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Application of a peptide bank from porcine brain in isolation of regulatory peptides

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Abstract

Over the past years, the introduction of biological assay systems, random peptide sequencing and orphan receptor screening has led to the isolation and identification of new regulatory peptides with potential clinical impact. We have developed a method for separating peptides into about 300 fractions from large amounts of porcine brain tissue. The preparation of this peptide bank consists of three steps including ultrafiltration followed by cation-exchange separation and reversed-phase chromatography. These fractions represent the peptide bank with desalted and lyophilized peptides from brain tissue. Molecular masses of the peptides in the fractions are determined by matrix-assisted laser desorption ionization MS and a mass data bank is subsequently generated. For systematic analysis of the peptides, a subsequent two-step purification procedure is followed by Edman sequencing resulting in the identification of different peptides. A survival assay with a neuronal cell line revealing the stimulatory and inhibitory activities is applied as a model to test the 300 fractions. This primary screen indicates that the biological activities of the extracted peptides are easily characterized and, moreover, can be related to the biochemical entities. We conclude that the established peptide bank is an efficient and useful tool for the isolation of regulatory brain peptides applying different purification strategies.

Keywords: Peptides

1. Introduction

The importance of peptides in the diagnosis and therapy of human diseases has been known for a long time. The use of insulin for the therapy of diabetes mellitus is exemplary for the proof of efficacy of a peptide. The same is true for other diseases treated with peptides, e.g., growth hormone for dwarfism [1,2], factor VIII for hemophilia A [3], or erythropoietin for anemia [4]. Up to now, these diseases can only be treated by the use of analogs of the corresponding endogenous peptides. Therefore, isolation and characterization of peptides represent an attractive approach to find new substances with direct clinical impact or to identify new targets for the development of low-molecular-mass drugs.

Since blood plasma is a comprehensive source for regulatory peptides, we previously used large amounts (up to 10 000 l) of human blood ultrafiltrate (hemofiltrate) to generate a peptide bank [5]. Using this large-scale preparation of circulating human peptides, we succeeded in isolating new targets which might have an impact for drug development [6–8]. In the last few years, approaches such as

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random peptide sequencing [9], use of biological assay systems or orphan receptor screening [10–13] have led to the identification and functional characterization of new peptides from brain tissue. Orphanin FQ/nociceptin, for example, was isolated from porcine hypothalamic extracts and activates an opioid like G protein-coupled receptor [10,11]. This peptide acts as a modulator in the brain probably influencing nociceptive and locomotor behavior. The orexins were isolated from rat brain and are discussed to play a mediating role in the central feedback mechanism that regulates feeding behavior [12]. Recently, a new factor was isolated from bovine brain which triggers the prolactin release from the pituitary [13]. These recent isolations clearly demonstrate that the brain is still a comprehensive source for the isolation and identification of new regulatory peptides. With the goal to isolate further undiscovered peptides, we established a peptide bank from porcine brain suitable for use in different purification strategies. This bank contains peptides in quantities which are sufficient for functional analysis and determination of their primary structure.

2. Experimental

2.1. Peptide extraction

Porcine brain (10 kg) obtained from the slaughterhouse in Gleidingen, Germany, is processed immediately after slaughtering and extracted in ice-cold 0.5 *M* acetic acid containing 8 m*M* ascorbate and 0.8 m*M* ethylenediaminetetraacetic acid (EDTA). The extract is homogenized with a waring-blendor (step: high) for 15–30 s. The homogenate with a final volume of 30 l is stirred at 100 rpm overnight at 4°C. After centrifugation (Sigma 6K10, rotor 12.500, 15 600 *g*, 35–40 min), the supernatant is filtered (4–7 μm, paper No. 597, Schleicher and Schuell, Dassel, Germany). The ultrafiltration of the 18 l filtrate is performed by a Sartocon-Mini (0.1 m^2, polysulfone membrane) using ultrafilters with a specified M_r cut-off of 50 000. Filtration is driven by a transmembranous pressure gradient of 1 bar at a temperature of 5°C and a feed rate of 100 l/h. The permeate flux was at 6 l/h. Ultrafiltration was stopped after generation of 43 l permeate by concentration and diafiltration with the extraction buffer.

