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Identification by fast atom bombardment mass spectrometry of insulin fragments produced by insulin proteinase

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We describe the isolation by reversed-phase h.p.l.c. of a number of products of the degradation of insulin by insulin proteinase and their direct analysis by fast atom bombardment mass spectrometry (f.a.b.-m.s.). Various semisynthetically labelled insulins were used, including [[$^2H_2$]Gly^{A1}]insulin and [[^{18}O]Lys^{B29}]insulin. The results obtained confirm and extend the results obtained by non-mass-spectrometric methods [Davies, Muir, Rose & Offord (1988) Biochem. J. **249**, 209–214, and papers cited therein]. Cleavage sites were identified between positions A13-A14, A14-A15, B9-B10, B13-B14, B24-B25 and B25-B26. The advantages and disadvantages of the application of f.a.b.-m.s. to such studies are discussed.

INTRODUCTION

The degradation of insulin *in vitro* by insulin proteinase produces at least seven fragments, which are not easy to resolve completely in a primary separation step, given the rather small amounts available (Davies *et al.*, 1986, 1987; Varandani & Shroyer, 1987). Identification of the degradation products, which is complicated to some extent by the two-chain nature of the substrate (insulin), has been performed by three quite different approaches. One approach involves a combination of *N*-terminal analysis and amino acid analysis of unlabelled insulin fragments (Duckworth *et al.*, 1979; Varandani & Shroyer, 1987). A second approach involves the use of ^{3}H-labelled insulins as substrates, and comparison with standards during separations involving combinations of electrophoretic and chromatographic systems with and without cleavage of disulphide bonds by performic acid oxidation (Muir *et al.*, 1986; Davies *et al.*, 1986, 1987). A third approach involves the use of radioiodinated insulin as substrate followed by Edman degradation to identify in which cycle radioactivity is released (Hamel *et al.*, 1986). Difficulties with the first approach include the need to isolate, in sufficient yield, pure fragments, since mixtures may give rise to ambiguous assignments (this problem may be alleviated to some extent by microscale sequencing, but is aggravated by the possibility of cyclization of *N*-terminal glutamine to pyroglutamyl). Difficulties with the second approach include the time and expense involved in the preparation of adequately labelled insulins and in preparing a rather large number of defined fragments as standards to study their behaviour under a variety of conditions. (Once these substrates and standards have been prepared, of course, further experiments are simplified; so far we have used insulins labelled specifically with ^{3}H at the B1 and the A1 positions). Difficulties with the third approach include the possibility of pyroglutamyl formation, varying yields upon Edman degradation of different peptides leading to errors in quantification, the inability to identify cleavage sites *C*-terminal to the label employed, and the fact that the substrate used is not authentic insulin: an iodoinsulin is much more different from authentic insulin than is a ^{2}H or ^{3}H-labelled insulin.

It is clear from the above that small quantities of peptide mixtures are produced by insulin proteinase from a substrate of known amino acid sequence (insulin) and must be analysed in order to determine the sites of cleavage. A technique which has become known as fast atom bombardment mass spectrometry (f.a.b.-m.s.) is well suited to this task (Barber *et al.*, 1981; Williams *et al.*, 1982).

We have applied f.a.b.-m.s. to the study of the products of the degradation of insulin with insulin proteinase. For one experiment we used insulins carrying stable-isotope substitutions in specific positions, and included small amounts of ^{3}H-labelled insulins for control purposes. The results obtained confirm and extend those described in the previous paper (Davies *et al.*, 1988). The advantages and disadvantages of the use of f.a.b.-m.s. in such experiments are discussed.

MATERIALS AND METHODS

All reagents and solvents were of analytical grade or better unless otherwise stated.

Insulin of human sequence labelled with ^{18}O at Lys-B29 was prepared as previously described (Rose *et al.*, 1984), except that procine insulin and the trypsin catalyst were lyophilized from 99 atom % $H_2{}^{18}O$ prior to reverse proteolysis; this raised the incorporation of ^{18}O into Lys-B29 to about 80%. Insulin of porcine sequence labelled at Gly-A1 with [$^2H_2$]glycine was prepared essentially as described for the corresponding ^{3}H-labelled analogue (Davies & Offord, 1985). Degradation of insulin by insulin proteinase was carried out as described by Muir *et al.* (1986) except that bovine serum albumin was omitted from the digestion medium, and the enzyme was purified beyond the second $(NH_4)_2SO_4$ precipitation by successive chromatography on DEAE-Sephacel and phenyl-Sepharose [A. V. Muir, unpublished work, based on the method of W. C. Duckworth (personal communi-

Abbreviation used: f.a.b.-m.s., fast atom bombardment mass spectrometry.

