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Matrix-assisted laser desorption/ionisation mass spectrometry guided purification of human guanylin from blood ultrafiltrate

Michael Schrader*, Michael Jürgens, Rüdiger Hess, Peter Schulz-Knappe, Manfred Raida, Wolf-Georg Forssmann

Lower Saxony Institute for Peptide Research (IPF), Feodor-Lynen-Strasse 31, D-30625 Hannover, Germany

Abstract

The purification of the human peptide hormone guanylin 22-115 from blood ultrafiltrate (hemofiltrate, HF) was achieved using matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) as the assay system. Screening a peptide bank generated from 5000 l HF guanylin 22-115 was detected by its molecular mass when adequate conditions for MALDI-MS analysis were chosen. The sensitivity was even better than of the established biological assay system. In addition, the susceptibility towards solvents and salts is strongly reduced. 1.2 mg of the peptide hormone was purified from 10% of the starting material. © 1997 Elsevier Science B.V.

Keywords: Matrix-assisted laser desorption/ionisation mass spectrometry; Peptides; Proteins; Hormones; Guanylin

1. Introduction

Human guanylin 22-115 (guanylate cyclase activating peptide I, GCAP-I) is a member of a peptide hormone family involved in the regulation of intestinal chloride secretion. Originally, guanylin was isolated from rat intestine as a peptide of 15 amino acid residues, screening its biological activity to raise intracellular cGMP-levels in colonic tumour cells via its specific receptor guanylate-cyclase C [1]. In human hemofiltrate (HF), a circulating bioactive form of this peptide (guanylin 22-115) [2] and of uroguanylin (uroguanylin 89-112 [3]) were discovered using the same assay system.

Human HF is generated during blood ultrafiltration of patients with chronic renal failure. Since molecules with about M_r<20 000 pass the filter, HF is used as a source for the isolation of peptide hormones [4].

Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) is an established tool to achieve a fast and sensitive analysis for peptides and proteins [5,6]. Even in complex mixtures, it is possible to detect a single peptide specifically by its molecular mass.

The aim of this investigation was to test whether the isolation of a known peptide hormone from HF can be performed with MALDI-MS as an assay system. As we required milligram amounts of guanylin 22-115, this peptide was chosen as an example for this approach. The starting material was 5000 l of HF. Aliquots from a peptide bank fractionated according to our standard procedure [7] were screened for the average molecular mass of guanylin 22-115 (M_r=10 337). Finally, we were able to purify

*Corresponding author. Tel. (+49-511) 5466-214; Fax: (+49-511) 5466-102; E-mail: 100140,2162@compuserve.com

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guanylin 22-115 to homogeneity in consecutive chromatographic steps monitored by MALDI-MS.

2. Experimental

2.1. Human peptide bank

Human HF was obtained from the Nephrologisches Zentrum Niedersachsen (Hannoversch-Münden, Germany) and processed in a standardised procedure as described in detail [7]. The peptides from five batches of 1000 l HF were extracted by cation-exchange chromatography (TSK SP 650(M), 40–90 μm, 1000 Å, Merck, Darmstadt, Germany). The collected extracts from 5000 l HF were separated on a 200×250 mm column filled with the same cation-exchange material by increasing the pH of the eluting buffers (1: 0.1 *M* sodium citrate, pH 3.6, 2: 0.1 *M* sodium acetate, pH 4.5; 3: 0.1 *M* sodium malate, pH 5.0, 4: 0.1 *M* sodium succinate, pH 5.6, 5: 0.1 *M* sodium phosphate, pH 6.6, 6: 0.1 *M* sodium phosphate, pH 7.4, 7: 0.1 *M* ammonium carbonate, pH 9.0).

The resulting seven pools were further separated by reversed-phase chromatography (125×100 mm column, Source RPC, 15 μm, Pharmacia, Freiburg, Germany).