2.2. Cation-exchange subfractionation

The ultrafiltrate (43 l) is immediately cooled to 4°C and acidified to pH 3 to prevent bacterial growth and proteolysis. After pH adjustment to pH 2.44 with hydrochloric acid, the permeate was diluted with deionized water to a conductivity of 3.1 mS/cm. Using the autopilot chromatography system (PerSeptive Biosystems, Freiburg, Germany), the sample is applied at a flow-rate of 0.5 l/min onto a strong cation exchanger (Fractogel TSK SP 650 (M), 250× 32 mm, Merck, Darmstadt, Germany) followed by a wash step using 0.01 *M* HCl. Stepwise elution is performed using six consecutive buffers with increasing pH. Conductivity, pH and absorbance at 280 nm are monitored.

The buffers used are as follows: 0: 0.01 *M* hydrochloric acid, pH 2.5, C=1.0 mS/cm; I: 0.1 *M* citric acid monohydrate, pH 3.6, C=2.9 mS/cm; II: 0.1 *M* acetic acid+0.1 *M* sodium acetate, pH 4.5, C=4.0 mS/cm; III: 0.1 *M* malic acid, pH 5.0, C=6.2 mS/cm; IV: 0.1 *M* succinic acid, pH 5.6, C=6.1 mS/cm; V: 0.1 *M* sodium dihydrogenphosphate, pH 6.6, C=4.9 mS/cm; VI: 0.1 *M* ammonium acetate, pH 7.0, C=3.5 mS/cm.

The void volume and the six fractions, designated as pH pools 0–VI, are collected and subjected to the second purification step.

2.3. Reversed-phase fractionation

The void volume and each of the six pH pools are directly loaded onto a 300×47 mm reversed-phase column (15–30 μm, 300 Å, Vydac, Hesperia, CA, USA). Following sample application, the column is washed with two column volumes of solvent A (0.01 *M* HCl) and eluted in a 52.5 min linear gradient at 40 ml/min from 7% to 57% B (solvent B: 80% acetonitrile, 0.01 *M* HCl). Fractions of 50 ml are collected and aliquots are subjected to matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) as well as to a bioassay.

2.4. MALDI-MS

Aliquots (1 μl) of the samples from the reversed-phase chromatography are applied on a stainless steel multiple sample tray as a mixture with either sinapinic acid or α-cyanohydroxycinnamic acid using the dried drop technique [14]. Measurements are performed in linear mode with a LaserTec RBT MALDI-MS (Perseptive/Vestec, Houston, TX, 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 up to 64 laser shots are automatically accumulated per sample position. The time-of-flight data are externally calibrated for each sample plate and sample preparation. Data acquisition and analysis are performed using the software supplied by Perseptive Biosystems [GRAMS (386 level II, version 3.0, Galactic Industries, Salem, USA)]. Accuracy of mass measurement is within 0.5%.

2.5. Further liquid chromatography

The 300 fractions of the peptide bank are applied to an analytical reversed-phase (RP)-C_{18} column (5 μm, 300 Å, 250×10 mm, Vydac; flow-rate: 1.8 ml/min; solvent A: 0.1% trifluoroacetic acid; solvent B: 80% acetonitrile, 0.1% trifluoroacetic acid; gradient: linear gradient from 5 to 35% B in 60 min). Fractions of 1 min are collected and further purified using an analytical RP-C_{18} column (YMC-120-ODS-AQ; 3 μm, 100×0.5 mm, Schermbeck, Germany; flow-rate: 5 μl/min; solvent A: 0.1% trifluoroacetic acid; solvent B: 100% acetonitrile, 0.085% trifluoroacetic acid; gradient: linear gradient from 10 to 70% B in 120 min). After four steps of separation, including cation-exchange and reversed-phase chromatography, for generation of the peptide bank, the peptides are highly purified for sequence analysis.

