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PO Box 117
221 00 Lund
+46 46-222 00 00

A novel mass spectrometric approach to the analysis of hormonal peptides in extracts of mouse pancreatic islets

Margareta Ramström¹, Charlotte Hagman², Youri O. Tsybin², Karin E. Markides¹, Per Håkansson², Albert Salehi³, Ingmar Lundquist³, Rolf Håkanson³ and Jonas Bergquist¹

¹*Institute of Chemistry, Department of Analytical Chemistry, ²Division of Ion Physics, The Ångström Laboratory, Uppsala University, Sweden; ³Department of Pharmacology, Institute of Physiological Sciences, Lund University, Sweden*

Liquid chromatography mass spectrometry (LC-MS) is a valuable tool in the analysis of proteins and peptides. The combination of LC-MS with different fragmentation methods provides sequence information on components in complex mixtures. In this work, on-line packed capillary LC electrospray ionization Fourier transform ion cyclotron resonance MS was combined with two complementary fragmentation techniques, i.e. nozzle-skimmer fragmentation and electron capture dissociation, for the determination of hormonal peptides in an acid ethanol extract of mouse pancreatic islets. The most abundant peptides, those derived from proinsulin and proglucagon, were identified by their

masses and additional sequence-tag information established their identities. Interestingly, the experiments demonstrated the presence of truncated C-peptides, *des*-(25–29)-C-peptide and *des*-(27–31)-C-peptide. These novel findings clearly illustrate the potential usefulness of the described technique for on-line sequencing and characterization of peptides in tissue extracts.

Keywords: liquid chromatography; Fourier transform ion cyclotron resonance mass spectrometry; electron capture dissociation; pancreas; peptides.

Mass spectrometry is a rapid, sensitive and reliable method for the analysis of complex samples. Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) provides the combination of high sensitivity, ultra high resolution and mass accuracy [1,2] and has proved to be of importance in studies of biomolecules [3].

When examining complex samples, such as body fluids and tissue extracts, it is advantageous to use on-line separation, such as liquid chromatography (LC) [4] or capillary electrophoresis [5], prior to MS. The separation of molecules in time decreases the complexity of the spectra, reduces the ion suppression in the spray and results in a preconcentration of the analytes. Several reports have described the advantages of combining LC with electrospray ionization (ESI) FTICR MS. For example, attomole detection limits have been reported for peptide analysis [6]. Enzymatic digests of proteins from prokaryotic and mammalian cells [7–10] and of proteins in cerebrospinal fluid [11] have been successfully analysed by the LC-FTICR MS approach. Furthermore, the chromatographic separation of analytes prior to MS opens up the possibility to apply on-line

fragmentation of components in complex mixtures. The information obtained from such experiments can be used to help identify unknown components and to characterize post-translational modifications. Different fragmentation methods, such as infrared multiphoton dissociation [12,13], electron capture dissociation (ECD) [14,15], and collision induced dissociation (CID) [13,16] have been combined with LC-FTICR MS for the examination of peptide or protein mixtures.

MS has proven to be of great value when analysing endocrine peptides of biological importance [17,18]. Here we present a novel mass spectrometric method for the detection and identification of peptides and proteins in tissue extracts. To illustrate the method, a sample of mouse pancreatic islets was subjected to on-line packed capillary LC combined with either nozzle-skimmer fragmentation or ECD ESI FTICR MS. The rationale behind the study is that a peptide hormone can be expected to make up a substantial proportion of the peptides in an endocrine cell. Insulin is the predominant and best known peptide in such extracts. To our knowledge, the LC-ECD FTICR MS approach has not previously been applied to the analysis of tissue extracts.

Nozzle-skimmer fragmentation and ECD represent two different classes of ion fragmentation techniques. In nozzle-skimmer fragmentation, or up-front collision-induced dissociation, the ions are accelerated into other molecules in the high pressure region (2 Torr) of the mass spectrometer by applying a high voltage between the nozzle and the skimmer. After each collision the internal ion energy is increased until it equals the dissociation energy of the weakest bond and fragmentation is induced [19,20]. ECD is so far only implemented in FTICR MS [21]. In this method, ion fragmentation is a result of reactions with low-energy

Correspondence to J. Bergquist, Institute of Chemistry, Department of Analytical Chemistry, Uppsala University, PO Box 599, SE-751 24 Uppsala, Sweden.

