3).
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10. The irreversibility of peptide detachment was confirmed by injecting 1.0 x 10^{-12} mol of bradykinin into the 16 ml of CPB solution, dipping a freshly prepared NC foil into the solution, and then analyzing the surface of the NC foil mass spectrometrically. Under these conditions no significant peak is observed which corresponds to protonated bradykinin.
11. The presence of a genetic variant in the porcine proinsulin sample was confirmed by direct mass spectrometric detection of the doubly and triply protonated intact variant molecule. The site and nature of the variation was deduced from additional mass spectrometric measurements and sequence analysis of chromatographically separated enzymatically generated fragments of porcine proinsulin. A detailed description of the nature of the variant is in preparation.
12. The trypsin catalyzed hydrolysis reaction was carried out in a 1:1 mixture of $H_2{}^{16}O$ and $H_2{}^{18}O$. Tryptic fragments which do not contain the C-terminus of the original peptide incorporate oxygen during hydrolysis and so yield peak doublets separated by 2 daltons in the mass spectrum. On the other hand, tryptic fragments which contain the C-terminus of the original peptide do not incorporate oxygen during hydrolysis and thus yield singlet peaks in

the mass spectrum.

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