2.6. Peptide sequence analysis

For automatic Edman degradation of pure peptides, a pulse-liquid sequencer (Applied Biosystems 473A or 494A) is used with a fast cycle program and a micro cartridge. Seven to 25 amino acid residues are sequenced to characterize the primary structure and confirm the peptide by a database search in the available protein and DNA databases.

2.7. Survival assay with neuronal cells

In the search for new proliferative and survival factors from the brain peptide bank, we used specific cellular assay systems for screening. Survival of the cells is analyzed by a viability assay of the rat adrenal pheochromocytoma cell line PC-12 [15,16]. Briefly, 7000 cells are plated in 96-well plates in serum-free RPMI 1640 (Sigma, Deisenhofen, Germany) containing 0.01% (v/v) penicillin/streptamycin, 1 m*M* L-glutamine, and aliquots of the porcine brain fractions are added. As positive controls nerve growth factor (NGF) (100 ng/ml), insulin-like growth factor-1 (IGF-I) (100 ng/ml), and 10% (v/v) fetal calf serum (FCS) are used. After incubation for 48 h at 37°C in a humidified atmosphere with 5% CO_2, WST-1-substrate (Boehringer Mannheim, Germany) is added and absorbance at 405 nm is measured by an enzyme-limked immunosorbent assay (ELISA) reader after 5 h.

3. Results and discussion

In order to systematically isolate regulatory peptides circulating in the blood stream, we previously introduced a peptide bank from human hemofiltrate [5]. Following different purification strategies, e.g., biological assays or random peptide sequencing, we have succeeded in isolating various peptides from this source over the last few years [6–8,17]. Even though circulating peptides represent an attractive target for isolation and further characterization, we extended this concept to generate alternative peptide banks to isolate targets not secreted in the extracellular/plasma compartment. Many neuromodulatory peptides synthesized in the brain act in locally restricted areas [18–21] and therefore do not appear in the plasma. Moreover, there is a controversy about the passage of peptidergic neuromodulators via the blood–brain barrier [22–24]. Recent isolations from brain tissue using the strategy of orphan receptor screening yielded the characterization of promising peptides [10–13]. These reasons incited us to establish the brain peptide bank using extraction and

purification strategies which are discussed in the following.

3.1. Tissue extraction

Immediately after dissection, the tissue is transferred into ice-cold acetic acid and homogenized. Extraction, including ultrafiltration, is subsequently carried out. Calculation of peptide/protein content is performed by amino acid analysis. A total amount of 40 g peptide/protein is extracted from the initial amount of 10 kg brain tissue. The wet mass of one porcine brain is 100 g and a total of 400 mg of peptide/protein content per brain is obtained. In contrast to our method, the initial step performed by another group [25] is the storage of the brain in liquid nitrogen followed by homogenization and centrifugation procedure. However, the peptide crude extract obtained by Karelin et al. [25] is comparable to ours.

3.2. Peptide bank generation

3.2.1. Cation-exchange chromatography

Isolation of regulatory peptides from the hemofiltrate peptide bank [5] as a proof of concept convinced us to apply a similar strategy to establish the brain peptide bank. Even though the compound composition in hemofiltrate and brain tissue is very different, the use of a strong cation exchanger proved to be suitable again for peptide extraction from brain. In order to check the binding and elution pattern of this procedure, analytical separation steps were carried out. The peptide concentrate from brain was diluted and acidified for binding to the cation-exchange column. An acceptable separation for acidic and basic peptides was achieved by stepwise elution using different pH buffers covering a pH spectrum from 2.6 up to 7.0. Based upon the findings from analytical experiments, we forwarded the peptide extract for the preparative approach also on the