Fax: + 46 18 4713692, Tel.: + 46 18 4713675,

E-mail: jonas.bergquist@kemi.uu.se

Abbreviations: ACN, acetonitrile; ECD, electron capture dissociation; FTICR MS, Fourier transform ion cyclotron resonance mass spectrometry; GRPP, glicentin-related pancreatic peptide; HAc, acetic acid.

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electrons that take place in the cell at ultra-high vacuum (10^{-10} – 10^{-9} Torr). The advantage of ECD compared to other fragmentation methods is that stronger bonds can dissociate prior to weaker bonds. At the present stage of method development the efficiency of nozzle-skimmer fragmentation exceeds that of ECD. By applying both nozzle-skimmer fragmentation and ECD to the pancreatic islet extract we expect to achieve complementary sequence information on the peptides under study.

Materials and methods

Animals

Female mice of the NMRI strain (B & K Universal, Sollentuna, Sweden), weighing 25–30 g were used throughout the experiments. They were fed a standard pellet diet and tap water *ad libitum*. Appropriate measures were taken to minimize pain and discomfort for the mice, which were maintained in accordance with the National Institutes of Health Guide for the care and use of Laboratory Animals. All experiments were approved by the regional ethical committee for animal research (Lund, Sweden).

Islet isolation and sample handling

Preparation of isolated pancreatic islets from the mouse was performed by retrograde injection of a collagenase solution via the bile-pancreatic duct [22]. Islets were collected by hand-picking under a stereomicroscope at room temperature as previously described [23]. After washing, 2000–2200 islets (approximately 7 million islet cells) were transferred to 0.5 mL of acid ethanol, sonicated and extracted overnight at +4 °C [23]. After centrifugation, the pellet was discarded and a sample of the supernatant was withdrawn for radioimmunoassay of insulin and glucagon [24–26]. The supernatant contained approximately 25 µg insulin and 5 µg glucagon, which corresponded to 13.2 ng insulin and 2.3 ng glucagon per islet.

A volume of 20 µL of the supernatant was centrifuged to dryness, using a Speedvac® system ISS110 (Savant Holbrook, NY, USA), in a siliconized Eppendorf tube (Costar, Corning Inc., NY, USA). The dried material was then dissolved in 1% acetic acid (HAc), and the sample was desalted on a ZipTip® C₁₈ column (Millipore Corporation, Bedford, MA, USA). This procedure has been described in detail elsewhere [11]. To get an overview of the most abundant proteins and peptides in the sample, a direct infusion mass spectrum was recorded. The samples were then analysed using packed capillary LC-FTICR MS combined with nozzle-skimmer fragmentation or ECD.

Direct infusion FTICR MS

The desalted peptides were electrosprayed to a Bruker Daltonics BioAPEX–94e 9.4 T Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Billerica, MA, USA) [27] using a Black Dust (polyimide-graphite) sheathless electrospray emitter [28], inner diameter 50 µm. A flow rate of approximately 100 nL·min^{−1} was applied, the electrospray voltage used was −3.5 kV.

Packed capillary LC-FTICR MS

Reversed-phase LC was performed using a 10-cm long in-house packed C₁₈ column, I.D. 200 µm. The packing material used was ODS-AQ, diameter 5 µm (YMC Europe, Schermbek, Germany). Two JASCO 1580 HPLC-pumps (JASCO, Tokyo, Japan) delivered the mobile phase A: acetonitrile(ACN)/H₂O/HAc (5 : 94.5 : 0.5, v/v/v), and the mobile phase B: ACN/H₂O/HAc (94.5 : 5 : 0.5, v/v/v). The program of choice was: isocratic elution using solvent A for 10 min, followed by the gradients 100–50% A in 54 min and 50–0% A in 6 min. This program had been optimized for separation of peptides in a previous study [11] and was also found to be appropriate for this experiment. Ten µL of the desalted sample was injected onto the column using a six-port injector valve (Valco Instruments Co. Inc., Schenkon, Switzerland). The peptides separated by LC passed a UV-detector before they were electrosprayed on-line to the FTICR MS using a Black Dust emitter as described for the direct infusion experiments.