2.2. Further liquid chromatography

The RP-HPLC with the selected fractions from the peptide bank was performed with a preparative 300×47 mm column (Bakerbond C_{18}, 15–30 μm, 300 Å, Waters, Milford, MA, USA). 10 m*M* HCl and 80% (v/v) acetonitrile in water and 10 m*M* HCl were the solvents in a gradient elution. In the cation-exchange chromatography, a 50×10 mm column (TSK SP 650(S), 20–50 μm, 1000 Å, Merck) was used. One solvent was 10% (v/v) methanol in 0.2 *M* acetic acid, whereas the elution solvent was 10% (v/v) methanol in 0.5 *M* acetic acid and 1 *M* ammonium acetate. A 250×4.6 mm C_{18} column (5 μm, 300 Å, Vydac, Hesperia, CA, USA) served for the final purification. The solvents were the same as in the first step. All runs were performed at ambient temperature.

2.3. MALDI-MS

Measurements were performed in linear mode with a LaserTec RBT II (PerSeptive Biosystems/Vestec, Houston, TX, USA). The instrument is equipped with a 1.2 m flight tube and a 337 nm nitrogen laser. Positive ions were accelerated at 30 kV and up to 256 laser shots accumulated. The time-of-flight data were externally calibrated for each sample plate and different sample preparations.

Polished stainless-steel sample plates with 100 sample positions were used. 1 ml matrix solution and HPLC fraction each were employed. The "dried drop" crystallisation technique [8] was applied by mixing on the plate with a pipette and accelerated air-drying. 3,5-Dimethoxy-4-hydroxycinnamic acid ("sinapinic acid"; Fluka, Neu-Ulm, Germany) and α-cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid (Aldrich, Deisenhofen, Germany) were used as matrices. Acetonitrile–0.1% aqueous trifluoroacetic acid (TFA) (1:1, v/v) was chosen as solvent for the matrices (typically 5–10 μg/μl).

2.4. T84 cell cGMP bioassay

The bioassay for the detection of GC-C activating peptides was carried out as described in detail [1,2]. Human colon carcinoma (T84) cells were used at passages 34–45. The cells were incubated for 60 min with aliquots derived from the chromatographic fractions, in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 1 m*M*; Sigma, Deisenhofen, Germany). The effects on intracellular cGMP content were compared with those of synthetic human guanylin (IPF, Hannover, Germany) and *E. coli* heat-stable enterotoxin (STa; Sigma, Deisenhofen, Germany). cGMP concentrations were measured using a specific radioimmunoassay after cell disruption.

3. Results and discussion

The molecular mass is a specific parameter that can be used to determine peptides in mixtures. In a crude mixture, two prerequisites for the identification of a selected peptide by MALDI-MS have to be fulfilled. First, the sample preparation should be

suitable for the peptide. Choice of matrix material, especially, can lead to different specificities, but also solvents and crystallisation technique have an impact. An optimisation of the sample preparation to analyse complex mixtures has been performed [9]. Secondly, the other components must not suppress the signal of the selected peptide.

In a pre-screening experiment, the seven pools of the stepwise elution from a cation-exchange column with increasing pH of the eluting buffers were analysed by MALDI-MS. As a result, a peak with the expected average molecular mass of guanylin 22-115 (M_r=10 337) was identified in the first pool using sinapinic acid as matrix (Fig. 1). This was in agreement with results from the bioassay. With α-cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid the signal of guanylin was suppressed by other components in the first pool containing about 5% of the blood peptide content. These results were confirmed by analysis of more purified samples using these matrix compounds, where sinapinic acid resulted in the highest sensitivity with regard to guanylin 22-115.

After the pre-screening, a human peptide bank [7] obtained from 5000 l HF (corresponding to about 300 g peptides and proteins) was screened identifying guanylin 22-115 in 5 of 46 fractions derived from a preparative reversed-phase chromatography with material from the first pool of the stepwise elution from the cation-exchange column. The middle three fractions (2.7 g in total) gave a higher relative signal intensity and were chosen for further purification. A preparative RP-HPLC was carried out as the next step. The collected fractions were also analysed with the biological assay system (Fig. 2) to verify the MALDI-MS results (Table 1). The biological assay system showed four fractions with the ability to stimulate cGMP production in T84 cells.