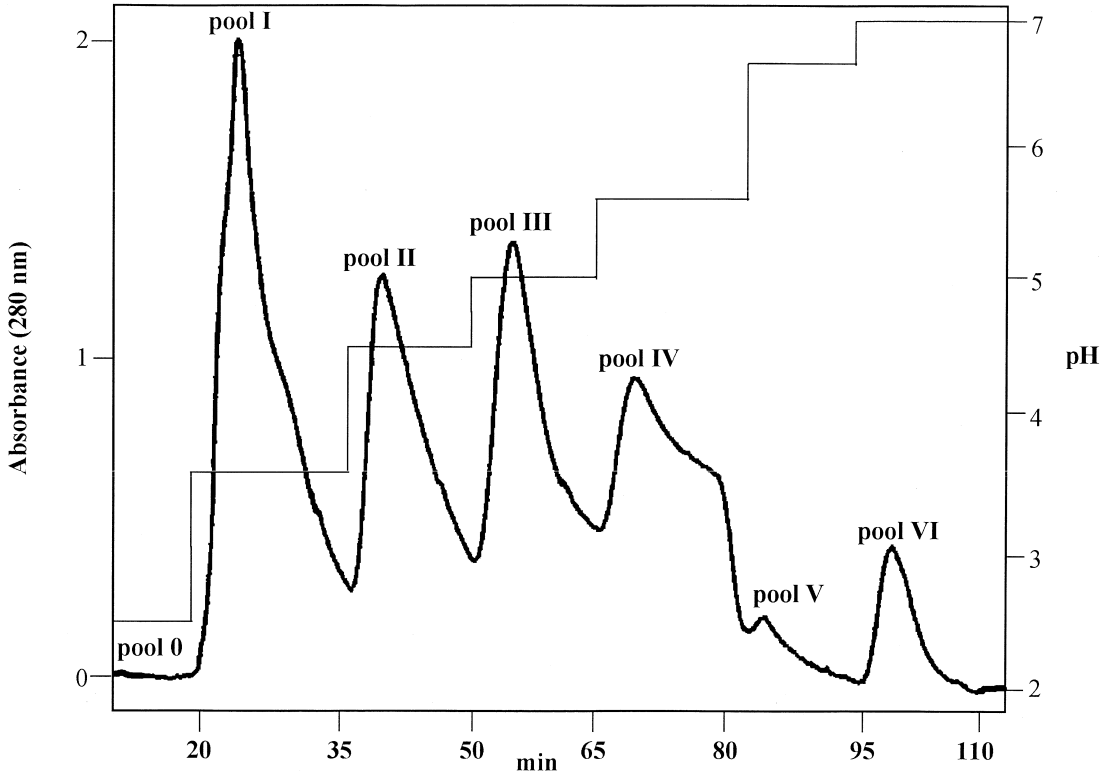

Fig. 1. Cation-exchange chromatography of the brain extract. Peaks 0 to VI represent the peptides of a batch of 10 kg brain tissue eluted with buffers of increasing pH as explained in Section 2.2.

cation-exchange resin. Stepwise elutions were performed and an elution profile as depicted in Fig. 1 was obtained.

3.2.2. Reversed-phase chromatography

The main purpose for subfractionation is to achieve a high ratio of specific activity/total protein content for regulatory peptides which are expressed in low concentrations. Further subfractionation reduces the likelihood of obscuring peptide activities due to the presence of functional antagonists or synergists in the identical fraction. Therefore, a widespread elution profile with an adequate separation has to be attempted. For our approach, the pH pools are fractionated using reversed-phase chromatography. Fractionation into 42 fractions per pH pool, as shown in Fig. 2, results in a total of about 300 salt-free fractions. After lyophilization, the generated fractions can be readily dissolved in aqueous solutions.

