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Proteolytic cleavage of glucagon-like peptide-1 by pancreatic β cells and by fetal calf serum analyzed by mass spectrometry

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Abstract

Fetal calf serum and a β-cell line exhibit a proteolytic activity essential for the biological function of glucagon-like peptide-1 (GLP-1). This process of cleavage was investigated using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). To generate processing products, GLP-1 was subjected to rat insulinoma m5F (RINm5F) cell cultures or to fetal calf serum (FCS). For detection of processing products, a standardized extraction method including ion-exchange batch extraction, ultrafiltration, gel filtration, and reversed-phase chromatography was used. The RP fractions were analyzed by MALDI-TOF-MS. Processed proteolytic products were detected by comparing the resulting mass spectra of cell media or FCS after 2 h incubation with GLP-1 (7–36) amide with these of 2 h controls. To perform the comparison of the resulting mass spectra, software (MASSSPECANALYST) based on Microcal Software, Origins C-like language LABTALK was developed. GLP-1 fragments were purified by RP-HPLC, and characterized by sequence analysis. As insulin is the major secretory product of β cells depending on GLP-1 stimulation, the insulin and insulin fragments of the cell culture supernatants were also analyzed by this method.

Keywords: Peptides; Insulin; Proteins; Glucagon-like peptide-1

1. Introduction

Glucagon-like peptide-1 (GLP-1) was first described by Lund et al. [1]. They sequenced the preproglucagon cDNA of anglerfish and identified two glucagon-like coding sequences arranged in tandem. The presence of Lys–Arg sequence flanking and glucagon-related sequences suggested that two peptides are formed in vivo by post-translational cleavage of this common precursor. Proglucagon is produced in both pancreatic and intestinal endocrine cells. Post-translational processing of the precursor yields different peptides in these organs [2,3]. In the intestine, the major products are glicentin (PG-1-69), oxyntomodulin (PG-33-69), GLP-1 and GLP-2 [4–6].

GLP-1 is involved in the regulation of many body functions. For example, the ability of an oral glucose load to increase insulin secretion at a higher level than an intravenous glucose load is due to the intestinal release of GLP-1 and gastric-inhibitory polypeptide (GIP) by intraluminal glucose stimulation [7–9]. GLP-1 is mostly secreted from the distal ileum in response to mixed meals [10]. GLP-1 stimulates insulin secretion [9].

Clinical studies of diabetic patients show that GLP-1 improves diabetes control [11]. A reduced insulin requirement was observed during GLP-1 treatment.

In the present study, we investigated the

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proteolytic processing of GLP-1 in cell media of β cells and by fetal calf serum using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The processing products may be of importance due to the possible generation of intrinsic biological activities that differ from those of GLP-1 (7–36) amide, e.g. they may be antagonists of GLP-1.

2. Methods

2.1. Cell culture

RINm5F cells were grown in plastic culture bottles with Roswell Park Memorial Institute (RPMI) 1640 medium, 10% fetal calf serum (FCS), 5 m*M* penicillin, 5 m*M* streptomycin, 37°C, air–CO_2 (95:5). Cell concentrations were determined by counting the cells in a Neubauer chamber.

2.2. Incubation of RINmF5 cells with GLP-1

Cells were seeded at a concentration of $0.5 \cdot 10^6$/ml in a 100-ml plastic culture bottle and grown for 48 h. The cells were washed three times with phosphate buffered saline (PBS). Incubation was performed with FCS-free RPMI 1640 medium. GLP-1 stimulation was performed with GLP-1 (7–36) amide at a concentration of 0.6 μ*M* for 2 h. Proteolytic activity of FCS was determined by incubation of 10% FCS-containing RPMI 1640 medium with GLP-1 (7–36) amide at a concentration of 0.6 μ*M* for 2 h in the absence of RINmF5 cells. The medium was collected and centrifuged at 200 *g*. The pH was adjusted to 2.7 using hydrochloric acid and diluted with water to a conductivity of 5 mS/cm.

2.3. Preparation of peptides from RINm5F cell supernatant

Centrifuged RINm5F cell culture medium was loaded on a cation-exchange column (50×4 mm, Fractogel TSK SP 650 M, Merck, Darmstadt). Batch elution of the peptides was achieved with 1 *M* ammonium acetate, pH 9.0. Purification and separation of the peptides is performed using reversed-phase C_{18} chromatography.

In experiments analyzing the proteolytic effect of FCS, the samples were ultrafiltered using an M_r 30 000 cut-off membrane (MiniPlate Amicon, Germany). The pressure gradient of 2 mbar was achieved by using a tube pump. The ultrafiltrate was loaded on a reversed-phase column for batch elution (Source 15 RPC, 125×4 mm, Pharmacia, Freiburg, Germany). The eluate was lyophilized. An equivalent of the lyophilisate was dissolved in 50 μl PBS, loaded on a gel filtration column (Superdex G75; 300×3 mm, Pharmacia) and eluted at a flow-rate of 40 μl/min. Collection of the eluate was started after 1.6 ml to exclude the high-molecular-mass proteins. The low-molecular-mass fraction was loaded on a reversed-phase column for separation (Zorbax MicroTech C_{18}, 150×1 mm, Sunnyvale, CA, USA). The generated fractions were subsequently subjected to MALDI-MS.