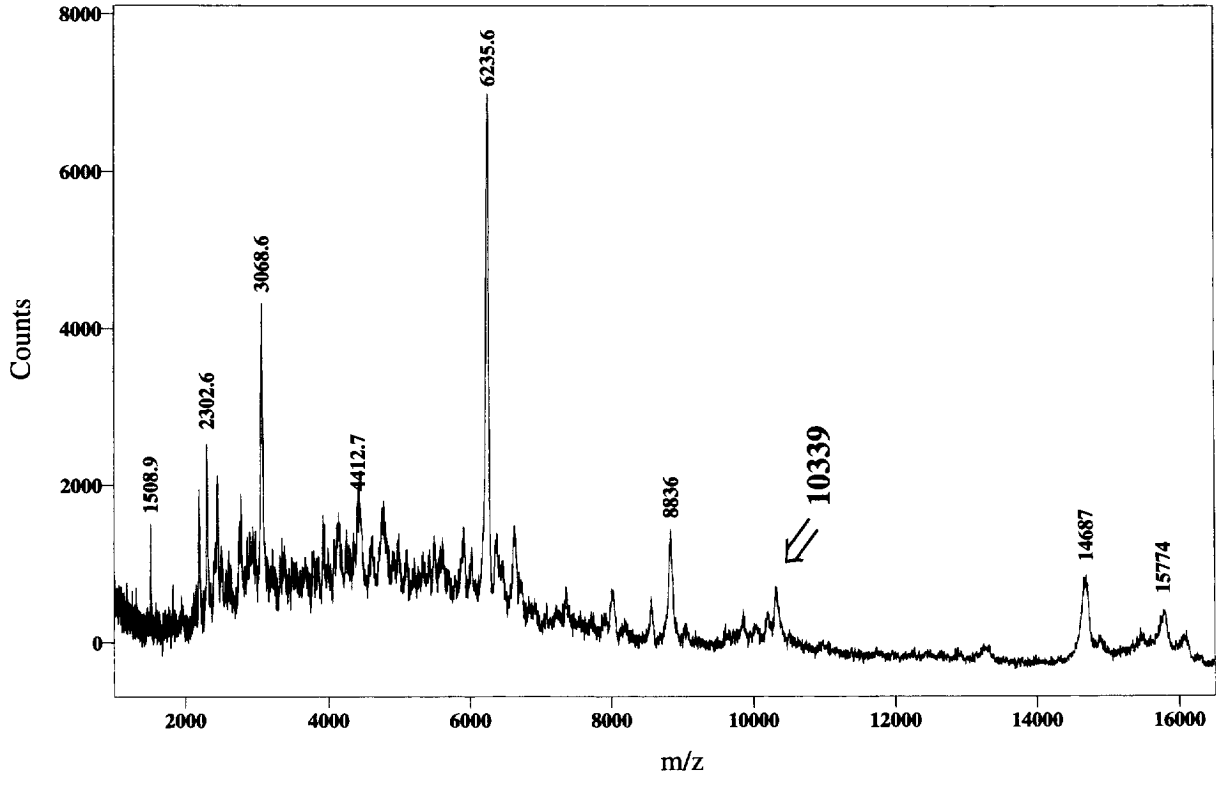

Fig. 1. MALDI spectrum of the first pool of a stepwise elution from a cation-exchange column representing about 5% of the peptide amount in hemofiltrate. A peak with the expected m/z (mass/charge) of 10 338 for single protonated guanylin 22-115 within the experimental error of 0.1% is detectable. An equivalent of 1 ml hemofiltrate in 1 μl solution was used. Sinapinic acid served as matrix.

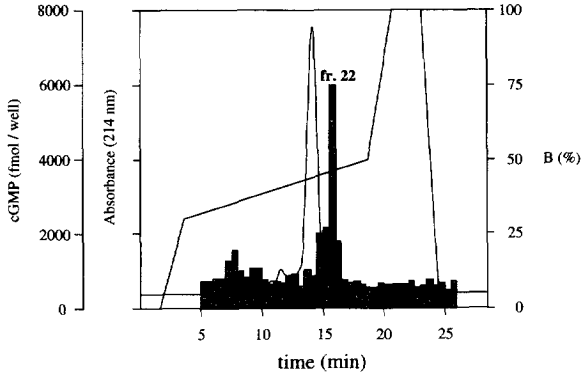

Fig. 2. Chromatogram and gradient of the preparative RP-HPLC in the second purification step and results of the cGMP-RIA (bars) after stimulation of T84 cells with an equivalent of 1 l hemofiltrate.