3.3. MALDI-MS

Following reversed-phase chromatography from all 300 fractions, MALDI-MS is performed and a mass database from the porcine brain peptides is generated. According to the composition and number of the peptides observed, we conclude that, similar to the hemofiltrate peptide bank [5], a typical pattern of masses is present. The number of detected peptides at this purification step (about 50–60 peptides) was comparable to that found in hemofiltrate [5], demonstrating the complexity of the peptide spectrum in the brain. Therefore, a further subfractionation is suitable to detect peptides present in the brain at low expression ranges. A typical MALDI-MS spectrum is shown in Fig. 3.

3.4. Isolation and identification of peptides

Over the last few years, we have developed the concept of peptide trapping for a systematic analysis

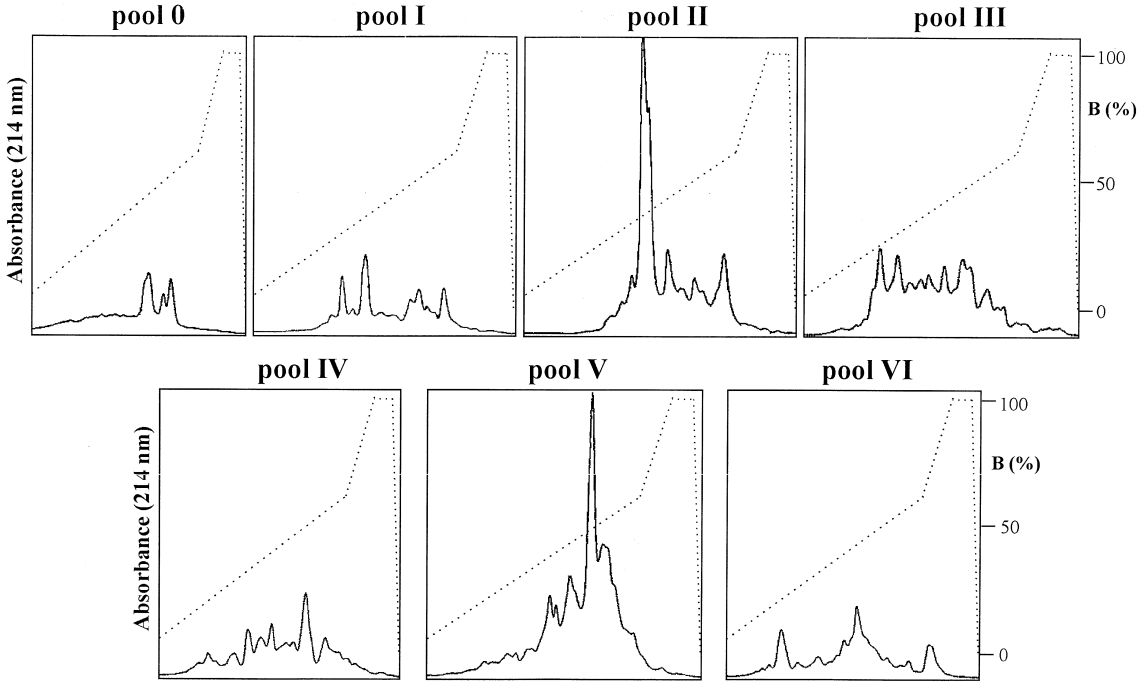

Fig. 2. Reversed-phase chromatography of the different pH pools. Numbers 0 to VI show the chromatograms and gradients of the individual pH pools. Forty-two fractions of 50 ml are collected and subsequently lyophilized. Approx. 300 fractions are used for peptide isolation according to the different strategies mentioned in the text. These fractions represent the peptide bank from porcine brain.