2.4. MALDI-MS

A 1-μl volume of each RP fraction and 1 μl of α-cyanohydroxycinnamic acid were applied to a stainless steel multiple sample tray as admixture using the dried drop technique. Measurements were performed in linear mode with a LaserTec RBT MALDI-MS system (Perseptive/Vestec, Houston, USA). The instrument is equipped with a 1.2-m flight tube and a 337-nm nitrogen laser. Positive ions are accelerated at 30 kV and 64 laser shots are automatically accumulated per sample position. The time-of-flight data are externally calibrated for each sample plate and sample preparation. For calibration a mixture of bradykinin (1061.24), human secretin (3040.46), and bovine insulin (5734.59) is used. Data acquisition and first analysis, implying an automatic Savitzky Golay smoothing, baseline correction, and peak labeling, is performed using GRAMS software supplied by the manufacturer.

2.5. Analysis of mass spectrometric data

For further analysis of the MALDI-MS data, new software MASSSPECANALYST based on Microcal Software Origins C-like language LABTALK has been

developed. To perform the comparison of the data, the spreadsheet files of the mass spectra produced by GRAMS software generated from the RP-HPLC fractions are used to prepare a single mass data table. Each mass data table is a list of the detected molecular masses of all fractions in a single preparation. With each molecular mass the according intensity, fraction number and MALDI-MS filename is listed. This database is the basis for further analysis. The data are reduced from ≈1 MB per MALDI mass trace to 70 KB per database.

The software compares two tables in consideration of the error of measurement, elution position of the molecular masses, and intensity of the molecular masses. These parameters are used to determine whether two mass values represent identical molecules. The error of measurement of the LaserTec RBT MALDI-MS is 2‰. The software generates 'mass identity spectra', which represent the molecular masses found in each of two peptide preparations and 'mass difference spectra', which represent the molecular masses found in only one of two peptide preparations.

2.6. Sequence analysis

Sequence analysis of the isolated peptides is performed by stepwise Edman degradation using a gas-phase automated sequencer (Model 470 A, Applied Biosystems, Weiterstadt, Germany). The resulting phenylthiohydantoin (PTH)-amino acids are identified by HPLC.

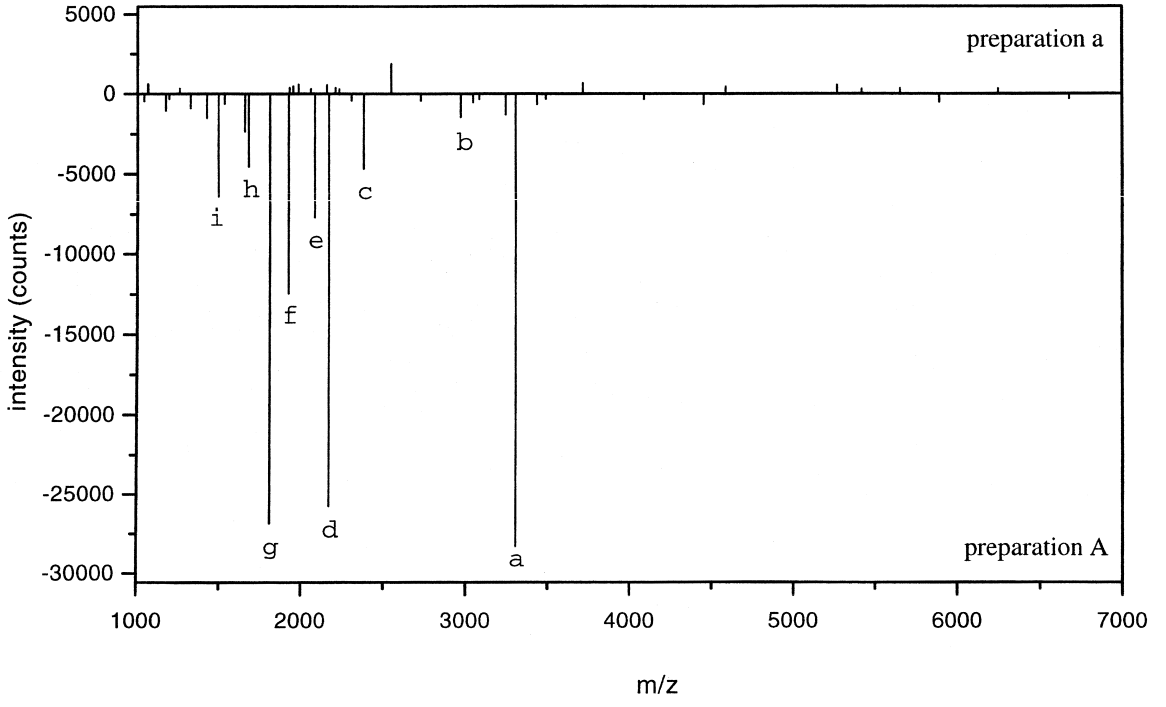

Fig. 1. Mass difference spectrum shows a comparison of the peptide content from unstimulated RINm5F cell supernatant (preparation a) with a preparation of supernatant from GLP-1 (7–36) amide-stimulated RIN m5F cells (preparation A). The masses found in one of the two preparations are shown. The mass data of both supernatants were compared by MASSSPECANALYST software. The letters a to i mark the mass spectrometric peaks representing GLP-1 proteolytic products. The crosses (+) in the legend refer to proteolytic products isolated and sequenced by Edman degradation. [a=3303: GLP-1 (7–36) amide (+); b=2968: GLP-1 (10–36); c=2378: GLP-1 (7–28); d=2170: GLP-1 (18–36) (+); e=2080: GLP-1 (19–36) (+); h=1678: GLP-1 (22–36) (+); g=1810: GLP-1 (21–36) (+); f=1923: GLP-1 (20–36); i=1493: GLP-1 (24–36)].