The MALDI-MS spectra gave a peak for guanylin 22-115 in these fractions (Fig. 3a) but also in neighbouring fractions (Fig. 3b) which exhibited no biological activity in the doses applied for the test. MALDI-MS is superior in sensitivity and much less of the sample is needed. The bioassay requires about 500 fmol of the peptide whereas only 10 fmol are enough to be detected by MALDI-MS. An advantage of the bioassay, however, is the possibility to quantify the amount of bioactive peptide, if only a single active compound is present. Nevertheless, comparison with the bioassay revealed that relative signal intensity in MALDI-MS correlated in a qualitative way with the content of guanylin 22-115 in positive fractions.

Three out of four fractions of the former step with the highest response for guanylin 22-115 were chosen considering their total peptide content (Fig. 2). A cation-exchange chromatography step was established to further separate the pooled fractions. The high sensitivity of MALDI-MS and low susceptibility towards salts allowed several combinations of buffers and eluting salts to be tried. Before selecting the final conditions, different gradients up to 1 *M* NaCl or NH_4Ac as eluting salts and with acetic acid or sodium phosphate buffers were tried with or without additional methanol and investigated. The identification of guanylin 22-115 in these fractions was again quickly and easily achieved (e.g., Fig. 4). Prior desalting was not necessary but would have been a prerequisite for correctly performing the bioassay. The concentration of guanylin 22-115 in the fractions had been increased significantly, for that reason it was also detectable with other matrix substances as is shown with α-cyano-4-hydroxycinnamic acid in Fig. 4. This demonstrates the wider applicability of this approach. If a peptide is purified to a certain degree, it will be detectable by MALDI-MS.

After applying ten cation-exchange chromatography runs, a final purification by gradient RP-HPLC was carried out with fractions from one run. This lead to a homogenous product showing no impurities in a routine capillary zone electrophoresis and the correct molecular mass in electrospray MS as well as the amino acid sequence of guanylin 22-115 in Edman degradation (experimental details to these methods, e.g., [10]).

So far, 10% of the material has been purified after 8 runs in the final step resulting in 1.2 mg of human guanylin 22-115. Therefore, a total of 12 mg is accessible from 5000 l HF.

The detection of peptide hormones is usually performed with biological or immunological assay systems [4]. With the availability of high sensitivity MALDI-MS an alternative for the purpose of guiding the isolation of known peptides from complex mix-

Table 1
Localisation of human guanylin 22-115 by the bioassay and MALDI-MS in fractions of the RP-HPLC shown in Fig. 2.

	Fraction No.											
	1–4	5	6	7–18	19	20	21	22	23	24	25	26–42
Bioassay results	–	+	+	–	–	+	+	++	+	–	–	–
MALDI-MS results	–	–	–	–	+	++	++	++	++	+	+	–

With MALDI-MS, the same fractions were found to be positive as with the biological assay system, but the peripheral fractions were also positive because of higher sensitivity of MALDI-MS. In fractions 5 and 6 the bioassay detected traces of human uroguanylin 89-112 [3]. Considering the total peptide content, fractions 21 to 23 were used for further purification.