Nozzle-skimmer fragmentation and ECD

The experimental parameters were optimized for fragmentation of the pancreatic peptides. Nozzle-skimmer fragmentation was performed on-line during the separation. In order to achieve extensive fragmentation the capillary potential was set to 340 V. The skimmer potential was 7.08 V and the hexapole offset was set to 2.5 V. ECD conditions were generated in alternating spectra while running the separation as described in detail elsewhere [14]. No isolation was used and all ions were subjected to the ECD conditions. The high rate ECD was realized using an electron injection system based on an indirectly heated cathode mounted on-axis [29]. The current through the cathode heater was set to 2.2 A. The cathode surface potential was −1 V and the anode potential was 10 V. Both end-plate potentials of the ion trap were set at 1.5 V and the duration of the electron pulse was 100 ms.

Data acquisition and handling

Primary data analysis was performed on a workstation running the xMASSTM software (Bruker Daltonics). In the direct infusion experiment, a spectrum of 200 scans, 512 K data points was collected. The spectrum was calibrated using a dataset of a sample of standard peptides. After calibration, the masses of the standard peptides differed by maximum 1.1 p.p.m. from the theoretical masses. In the LC-nozzle-skimmer FTICR MS experiment, 87 spectra of 256 K were recorded, each spectrum was generated during 16 s. In the LC-ECD FTICR MS run, 256 spectra of 128 K were collected in total, 10 s per spectrum. The peaks of interest were selected manually using the built-in function for peak picking in xMASS. The masses of the fragments were compared to lists of fragment ion masses generated from MS-product (<http://prospector.ucsf.edu/ucshtml/4.0/msprod.htm>) or BIOTOOLS 2.0 software (Bruker Daltonics).

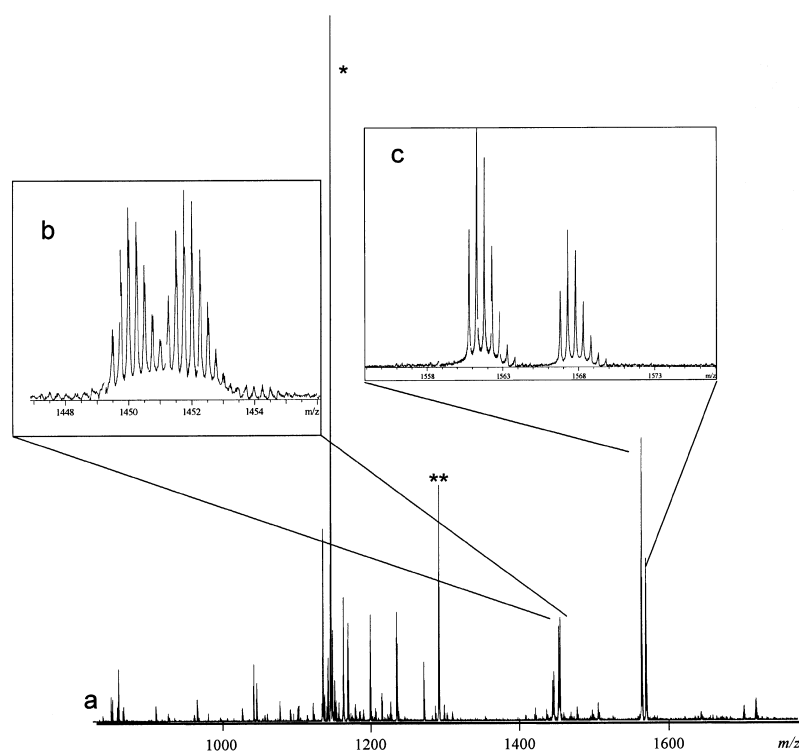


Fig. 1. A direct infusion FTICR mass spectrum of the extract from pancreatic islets (a), showing details of the insulin 1 and 2 (b) and C-peptides 1 and 2 (c) signals. The most abundant peptides turned out to be those from proinsulin. Insulin 1 and 2 and C-peptide 1 and 2 were identified, while the other prominent peaks represent the B-chain of insulin (*) and the truncated form of the C-peptide (**). High mass accuracy is provided using the FTICR technique, and mass measurement errors on the p.p.m. level were observed.