3. Results

3.1. Identification of rat-insulin-1, GLP-1 and their proteolytic fragments

Serum-free supernatant from unstimulated RINm5F cells as well as GLP-1 stimulated RINm5F cells were produced. To induce r-insulin secretion, 0.6 μ*M* GLP-1 was subjected to the RINm5F culture. Supernatants were subjected to cation-exchange chromatography and batch-eluted. The eluate was fractionated using RP-HPLC chromatography. Fractions were lyophilized and diluted in 100 μl sample buffer. A 1-μl volume of the fractions was subjected to MALDI-MS. The spreadsheet files were processed using the computer software MASSSPECANALYST to prepare a single mass data table for each run of chromatography. MASSSPECANALYST was used to compare the single mass data tables from unstimulated and stimulated RINm5F supernatants. Mass identity and mass difference spectra are shown in Figs. 1 and 2. In the mass identity spectrum, the mass of r-insulin (M_r 5816) was identified. Purification and amino acid sequencing of this mass confirmed r-insulin I. The insulin peak from GLP-1-stimulated RINm5F cells has an intensity 3 times higher than the unstimulated sample. The molecular mass of 2850 was identified as a proteolytic product of r-insulin I (25–49).

Applying the described technique, the culture medium of the GLP-1-stimulated RINm5F cells reveals the different cleavage products of GLP-1 (7–36) amide. According to MS data (Fig. 1), these are GLP-1 (7–36), GLP-1 (10–36), GLP-1 (7–28), GLP-1 (18–36), GLP-1 (19–36), GLP-1 (20–36), GLP-1 (21–36), GLP-1 (22–36), and GLP-1 (24–36). Furthermore, GLP-1 (7–36), GLP-1 (18–36), GLP-1 (19–36), GLP-1 (21–36), and GLP-1 (22–36) are identified by purification and amino acid sequencing (Fig. 1). The mass difference spectrum of Fig. 1 depicts the proteolytic products of GLP-1 (7–36) amide in the form of the most prominent peaks.

Incubation of GLP-1 in FCS (Fig. 6) reveals the

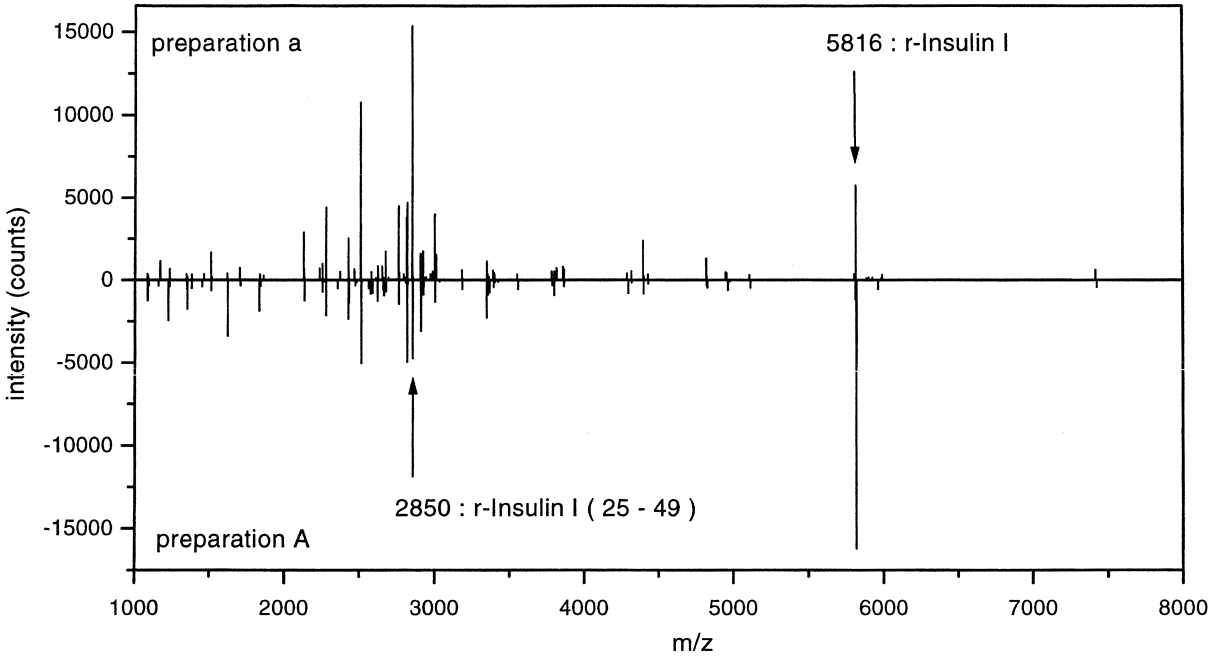

Fig. 2. Mass identity spectrum shows a comparison of the peptide content from unstimulated RINm5F cell supernatant (preparation a) with a preparation of supernatant from GLP-1 (7–36) amide-stimulated RIN m5F cells (preparation A). Comparing the mass data tables of preparation a and A, taking into consideration the error of measurement and the elution time of the peptides, the software MASSSPECANALYST produces this mass identity spectrum, demonstrating the detected masses found in both preparation.