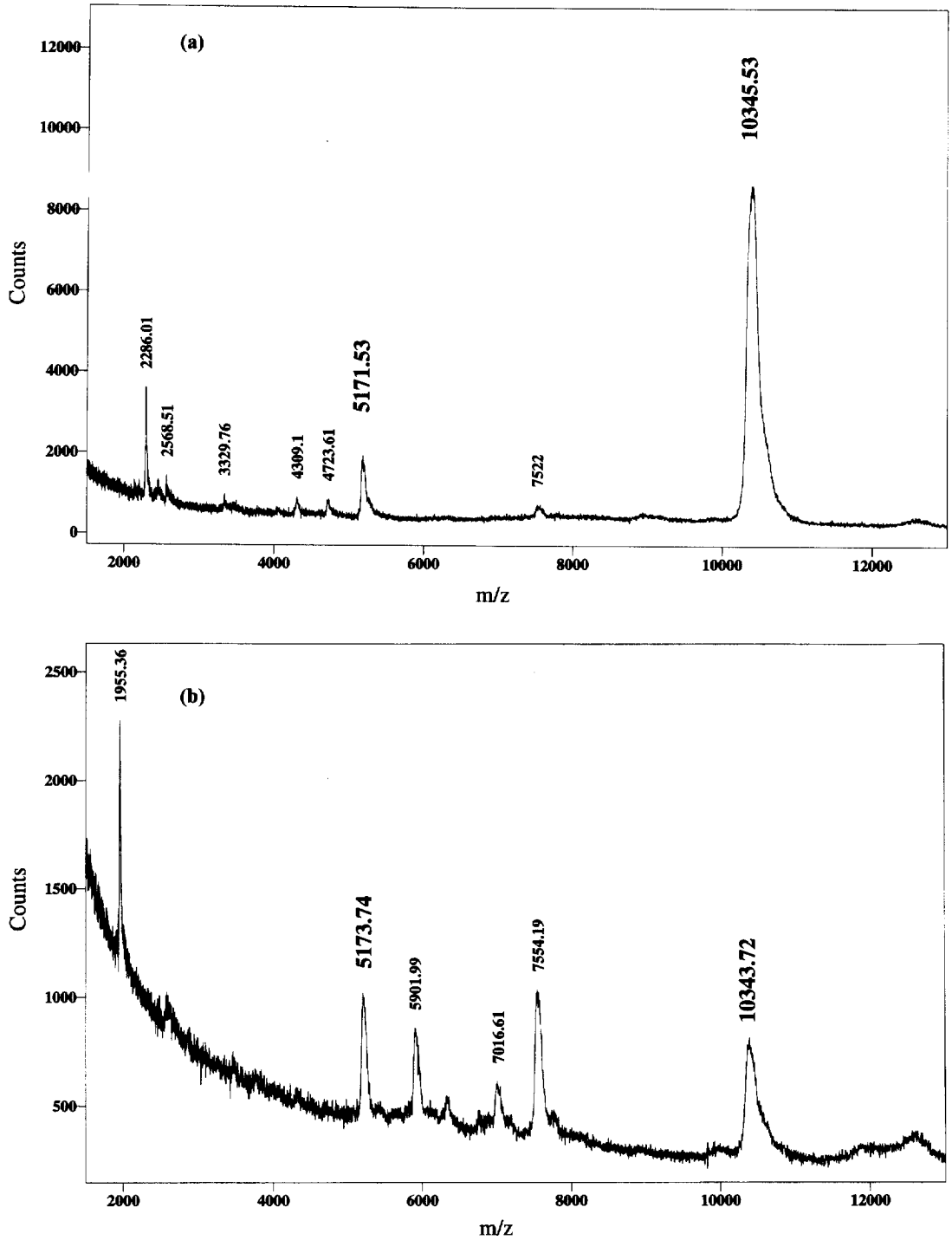

Fig. 3. MALDI spectrum of (a) fraction 22 which gave the highest response in the bioassay, (b) fraction 24 that is already negative in the bioassay (see Fig. 2). A peak with the expected m/z of 10 338 for single protonated guanylin 22-115 within the experimental error of 0.1% is detectable. Sinapinic acid served as matrix.