Results and discussion

Direct infusion

Initial direct infusion experiments were performed in order to get an overview of the many compounds of the pancreatic islet extract (Fig. 1a). The prominent signals in the spectrum originated from products of proinsulin. Two insulin variants are expressed in mouse, insulin 1 and 2 [30]. Masses corresponding to these two peptides were detected (Fig. 1b). The masses of the two intense signals to the right in the spectrum (Fig. 1c) matched those of the proinsulin-derived C-peptides 1 and 2. The mass measurement error in a well-calibrated FTICR mass spectrum is just a few p.p.m. The measured and theoretical masses of the peptides in this experiment are in good agreement (Table 1). Studies of rat β -cells have revealed specific endoproteolytic cleavage of the C-peptides, resulting in truncated C-peptides lacking the five

C-terminal residues, *des*-(27–31) C-peptides [31]. If such cleavage also takes place in β -cells from mice, two peptides of masses 2536.25 (from proinsulin 1) and 2576.30 (from proinsulin 2) should be expected in the spectrum. Examination of the spectrum indicated that this was indeed the case. The sequence of C-peptide 1 from mouse differs from the corresponding peptide from rat. Because C-peptide 1 in the mouse consists of 29 residues, the corresponding truncated form should be designated *des*-(25–29)-C-peptide. This peptide has to the best of our knowledge not been reported elsewhere. In the mouse, as in the rat, C-peptide 2 consists of 31 residues; the truncated form is *des*-(27–31)-C-peptide.

LC and on-line fragmentation

In order to detect less dominating peptides and to verify the identities of the components assigned by mass accuracy in the direct infusion experiment, on-line LC was applied in combination with either nozzle-skimmer fragmentation or ECD. Referring to the commonly used nomenclature [32] (Fig. 2), nozzle-skimmer fragmentation results mostly in *b*- and *y*-fragments, but *a*-fragments can also be generated. In ECD-experiments *c*- and *z*-fragments are produced. All peptides in Table 1 were also detected in the LC-FTICR MS experiments. The peptides derived from proinsulin 1 were separated from the corresponding proinsulin 2-derived peptides (Fig. 3), except for the two truncated C-peptides that coeluted. Intense signals of *b*- and *y*-fragments of both C-peptides and the truncated form of C-peptide from proinsulin 2 (Fig. 4) were detected in the LC-nozzle-skimmer experiments. The signal from the truncated form of C-peptide 1 was not intense and hence only very weak signals from the fragments were detected. Insulin 1 and 2 were detected, but no clear *b*- or *y*-fragments were observed.

Table 1. Measured and theoretical masses of the pancreatic islet peptides. The experimental masses agree well with the theoretical masses of the candidate peptides. Mass measurement errors at the p.p.m.-level were typically observed.

| Molecule | Theoretical mass (Da) | Measured mass (Da) | Error (p.p.m.) |
|---------------------------------|-----------------------|--------------------|----------------|
| Insulin 1 | 5799.6788 | 5799.6664 | 2.1 |
| Insulin 2 | 5793.6109 | 5793.5905 | 3.5 |
| C-peptide 1 | 3119.5621 | 3119.5623 | 0.06 |
| C-peptide 2 | 3131.5621 | 3131.5684 | 2.0 |
| <i>Des</i> -(25–29)-C-peptide 1 | 2536.2543 | 2536.2592 | 1.9 |
| <i>Des</i> -(27–31)-C-peptide 2 | 2576.2969 | 2576.2799 | 6.6 |

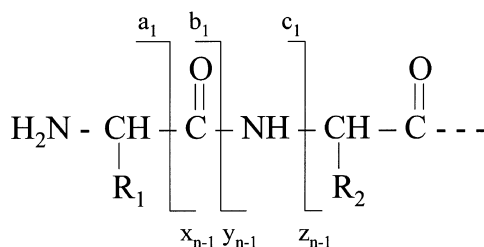


Fig. 2. Nomenclature of the peptide fragmentation. Depending on the cleavage site in the peptide chain, different fragments are produced. Nozzle-skimmer fragmentation results mostly in *b*- and *y*-fragments, while the ECD-process yields *c*- and *z*-fragments.