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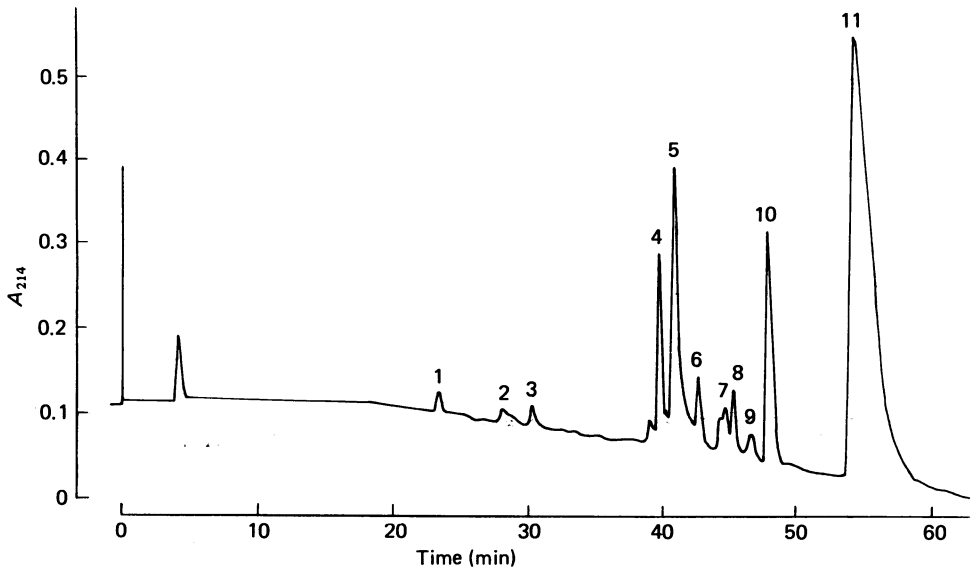

Fig. 1. H.p.l.c. profile of the degradation of porcine insulin by insulin proteinase

Conditions of the degradation, work up and chromatography are described under 'Materials and methods'.

cation)]. Enzyme thus purified gave the same pattern of fragments (A. V. Muir, J. G. Davies & R. E. Offord, unpublished results) for ^{3}H-labelled insulins as did the enzyme preparations previously employed (Davies *et al.*, 1988). In one experiment, 8 nmol of insulin, being a mixture of [$^2H_2$]Gly-A1- (porcine sequence) and [^{18}O]Lys-B29- (human sequence) labelled forms and containing 8.7×10^5 d.p.m. of ^{3}H-labelled insulins [an equimolar mixture of insulins of porcine sequence labelled with ^{3}H at Gly-A1, at Phe-B1, and at Ala-B30; Davies *et al.* (1987); J. G. Davies, K. Rose, C. G. Bradshaw & R. E. Offord, unpublished work] were digested with enzyme preparation in 100 ml of 50 mM-Tris/HCl, pH 7.5. The quantity of enzyme was chosen on the basis of preliminary experiments so as to give a degree of digestion of about 50% (see below). After incubation for 10 min at 37 °C, digestion was terminated by acidification to about pH 4 with acetic acid. In another experiment, 8 nmol of unlabelled zinc-free porcine insulin were digested. Precipitation (performed on a 1 ml digest containing ^{3}H-labelled insulin) with trichloroacetic acid (Muir *et al.*, 1986) showed an extent of degradation of about 50%. The digest was concentrated by passing the acidified digest through a C_{18} Sep-Pak (Waters Associates) previously washed with methanol and equilibrated with 0.1% trifluoroacetic acid. After washing with 4 ml of 0.1% trifluoroacetic acid, peptides were eluted with 2 ml of 0.1% trifluoroacetic acid/acetonitrile (1:1, v/v). Solvent was removed under vacuum in a centrifuge (SpeedVac, Savant Instruments), and the sample was redissolved in about 450 μl of 0.1% trifluoroacetic acid for injection onto h.p.l.c. H.p.l.c. was performed with a Waters system (Wisp 710B injector, M6000A and M45 pumps, model 680 gradient programmer, model 440 absorbance detector, extended wavelength module at 214 nm). The column was a μBondapak C_{18} radial compression cartridge in a Z-module (Waters) operated at a flow rate of 1 ml/min. Solvent A was 1 g of h.p.l.c.-grade trifluoroacetic acid (Pierce) dissolved in 1 litre of h.p.l.c.-grade water (MilliQ system), and solvent B was h.p.l.c.-grade acetonitrile (Merck). The column was equilibrated with solvent A, the sample was injected, and, after 5 minutes at 100% solvent A, a linear gradient (1%/min for 50 min) of solvent B was applied. The effluent was monitored at 214 nm, and fractions absorbing at this wavelength were collected manually. After solvent removal in the vacuum centrifuge, samples were examined by mass spectrometry.

F.a.b.-m.s. was performed on an MS 50 S mass spectrometer (Kratos Analytical, Manchester, U.K.) with a full f.a.b. retro-fit (MScan Ltd.) and operated under control of a DS 55 M data system (Kratos). A mass spectrometric resolution of about 3000, an accelerating voltage of 4 kV and a scan rate of 30 s/decade were generally used. The f.a.b. gun was operated with xenon at about 9 kV and 7 μA. External calibration of the mass scale was achieved with a mixture of the iodides of sodium, rubidium and caesium (1:1:1 molar ratio). For analyses in positive-ion mode, the stainless steel target was loaded with 1 μl of a mixture of glycerol and 1-thioglycerol (2:1, v/v) followed by 0.5 μl of acetic acid; samples were taken up in 5 μl of 0.1% trifluoroacetic acid and about 1 μl was applied to the loaded target. For analyses in negative-ion mode, triethylamine replaced the acetic acid and the samples were taken up in water (after evaporation under vacuum of the 0.1% trifluoroacetic acid used for analysis in positive-ion mode).