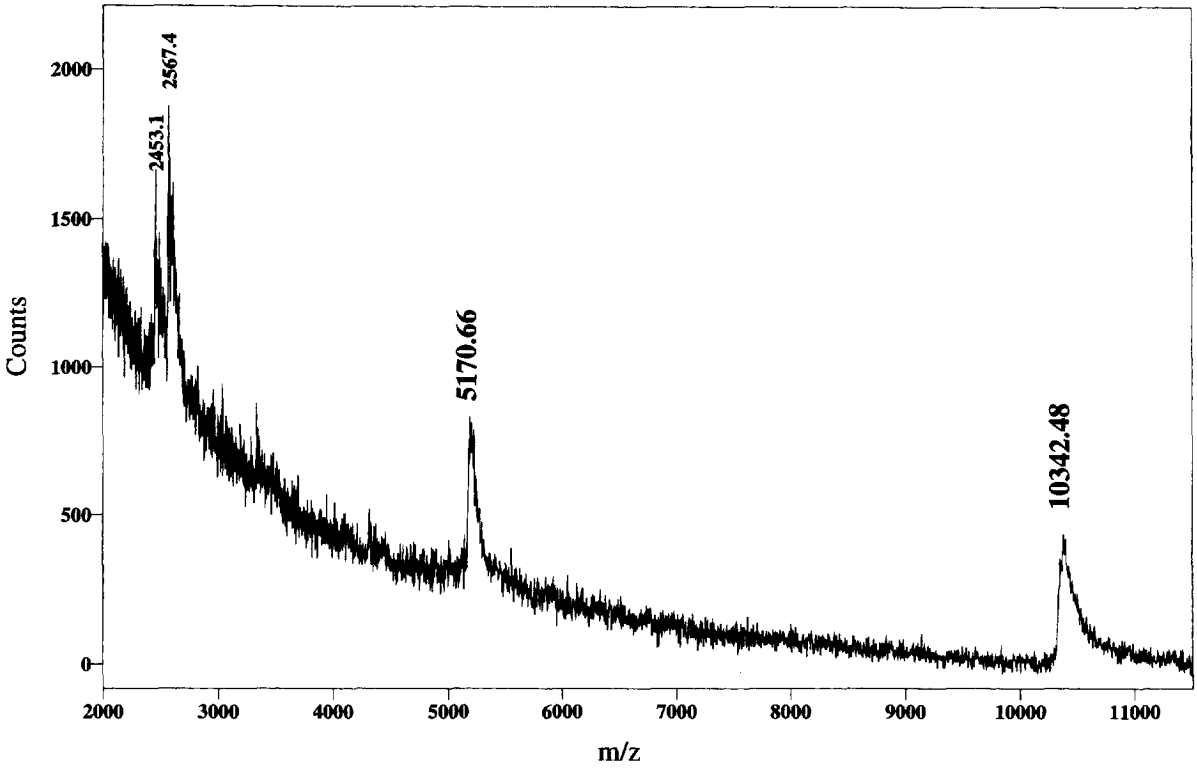

Fig. 4. MALDI-MS spectrum of a fraction from cation-exchange chromatography without prior desalting. Peaks for the single and double-protonated guanylin molecule are detectable within the experimental error of 0.1%. Theoretical values are m/z 10 338 and 5169. α-Cyano-4-hydroxycinnamic acid served as matrix.

tures is given. False positive results relying on crossreactivities of biological or immunological assays are not encountered with MALDI-MS, but a peptide which accidentally has nearly the same molecular mass as the target could be misleading. Hence, the more accurate determination with delayed extraction MALDI-MS [11] is of great importance.

4. Conclusions

Human guanylin 22-115 was identified by MALDI-MS even in crude fractions from human hemofiltrate. The purification of the peptide hormone was possible without other supporting assay systems. Using a human peptide bank, milligram amounts were obtained after three chromatographic steps.

With this approach, detection sensitivity for guanylin 22-115 was even higher than in the biological assay system. Moreover, MALDI-MS proved to be much less affected by solvents and particularly salts of chromatographic eluents used during different chromatographic steps (reversed-phase and ion-exchange chromatography).

Meanwhile, we apply MALDI-MS as a detection system to purify other peptide hormones. For peptides that are more difficult to display, pre-purification may be necessary with other assay systems. After a few initial steps, the pre-purified target peptide is detectable by MALDI-MS. Therefore, this principle is applicable for almost any peptide in mixtures. Generally, it is recommended to use several kinds of optimised sample preparations to improve the analysis of complex mixtures.

Our results clearly demonstrate that MALDI-MS

is a fast and flexible detection system for peptide hormones in complex biological mixtures.

Acknowledgments

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