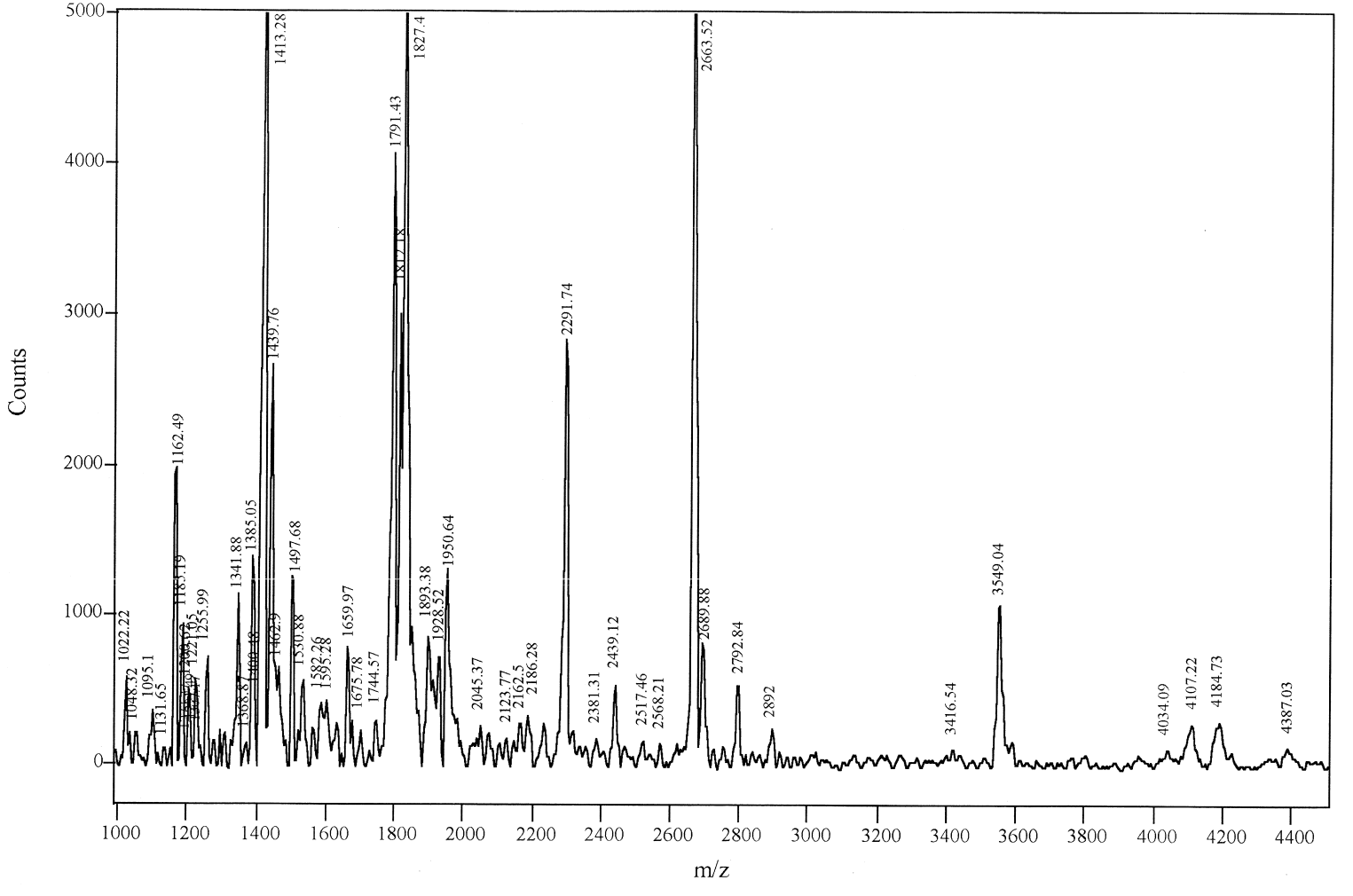

Fig. 3. Typical MALDI-MS spectrum of a brain peptide fraction 7 obtained from the RP chromatography of pH pool V.