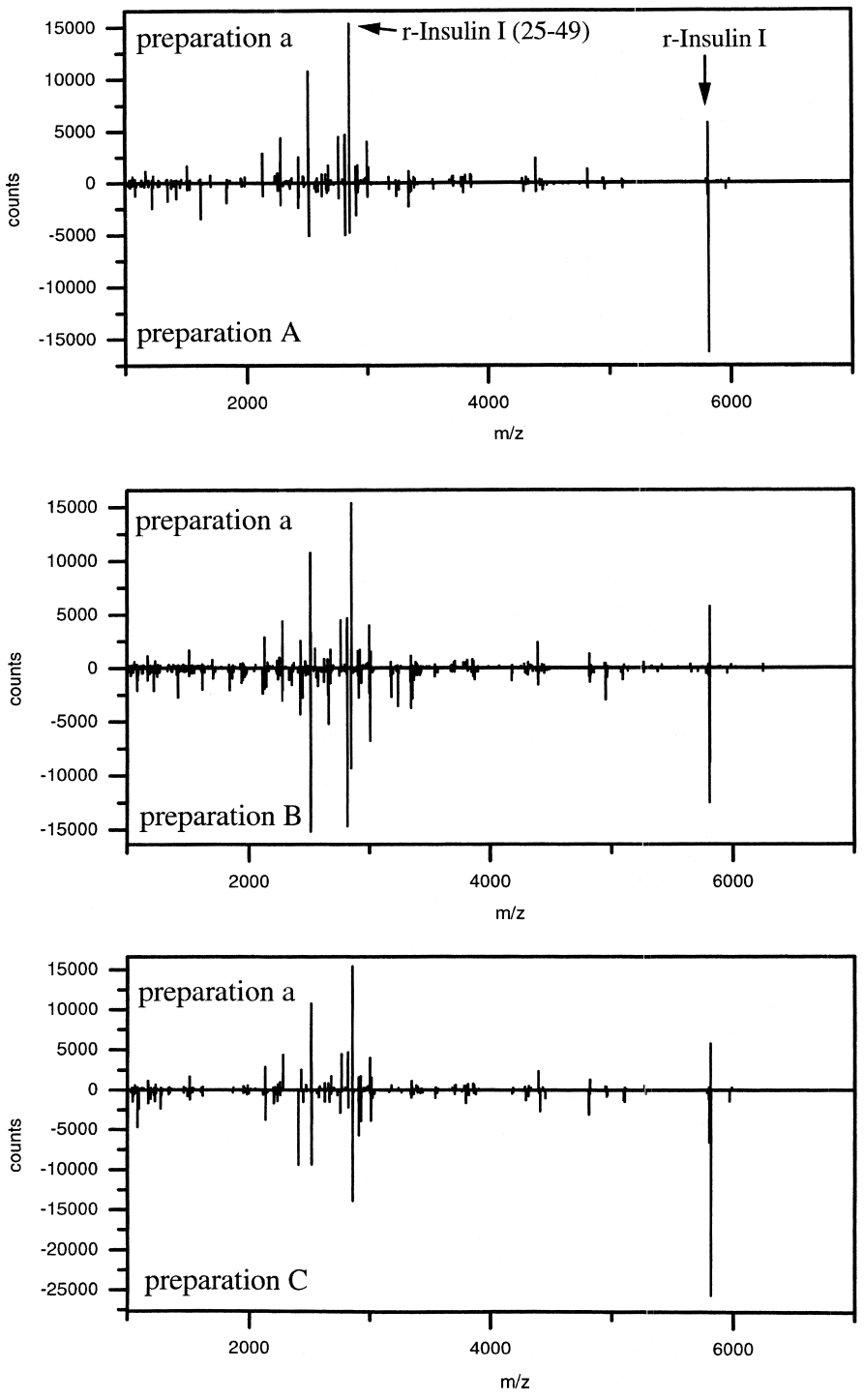

Fig. 3. Three mass identity spectra show a comparison of the peptide content from unstimulated RINm5F cell supernatant (preparation a) with three different preparations of supernatant from GLP-1 (7–36) amide-stimulated RINm5F cells (preparation A, B, C).