A simple computer program was written to generate the exact masses of all possible fragments of insulin of mass up to 1999.99999 (with and without disulphide bridges, with and without sodium cationization, and with and without linkage to one molecule of thioglycerol by formation of a mixed disulphide). The computation was continued beyond M_r 1999.99999 for fragments without disulphide bridges up to the mass corresponding to the complete chain. Tables (equivalent to 250 pages of typescript) are available on request in computer-readable form (four IBM PC-compatible diskettes). The tables were interrogated by computer to provide all possible sequences corresponding to a given mass.

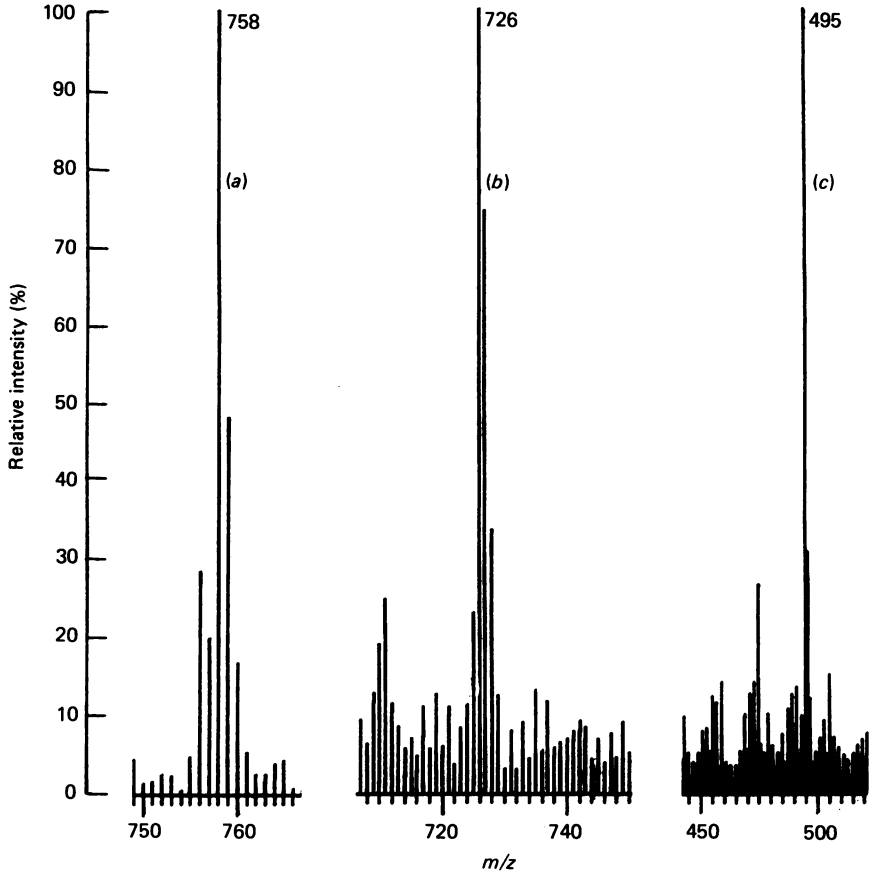

Fig. 2. Mass spectra of degradation products

(*a*) Positive ion spectrum of fraction 3i, B25–30 of human insulin labelled at Lys-B29 with ^{18}O to an extent of about 80 atom %. The protonated molecular ion of the unlabelled peptide appears at *m/z* 756 and that of the labelled peptide at *m/z* 758; (*b*) positive ion spectrum of fraction 3ii, B25–30 of porcine insulin, showing a protonated molecular ion at *m/z* 726; (*c*) negative ion spectrum of fraction 2; the signal at *m/z* 495 is most probably due to the deprotonated molecular ion of fragment B10–13 (see the text).

RESULTS AND DISCUSSION

Separation of the degradation products by h.p.l.c.

Fig. 1 shows the elution profile of the degradation of unlabelled porcine insulin. Fractions 1–8 were identified by f.a.b.-m.s. as fragments of insulin, as is discussed below. The information from two experiments, one of which involved the use of insulins labelled with stable isotopes, has been pooled in the discussion which follows. For this reason, a given fraction number always refers to the same region of the chromatogram as indicated in Fig. 1, but the sample in question will sometimes have come from a different run to the one illustrated in Fig. 1.

Mass spectrometry

Fractions 1 and 3 were found to contain residues B26–30 and B25–30, respectively. A computer-assisted search of the possible insulin fragments showed several possibilities for the masses found, 579 and 726 in positive ion mode, respectively. The assignment B26–30 and B25–30 was confirmed in an experiment where an equimolar mixture of porcine and human insulin (the latter labelled at Lys-B29 with ^{18}O to an extent of about 80 %) was degraded, resulting in the production of twin peaks (1i and 1ii; 3i and 3ii) for these two peptides, the threonine-containing peptide eluting earlier than the alanine-containing peptide, as expected. Figs. 2(*a*) and 2(*b*) show the mass spectrum of B25–30, threonine- and alanine-containing, respectively (*m/z* 758 and 726, respectively, taking into account the ^{18}O).