Instead, peaks corresponding to loss of water were detected. This reflects the difficulty of fragmenting large biomolecules with disulfide bridges by CID [33].

Two other signals of importance were detected whose masses agreed with those of glucagon and glicentin-related pancreatic peptide (GRPP), 3480.62 Da and 3438.41 Da, respectively. Both peptides are products of the glucagon precursor. Intense doubly and triply charged *b*-ions and doubly charged *y*-ions from GRPP were detected from the 3438 Da peptide (Fig. 5), which gave good sequence coverage of the peptide. No clear fragment peaks were detected from the peptide assumed to be glucagon. However, the theoretical mass of glucagon and the measured mass of the peptide differ by only 1.8 p.p.m.

In the ECD experiments, ECD and normal spectra were recorded alternately. The online-LC-ECD approach is rather novel; in fact this is the first time that it has been applied to a complex biological sample. In general, the fragmental ion peaks detected in these experiments had lower signal intensity than those in the nozzle-skimmer

Fig. 3. Mass chromatograms of two interesting regions from the LC-FTICR experiments. Insulin 1 and 2 (a), and also the two C-peptides (b) were separated in time. This permitted effective fragmentation of the peptides, and excluded overlap of the sequence tag information from different peptides.

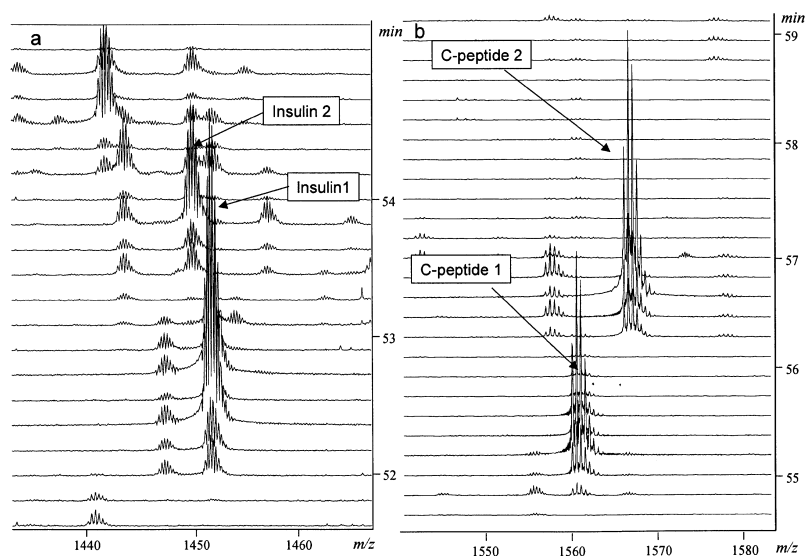
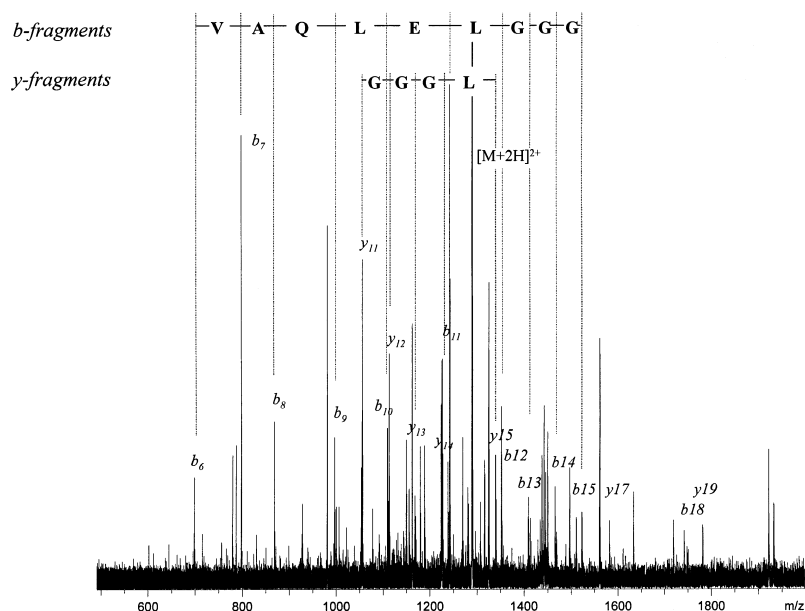


Fig. 4. Des-(27–31)-C-peptide 2 as observed by on-line LC FTICR MS combined with nozzle-skimmer fragmentation. The sequence information obtained in this experiment is enough to prove the identity of the peptide, and hence the presence of *des*-(27–31)-C-peptide in extracts of mouse pancreatic islets.