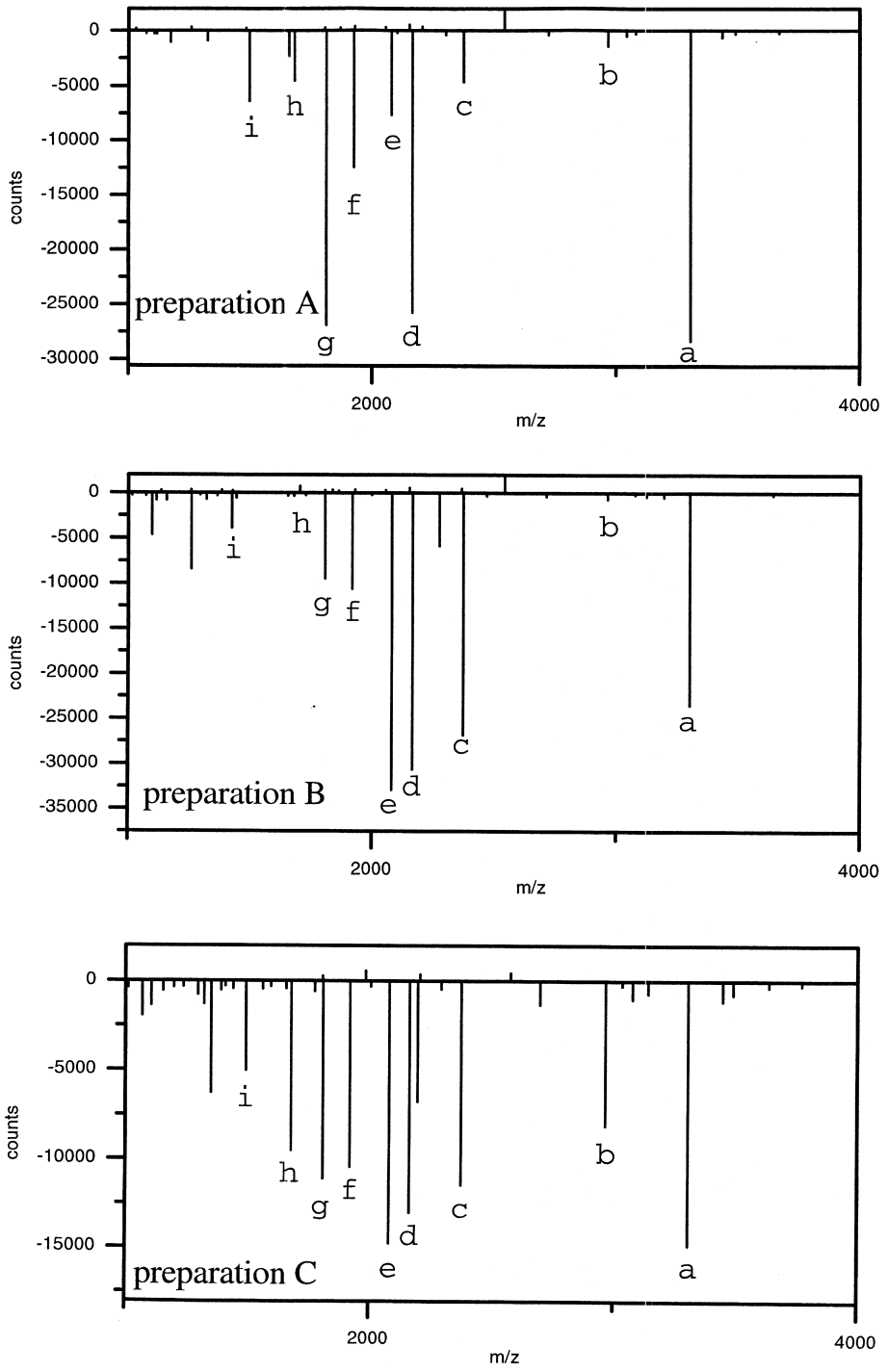

Fig. 4. Three mass difference spectra show a comparison of the peptide content from unstimulated RINm5F cell supernatant (preparation a) with three different preparations of supernatant from GLP-1 (7–36) amide-stimulated RINm5F cells (preparations A, B, C). (Letters refer to the legend in Fig. 2).

mass difference spectrum demonstrating molecular masses, which may represent GLP-1 (22–36), GLP-1 (21–36), GLP-1 (19–36), GLP-1 (9–34), GLP-1 (9–36), GLP-1 (8–36), and GLP-1 (7–36). These peaks presenting the cleavage fragments of GLP-1 are predominant in the mass difference spectrum.

3.2. Reproducibility of the method

For assessment of the reproducibility of the method, three preparations (preparation A, B, C) of RINm5F cell supernatant incubated with GLP-1 (7–36) amide are compared with an unstimulated sample (preparation a) by producing mass identity and mass difference spectra (Figs. 3 and 4). The mass of r-insulin I and the proteolytic fragment (25–49) are detected in each preparation. In all preparations, the insulin peak from GLP-1-stimulated RINm5F cells is prominently higher when compared to the unstimulated sample. The identified proteolytic fragments of GLP-1 (7–36) amide are present in all mass difference spectra.

For further examination of the reproducibility of the method, 23 molecular masses with high intensity found in a preparation of stimulated RINm5F cell supernatant have been selected and their occurrence has been determined in five other preparations. Fig. 5 reveals that 16 out of 23 selected molecular masses are found in all preparations, 2 molecular masses were found in 5, 3 molecular masses are found in 4, and 1 molecular mass is found in only 3 preparations. Missing molecular masses are in a molecular mass range >3500. With the exception of the molecular mass of 5818-representing r-insulin-1- the molecules >4000 are detected only with intensities <2000.