Fraction 2 gives a mass spectrum in negative-ion mode containing a strong signal at *m/z* 495 (Fig. 2*c*). A computer-assisted search of all possible polypeptide fragments of insulin produced three possibilities: B16–19, B2–5 and B10–13, with masses 495.226, 495.231 and 495.256, respectively. Only B10–13 fits with the degradation scheme presented below.

Fractions 1–3 represent small (tetra- to hexa-peptide) fragments and so absorb at 214 nm, in molar terms, much less than do the larger fragments. This should be borne in mind when using Fig. 1 to assess their importance relative to that of later fractions.

Fraction 4 was found to correspond to the major degradation product as deduced from the results of non-mass-spectrometric experiments (Muir *et al.*, 1986; Davies *et al.*, 1986; Varandani & Shroyer, 1987). The positive-ion mass spectrum shows signals at *m/z* 1004 and 1353, corresponding to B1–9 and A1–13, respectively (Fig. 3). The losses of 15 and 16 mass units from *m/z* 1004, giving rise to the signal at *m/z* 989 and 990, are not unusual (see, for example, Figs. 2*b* and 10, where similar

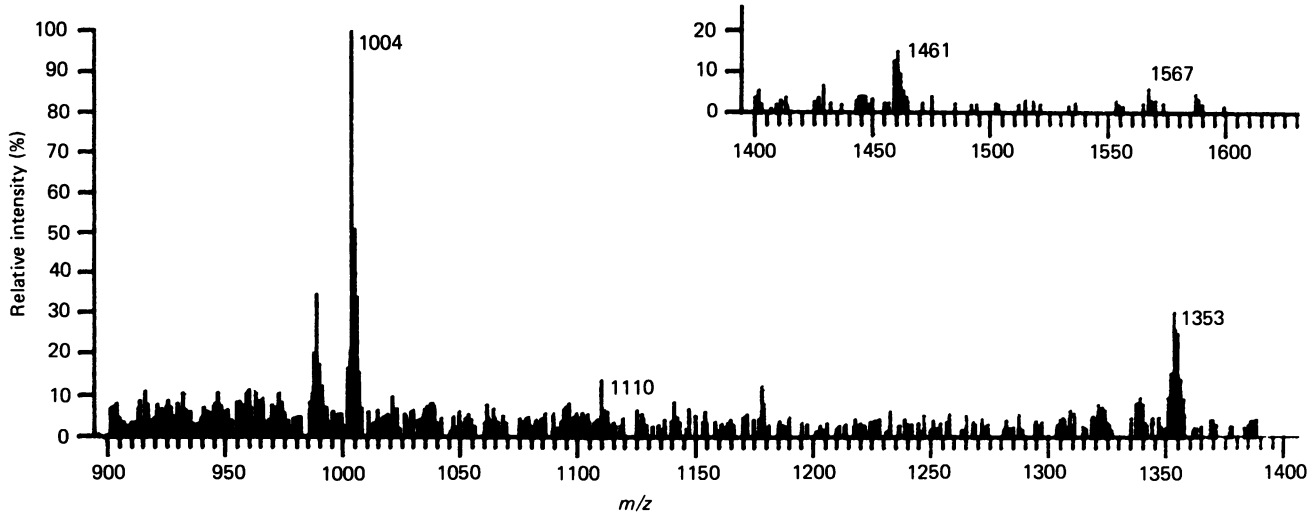

Fig. 3. Positive-ion mass spectrum of fraction 4

m/z 1004 corresponds to the protonated molecular ion of fragment B1–9, and *m/z* 1353 corresponds to the protonated molecular ion of A1–13; see text for further interpretation of this spectrum.