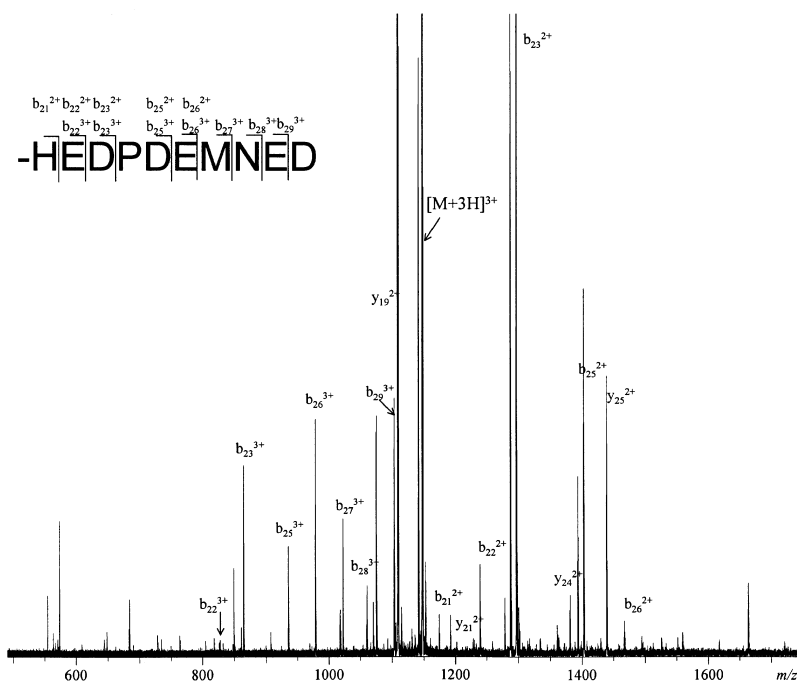


Fig. 5. Nozzle-skimmer fragmentation of GRPP. The inset shows the C-terminal of GRPP. Extensive fragmentation of this part of the peptide yielded several consecutive doubly and triply charged *b*-fragments resulting in high sequence coverage. A few doubly charged *y*-fragments were also observed in the experiment.

experiments. The detected fragments were mostly *c*-fragments. Fragmentation of C-peptide 2 and GRPP was observed. Also, *c*-fragments from insulin 1 and 2 were detected and possible *z*-fragments from insulin 2. These fragments were all from the insulin B-chain. All results from the LC-fragmentation experiments are summarized in Table 2.

The results from the LC-nozzle-skimmer and LC-ECD-experiments are complementary. For some of the peptides, sequence information was obtained by both methods. Nozzle-skimmer fragmentation gave rise to more intense fragment ion signals than ECD. Sequence information from

some of the peptides could not be obtained in the ECD-experiments, due to the lower fragmentation efficiency of the present available method. Nozzle-skimmer fragmentation of proteins with multiple disulfide bonds is difficult to achieve. On the other hand, the ECD process allows fragmentation of such molecules, and so ECD-fragments from both insulin molecules were detected.

Biological relevance of the detected peptides

In this study we have analysed an extract of mouse pancreatic islets. Because insulin-producing β -cells make

Table 2. Pancreatic islet peptides and the detected fragments from the LC-nozzle-skimmer and LC-ECD FTICR MS experiments. The length of the peptides and the number of detected fragments in the fragmentation experiment reflects the sequence-tag information obtained for each peptide. If the charge state differs from 1+, this is indicated in parentheses.