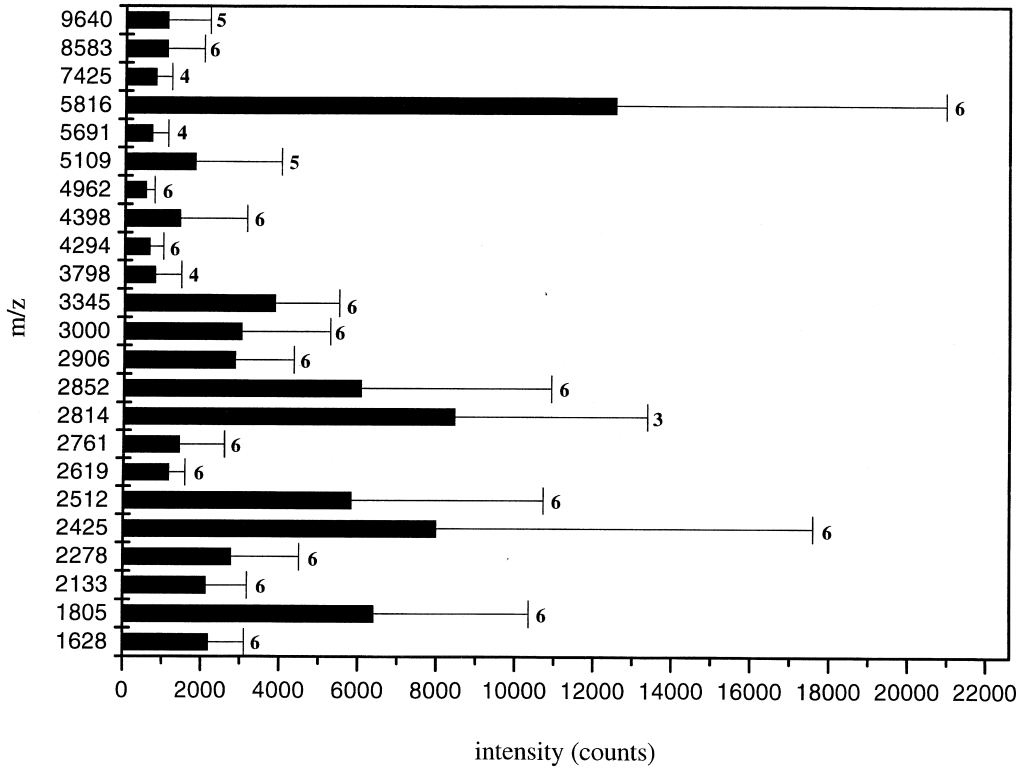

Fig. 5. Mass intensities (mean value and standard error) of masses identified in six preparations. The mass values represent detected MALDI-MS peaks, which appear in different preparations of GLP-1 (7–36) amide incubated supernatant of RIN m5F cells. The columns show the mean intensities of detected masses. The numbers next to the error bars indicate the number of appearances of each mass in six preparations.