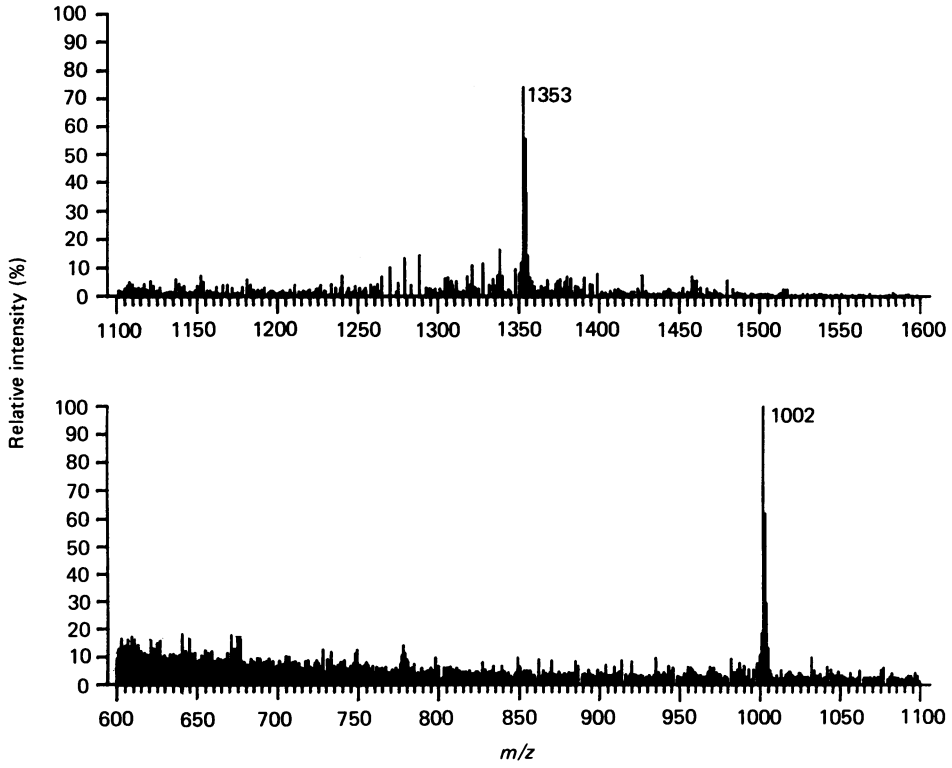

Fig. 4. Negative-ion mass spectrum of fraction 4

m/z 1002 corresponds to the deprotonated molecular ion of fragment B1–9, and *m/z* 1353 corresponds to the deprotonated molecular ion of A1–13; see the text for further interpretation of this spectrum.

losses are evident). *m/z* 1353 corresponds to A1–13 having the intrachain disulphide bond present. In positive ion mass spectra of Cys-containing peptides, under the conditions used, partial reduction of disulphide bonds occurs, with partial mixed disulphide formation with thioglycerol. This phenomenon assists spectral interpretation by indicating the presence of Cys, and is responsible for the signals at *m/z* 1004 (reduction of the interchain disulphide bond), 1110 (mixed disulphide of 1004 with the thioglycerol matrix), 1461 (reduction of the intrachain bridge of 1353 and mixed disulphide formation between one of the three Cys-SH and thioglycerol) and 1567 (reduction of the intrachain bridge of 1353 and two mixed disulphides with thioglycerol). Further confirmation comes from the negative-ion mass spectrum (Fig. 4), which is obtained in the presence of triethylamine and

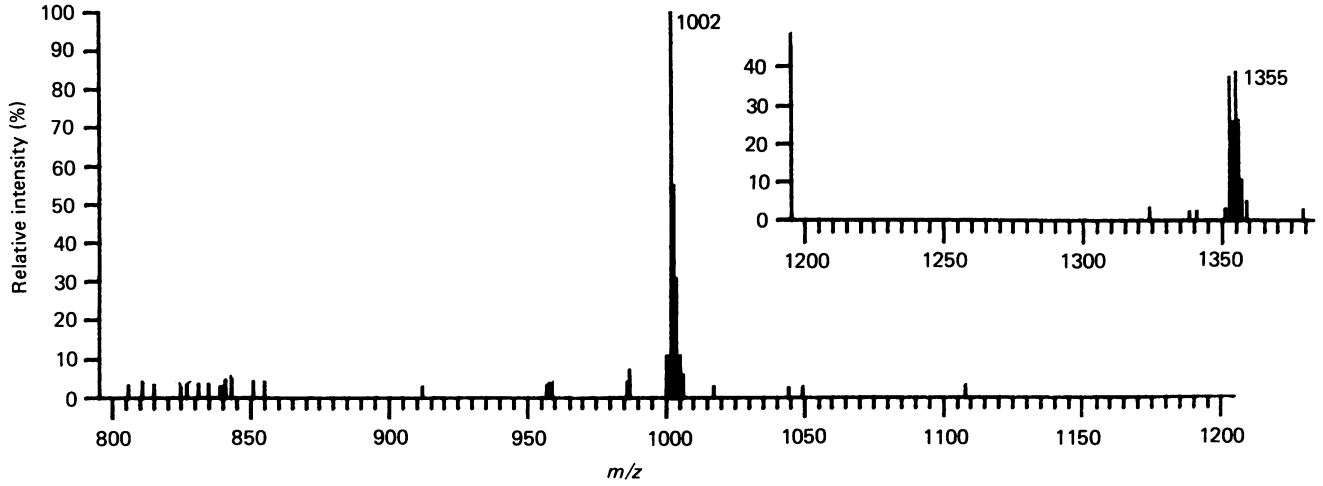

Fig. 5. Negative-ion mass spectrum of fraction 4 from the degradation of a labelled insulin mixture

Degradation of a mixture of insulins containing equimolar Gly-A1 and [$^2H_2$]Gly-A1 produced a fraction 4 where the label is clearly visible, being responsible for the pair of signals at *m/z* 1353 and 1355 (due to A1–13; see legend to Fig. 4).