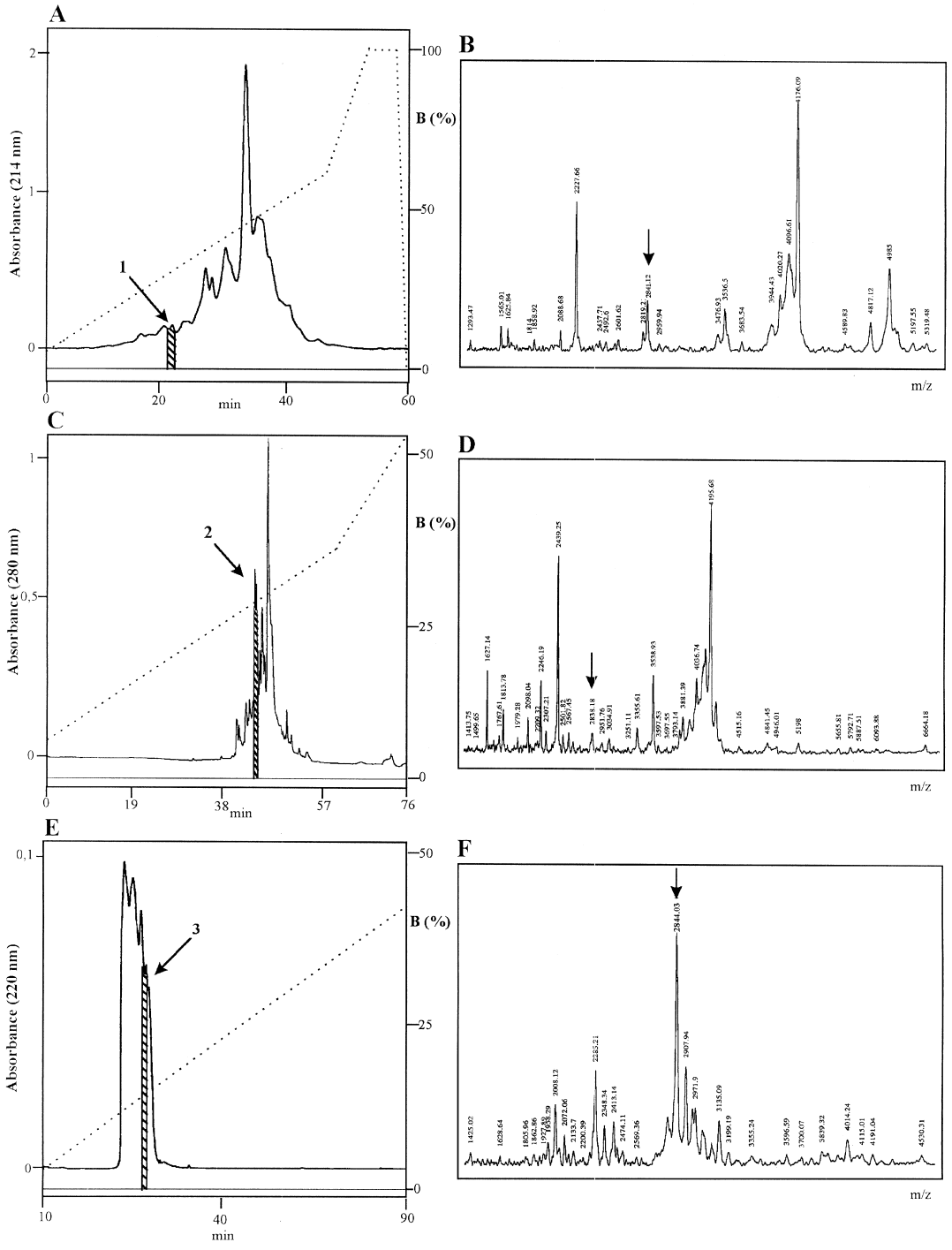

Fig. 4. Purification of profilin 1 from porcine brain guided by MALDI-MS analysis. (A) RP-HPLC fractionation of pH pool V. (B) MALDI-MS analysis of the marked fraction (No. 1 in A) reveals the mass of a suspected peptide (M_r 2841). Further separation of the identified fraction is performed by additional chromatographic steps. (C) Analytical RP-HPLC of fraction No. 1 (A). (D) MALDI-MS analysis of the marked fraction (No. 2 in C) reveals the mass of 2838. (E) Analytical RP-HPLC of fraction No. 2 (C). (F) MALDI-MS analysis reveals the mass (M_r 2844) of the peptide in fraction No. 3 (E). Sequence analysis indicates purification of a fragment of porcine profilin 1 (113–137). The molecular mass of 2844 is in accordance with the amino acid sequence obtained.