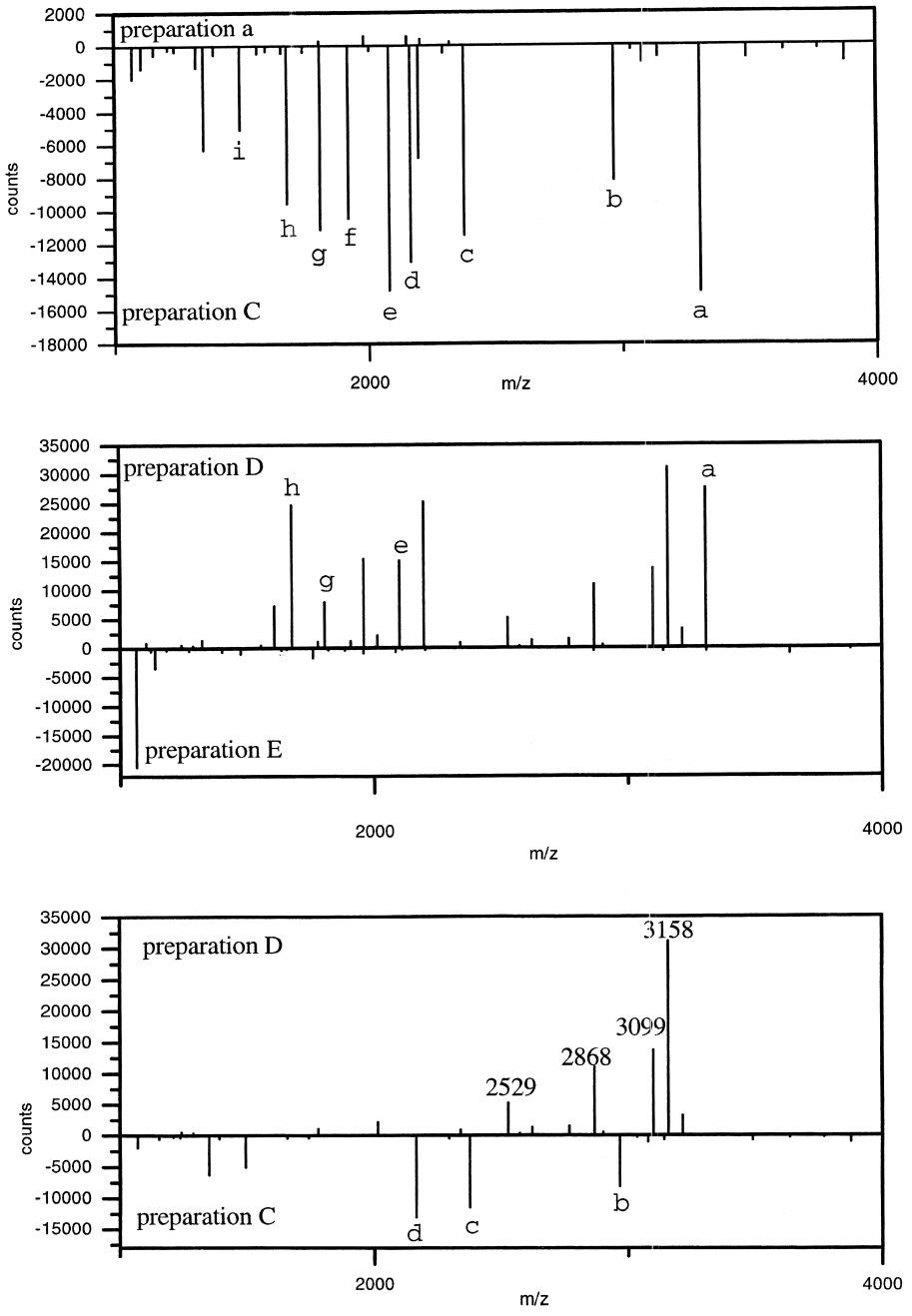

Fig. 6. Identification of cleavage products of GLP-1 (7–36) amide generated in fetal calf serum. The upper mass difference spectrum shows a comparison of the peptide content from unstimulated RIN m5F cell supernatant (preparation a) with a preparation of GLP-1 (7–36) amide stimulated RIN m5F cell supernatant. The second mass difference spectrum shows a comparison of fetal calf serum containing GLP-1 (7–36) amide (preparation D) and 10% fetal calf serum containing media (preparation E). Marked mass spectrometric peaks a, e, g, and h may represent GLP-1 (7–36) amide, GLP-1 (19–36), GLP-1 (21–36), and GLP-1 (22–36) respectively. The last mass difference spectrum shows a comparison of the data gained from both upper spectra. The numbers indicate the detected masses. By using MACBIOSPEC, the only possible cleavage product of GLP-1 for 3158 is GLP-1 (8–36), for 3099 GLP-1 (9–36) and for 2868 GLP-1 (9–34). Marked mass spectrometric peaks b, c, and d refer to GLP-1 (10–36) GLP-1 (7–28), and GLP-1 (18–36), respectively.

3.3. Quantification of GLP-1 (7–34) amide by MALDI signal

To determine the dependence of peptide concentration in the sample and signal intensity in MALDI-MS, different concentrations of GLP-1 (7–34) amide are measured using CHC matrix. 60 fmol/μl of GLP-1 (7–34) amide are necessary to give a reliable determination of the peptide in MALDI-MS (Fig. 8).

4. Discussion

This study was initiated to investigate the proteolytic cleavage of GLP-1 by RINm5F cells and in FCS using MALDI-MS. To carry out this study a new tool, the computer program MassSpecAnalyst was developed to compare the MALDI mass data gained from the chromatographic fractions of RINm5F cell culture supernatant.

GLP-1 fragments	Detected M_r	Theoretical average M_r	Sequenced by Edman degradation
GLP-1 (7-36)	3305.5	3298.6	+++
GLP-1 (7-28)	2377.9	2374.5	
GLP-1 (8-36)	3158.3	3161.5	
GLP-1 (9-36)	3099.4	3090.4	
GLP-1 (9-34)	2868.1	2877.2	
GLP-1 (10-36)	2968.4	2961.3	
GLP-1 (18-36)	2168.6	2166.5	+++
GLP-1 (19-36)	2081.4	2079.4	+++
GLP-1 (20-36)	1923.8	1916.2	
GLP-1 (21-36)	1810.1	1803.1	+++
GLP-1 (22-36)	1678.6	1673.1	+++
GLP-1 (24-36)	1492.9	1488.8	

Fig. 7. (A) Amino acid sequence of GLP-1 (7–36) amide. Arrows (↑) indicate the identified processing sites of GLP-1. (B) Detected molecular masses and theoretical average molecular masses of the identified GLP-1 fragments. Identified GLP-1 fragments using amino acid sequencing, are indicated in the right column.

Using this method, eight different GLP-1 proteolytic fragments were identified in RINm5F cell culture medium. Four of these were isolated and amino acid sequencing confirmed the identity of molecular masses and GLP-1 fragments. GLP-1 fragments induced a high MALDI-MS intensity. Four of the molecular masses were not sequenced, but the high intensity of the peaks as well as the molecular mass itself suggest that the peaks represent GLP-1 (10–36) and GLP-1 (7–28) GLP-1 (20–36), and GLP-1 (24–36).