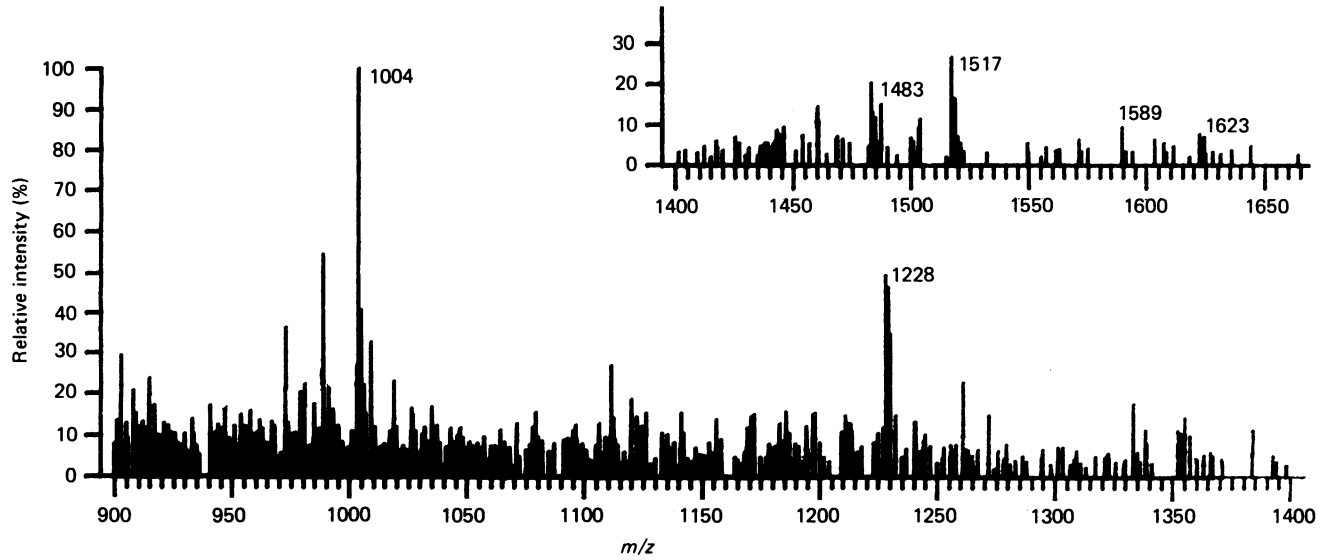

Fig. 6. Positive-ion mass spectrum of fraction 5

See the text for the interpretation of this spectrum.

thioglycerol (see the Materials and methods section); the conditions tend to reduce disulphide bonds and yield fewer signals. In this spectrum we see intense signals at *m/z* 1002 and 1353, due to the fully reduced, negatively charged forms of peptides B1–9 and A1–13, respectively. (Corresponding ions in positive- and negative-ion mode should have an *m/z* difference of 2 mass units, but the complete reduction of the intrachain bridge abolishes this difference for the peptide A1–13 by adding two hydrogen atoms.) A computer-assisted search of the possible fragments of insulin produced B1–9 as the only candidate for *m/z* 1002 (1004 in positive ion mode). There are five possible insulin fragments that could correspond to the signal found at *m/z* 1353 in positive-ion mode, but only one possibility (A1–13) exists for the signal found at *m/z* 1353 in negative ion mode. This conclusion was verified by the data obtained upon degradation of a mixture of insulins containing equimolar Gly-A1 and [$^2H_2$]Gly-A1: the corresponding fraction showed, as expected, a pair of signals at *m/z* 1353 and 1355 in the negative-ion mass spectrum (Fig. 5).

Fraction 5 was found to be a mixture (Fig. 6). Signals due to B1–9 (*m/z* 1004), B1–13 (*m/z* 1483), B14–24 (*m/z* 1228) and A1–14 (intrachain disulphide bond intact, protonated molecular ion 1516.6, plotted at *m/z* 1517) are evident. Assignments of *m/z* 1004, 1228 and 1517 were confirmed by mass spectrometry in negative ion mode, and the assignment of the signal at *m/z* 1517 was further confirmed by the appearance of a pair of signals 2 mass units apart when a mixture of insulins containing equimolar Gly-A1 and [$^2H_2$]Gly-A1 was used (results not shown). A computer-assisted search of the possible insulin fragments showed B14–24 to be the only possibility, giving rise to a signal close to *m/z* 1226 (exact