| Name | Measured and (theoretical) masses (Da) | Number of amino acid residues | Detected fragment ions | |
|---|--|----------------------------------|---|---|
| | | | LC-Nozzle-skimmer FTICR MS | LC-ECD FTICR MS |
| Insulin 1 | 5799.6788 (5799.6664) | 21 (A-chain) + 30 (B-chain) | Loss of water | c_3 - c_5 , z_6 - z_7 from the B-chain |
| Insulin 2 | 5793.5905 (5793.6109) | 21 (A-chain) + 30 (B-chain) | Loss of water | c_3 - c_5 from the B-chain |
| C-peptide 1 | 3119.5623 (3119.5621) | 29 | b_5 - b_{13} , b_{15} , y_5 - y_{14} | — |
| C-peptide 2 | 3131.584 (3131.5621) | 31 | b_7 - b_{15} , b_{18} , y_6 - y_7 , y_9 - y_{10} , y_{13} , y_{16} - y_{17} | c_9 - c_{12} |
| <i>Des</i> -(25-29)-C-peptide 1 | 2536.2592 (2536.2543) | 24 | b_{10} - b_{11} | — |
| <i>Des</i> -(27-31)-C-peptide 2 | 2576.2799 (2576.2969) | 26 | b_6 - b_{15} , b_{23} - b_{25} (2+), y_{11} - y_{15} y_{17} , y_{19} | — |
| Glucagon | 3480.6219 (3480.6156) | 29 | — | — |
| Glicentin-related pancreatic peptide | 3438.4260 (3438.4184) | 30 | b_{21} - b_{26} (2+), b_{22} - b_{29} (3+), y_{19} , y_{21} , y_{24} , y_{25} (2+) | c_5 - c_8 , c_{11} - c_{12} |

up 60–80% of the islets and as insulin makes up almost 20% of the proteins in the β -cells, proinsulin-derived peptides can be expected to constitute quantitatively predominant components of the extracts. In the direct infusion experiment peptides from proinsulin 1 and 2 were detected, whose identities were further established by the LC-nozzle-skimmer fragmentation and LC-ECD experiments. Insulin was one of the first peptide hormones to be discovered [34]. It has a key role in regulating carbohydrate and fat metabolism. Also, it was the first small protein to be detected by mass spectrometry [35]. Detailed studies on the processing of proinsulin to insulin and C-peptides have been performed [36].

Previously, the C-peptide was thought to be merely a by-product of insulin biosynthesis. However, recent studies in diabetes patients and animal models have suggested that the C-peptides possess biological activity [37,38]. For example, C-peptide affects the intracellular level of calcium and specific binding of C-peptide to plasma membrane has been observed. During proinsulin conversion, insulin and C-peptides are produced in equimolar amounts. However, in rat pancreatic islets, the C-peptides undergo cleavage, resulting in *des*-(27–31)-C-peptides [31]. The results of the present study show that this is also the case in pancreatic islets from the mouse. The C-terminal pentapeptide of the rat and human C-peptides is thought to be critical for bioactivity and for binding of the C-peptide to its putative receptor. Hence, the role of the truncated peptide is not known, but it should differ significantly from that of the C-peptide itself. Interestingly, the truncated C-peptide is not a major secretory product from human islets [39].

Proglucagon is produced in the pancreatic α -cells and in the L-cells of the intestines. The post-translational processing of proglucagon is tissue-specific. In the pancreatic α -cells, the main products are glucagon, GRPP and the major proglucagon fragment [40,41]. Glucagon is known to cause an increase in the blood glucose level, while the functions of the two other fragments are poorly understood.

Conclusions

In this study LC-FTICR MS was combined with nozzle-skimmer fragmentation and ECD in order to characterize peptides in extracts of pancreatic islets of the mouse. Eight of the main components were identified by their masses, and additional sequence information was generated for seven of them. The identified components were peptides from proinsulin and proglucagon. Two truncated forms of the C-peptide 1 and 2, *des*-(25–29)-C-peptide and *des*-(27–31)-C-peptide, respectively, were identified in the sample.

Generally, the efficiency of nozzle-skimmer fragmentation exceeds that of ECD, and more sequence-tag information is generated in the nozzle-skimmer experiments. However, insulin, which contains internal disulfide bridges, was only fragmented when ECD was applied. To our knowledge, this is the first application of LC-ECD-FTICR MS to a biological sample. The results of these experiments illustrate the benefit of performing both fragmentation methods combined with on-line LC-FTICR MS. With improvement of the ECD efficiency, we believe that this combined approach will be useful in further attempts to identify, e.g. novel peptide hormones in tissue extracts.

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