Interestingly, in our study primarily N-terminally truncated fragments were found with proteolytic cleavage between amino acids 9 and 10, 17 and 18, 18 and 19, 19 and 20, 20 and 21, 21 and 22, and 23 and 24, whereas Hupe-Sodmann et al. [13] found only the C-terminal fragment GLP-1 (29–36). One explanation for this finding is the use of plasma membranes of RINm5F cells in the study of Hupe-Sodmann et al. [13] whereas in our study we used intact RINm5F cells.

Comparison of GLP-1 fragmentation in plasma and in RINm5F cell culture suggest that three GLP-1 fragments be exclusively produced in plasma (Fig. 6). These N-terminally truncated forms are suggested to be GLP-1 (8–36), GLP-1 (9–36), and GLP-1 (9–34). The other identified cleavage fragments appear in both preparations. Interestingly, GLP-1 (9–36) is suggested to be a cleavage product of the dipeptidylpeptidase IV which is known as a plasmatic enzyme and which inactivates the GLP-1 (7–36) amide [12]. In addition, it is suggested that it binds to the GLP-1 receptors and thus may act as a receptor antagonist [12].

Assessment of the reproducibility of the method used shows that r-insulin, an r-insulin fragment,

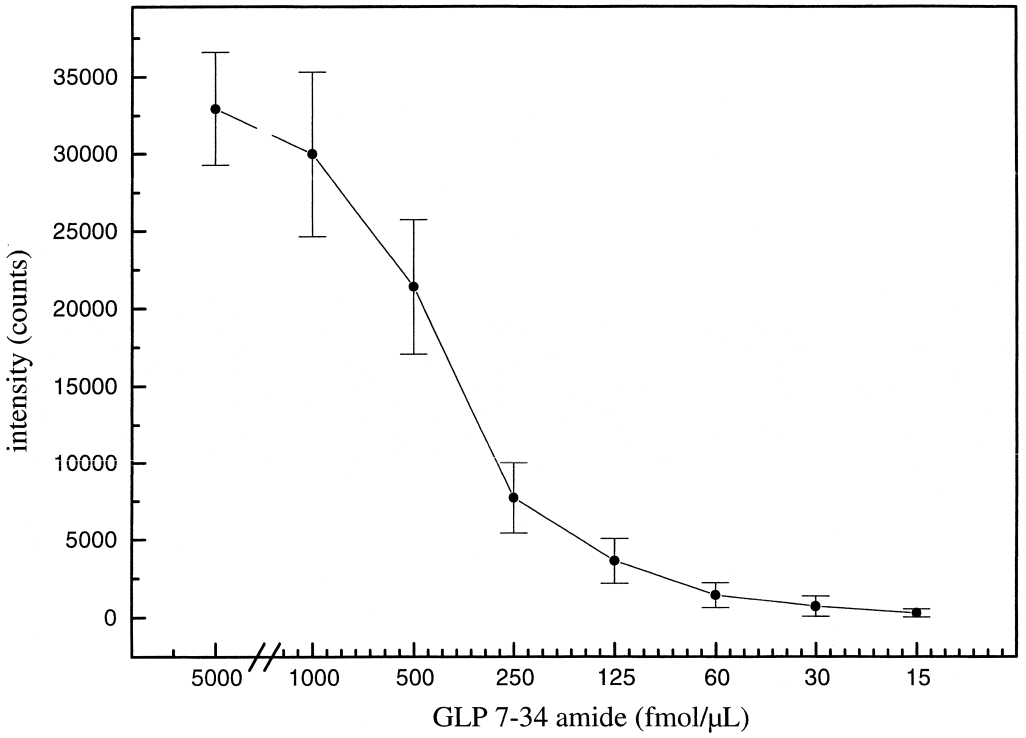

Fig. 8. Dependence of MALDI-MS signal intensity and GLP-1 (7–34) amide concentration. 1 μl GLP-1 (7–34) amide admixtured in 50% (v/v) acetonitrile, 0.1% trifluoroacetic acid were subjected to the multiple sample tray. Eight different concentrations of GLP-1 were analyzed by MALDI-TOF-MS using α-cyano-4-hydroxycinnamic acid as matrix. GLP-1 (7–34) amide concentrations ranged from 15 fmol/μl to 5 pmol/μl. Each data point represents the mean value and the standard error of eight trials.

GLP-1 (7–36) amide, as well as eight GLP-1 fragments are detected in three preparations (Figs. 3 and 4). Furthermore, of twenty-three molecular masses, sixteen are found in each of six preparations of RINm5F cell supernatants (Fig. 5). Seven masses are found only in three, four and five preparations. An explanation for the missing molecular masses as well as the high standard errors of the peak intensities is the sample preparation, which starts with incubation of the cell culture, followed by cation-exchange chromatography, RP chromatography, lyophilization of the samples and manual pipetting of the MALDI plates (Fig. 7). Measurement of GLP-1 (7–34) amide standards revealed a concentration dependence of the MALDI signal intensity suggesting that a quantification of the amount of peptide in a sample is possible (Fig. 8).

Our study shows a new strategy for the complex analysis of reduced MALDI-TOF-MS mass spectra. The application of this method allows the examination of the molecular fragments of regulatory peptides appearing during metabolism and processing in vitro by HPLC and MALDI-MS.

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