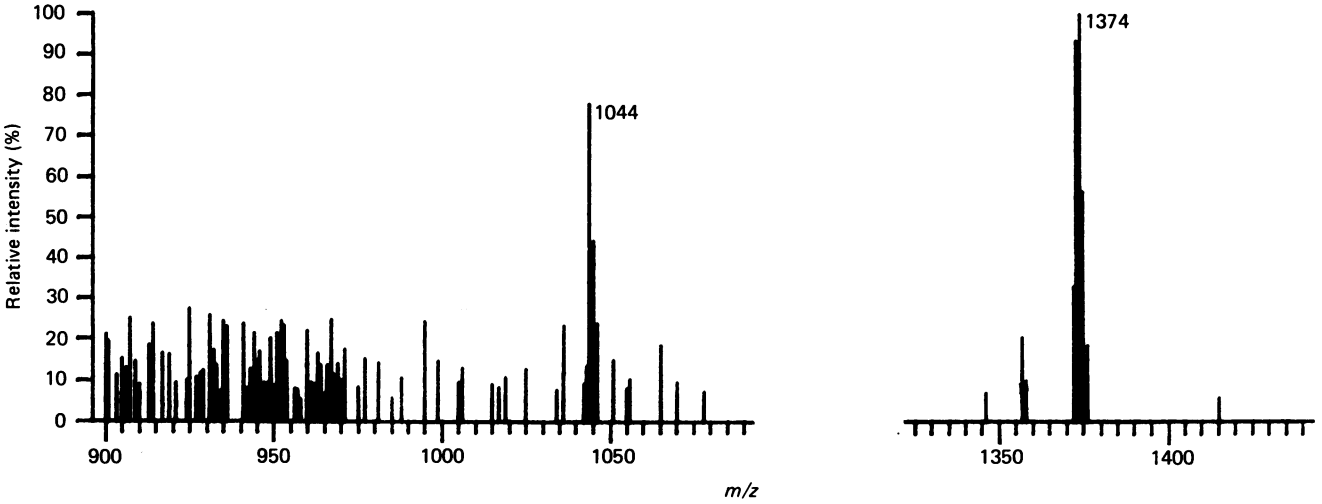

Fig. 7. Negative-ion mass spectrum of fraction 7

See the text for the interpretation of this spectrum.

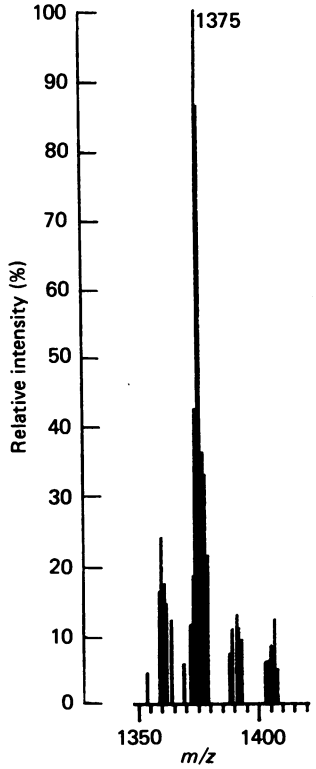

Fig. 8. Positive-ion mass spectrum of fraction 7

See the text for the interpretation of this spectrum.

mass 1225.6) in negative ion mode. It is not clear with which A-chain fragment the fragment B14–24 is associated; perhaps the ionization of the corresponding fragment was suppressed by the higher surface activity of other fragments present in this mixture, an effect previously documented (Clench *et al.*, 1985).

Fraction 6 was found (results not shown) to contain signals corresponding to A14–21 (m/z 1044 in negative ion mode) and B14–24 (m/z 1225.6 in negative ion mode).

Fraction 7 was found (Fig. 7) to contain signals corresponding to A14–21 (m/z 1044 in negative ion mode) and B14–25 (expected at m/z 1373 in negative ion mode). At masses approaching 2000, there is an almost equal chance that a molecule of an organic compound contains at least one atom of ^{13}C as no ^{13}C (isotopic abundance 1.1%). Even so, the intensity of m/z 1374 relative to that of m/z 1373 is greater than would have been predicted on a simple calculation of isotopic abundance. However, the spectrum shown in Fig. 7 had to be obtained with the instrument set at very high gain, and under these conditions counting statistics make the

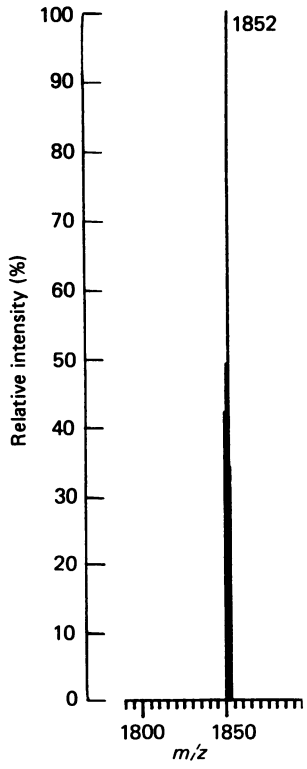

Fig. 9. Negative-ion mass spectrum of fraction 8

See the text for the interpretation of this spectrum.

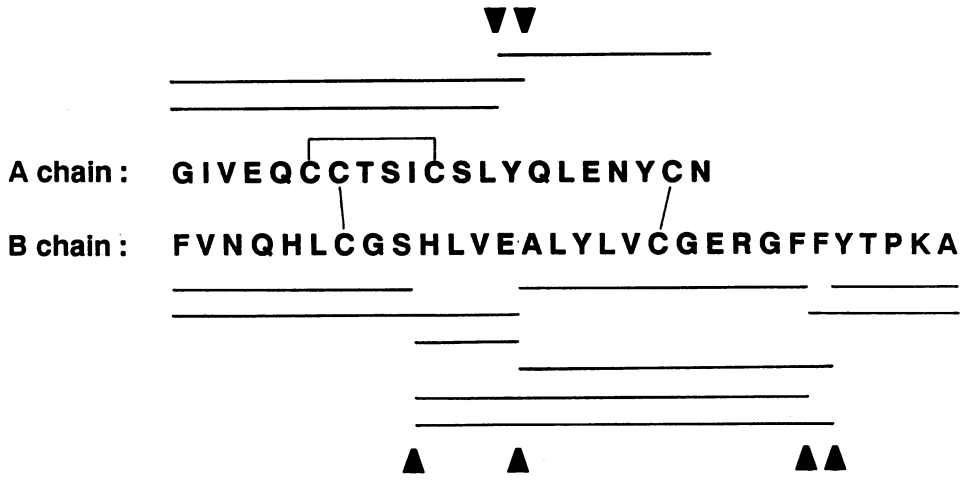

Fig. 10. Fragments identified by f.a.b.-m.s.

The sequence of porcine insulin is shown. The human sequence differs from this one by having Thr in place of Ala at position B30. Fragments identified by f.a.b.-m.s. are represented by lines covering the corresponding parts of the sequence, and the cleavage sites are marked with arrows.

relative intensities of peaks an unreliable guide. We conclude that ion statistics are responsible for the higher relative intensity of m/z 1374, which corresponds to the ^{13}C-containing species, over 1373. Fragment B14–25 was confirmed in positive ion mode (Fig. 8), where we see, as expected, a signal at m/z 1375.

Fraction 8 showed a group of signals in the region of m/z 1852 in negative ion mode (Fig. 9). A computer-assisted search of all possible insulin fragments produced only one candidate, the fragment B10–25. The expected mass of this fragment in negative ion mode is 1850.92, which would be plotted at m/z 1851. For the same reasons discussed above regarding Fig. 7, we conclude that ion statistics are responsible for the relative intensity of m/z 1852 being greater than that of m/z 1851.

No information was obtained from the small fraction 9. Either the material is non-peptide in nature or the masses of the fragments were beyond the mass range of our instrument at the sensitivity required for the analysis. If the latter possibility is correct, the small size of the h.p.l.c. peak permits us to conclude that it represents only quantitatively insignificant fragments: the larger the peptide, the greater its absorption at 214 nm.

Fraction no. 10 corresponds to the elution time of undigested insulin; precipitation of 1 ml of the initial digest, with trichloroacetic acid, had indicated a degree of digestion of about 50%, so it is not surprising to find a strong peak in this position. The broad fraction (no. 11) evident near the end of the gradient gives no result on f.a.b.-m.s. analysis and is due to the enzyme preparation: it is present in incubations performed with enzyme but without insulin, and such control incubations show no peaks that elute earlier than this broad peak.

Fragments found

Fig. 10 shows the cleavages identified by f.a.b.-m.s. Two sites of cleavage in the A-chain were identified, one between A13–14 and the other between A14–15. Four sites of cleavage in the B-chain was identified, between B9–10, B13–14, B24–25 and B25–26. In all, three A-chain fragments and nine B-chain fragments were identified (Fig. 10). We have accounted for the whole molecule in the form of fragments, although we do not exclude there being additional fragments. For example, the fragment A15–21, which might have been expected given the cleavages shown in Fig. 10, was not detected.

The results obtained confirm and extend previous work with insulin-degrading proteinase (Muir *et al.*, 1986; Davies *et al.*, 1986, 1988; Varandani & Shroyer, 1987). Varandani & Shroyer (1987) identified a fragment which had the expected *N*-termini and (approximate) amino acid composition of A1–13 and B1–9 linked by an interchain disulphide bond. We did not identify B1–10, which was found by Davies *et al.* (1988), possibly because the fragment was not present in high enough yield in the two digests which we examined. Fragment B1–3, also identified by Davies *et al.* (1988), could not have been detected in the experiments described here (expected in positive ion mode at m/z 379) since we did not acquire data below m/z 400. On the other hand, we identified eight fragments (A14–21, B10–13, B10–24, B10–25, B14–24, B14–25, B25–30 and B26–30) which were not identified in the above-cited paper because they do not contain one of the ^3H-labelled residues (A1 and B1). Six of these fragments would remain inaccessible even if insulin labelled with ^3H at B30 (the preparation and characterization of which is described in Davies *et al.*, 1987) were to be employed.

CONCLUSION

We have demonstrated that a combination of h.p.l.c., positive- and negative-ion fast atom bombardment mass spectrometry, with the use of stable isotope labelled polypeptide substrates, provides a powerful means for the study of the degradation *in vitro* of the polypeptide hormone insulin by insulin proteinase. Studies on the degradation of glucagon by insulin proteinase (L. A. Savoy, A. Muir, J. G. Davies, R. E. Offord & K. Rose, umpublished work) have shown that these methods are generally applicable. In addition, the possibility exists, when employing mass spectrometry, to add to the digest fragments labelled with stable isotopes (synthetic, or prepared by digestion of labelled insulins). These

fragments would serve as internal standards and permit one to determine accurate yields of fragments by correcting for losses during recovery.

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