Table 1
Fragments of proteins isolated from porcine brain

	Amino acid sequence	Protein precursor and the position of the identified fragment	Content (pmol/g tissue)	Anal. mass (MALDI-MS)
	Neurospecific proteins			
1	EPYYSTSYKRRYVETPRVH	Neurofilament triplet L protein 6–24	1.0–3.0	2425
	Other functional proteins			
2	FVGGNWKMNGRKXN	Triosephosphate isomerase (macmu/rabbit) 7–20	1.0–3.0	1626
3	FVGGNWKMNGRKXNL	Triosephosphate isomerase (macmu/rabbit) 7–21	>5.0	1736
4	FVGGNWKMNGRKXNLG	Triosephosphate isomerase (macmu/rabbit) 7–22	1.0–3.0	1813
5	YKVIPKS	Endoplasmic reticulum protein (human/rat) 47–53	1.0–3.0	835
6	MGKEGVHGGMINKKXYEMASHLRRS	Profilin I (bovine) 113–137	1.0–3.0	2844
7	FQVKGRRVVRATEVPVS	Gelsolin 180–196	>10.0	1933
8	RTQLVSNLKKEGSSHN	Aspartate aminotransferase 355–370	>20.0	1798
9	TFSYGRALQASALKAWGGKKENLKAA-QEEYVKRALANSLACQGKYTPS	Fructose-bisphosphate aldolase (human) 298–346	1.0–3.0	5260
10	VKVGVNGFGRIGRLVTRAAFNSGKVD	Glycerinaldehyde 3-phosphate dehydrogenase 1–25	>10.0	2721
	Hemoglobin			
11	VLSAADKANVKAAWGKVGGQAGAHG	α-Globin 1–25	1.0–3.0	2364
12	FLGFPTTKTYFPHFNLSHGSDQV	α-Globin 33–55	1.0–3.0	2641
13	FLGFPTTKTY	α-Globin 33–42	1.0–3.0	1174
14	ARRLGHDFNPDVQA	β-Globin 115–128	1.0–3.0	2425

of circulating peptides [9]. Using the elution position in high-resolution capillary LC (RP-C_{18}) and their precise molecular mass as determined by MALDI-MS or electrospray MS, several thousand peptides were unambiguously identified in the hemofiltrate peptide bank. Some of them turned out to be bioactive fragments of plasma proteins [26,27] as well as novel peptides [6–8]. According to the strategy of isolating peptides following their mass detected by MALDI-MS [28], we also purified and isolated several peptides from the brain peptide bank as a model for future work.

Based upon the MALDI-MS data of the fractions generated from pH pool V (Fig. 4A), the peptide with the indicated mass (see the arrow in Fig. 4B (M_r 2841)] in fraction No. 1 is further purified. Pursuing this mass, we isolated a fragment of profilin I in a two-step isolation procedure. Using an analytical RP-C_{18} separation and an analytical capillary LC, the results shown in Fig. 4C and E are obtained. Fractions of each isolation step are subjected to MALDI-MS and the fraction containing the identified mass [fraction No. 2: Fig. 4D (M_r=2838); fraction No. 3: Fig. 4F (M_r=2844)] is purified further. Sequence analysis of fraction No. 3 resulted in the identification of a fragment of profilin I (Fig. 4F) exhibiting a homology of 96% to the bovine sequence. This protein is discussed to play a role in actin polymerization and depolymerization during axonal elongation [29]. Different mass measurements of the same fragment by MALDI-MS during the purification procedure are due to the detection accuracy as indicated above.

So far, this strategy has been effective for the isolation of further peptides from the brain peptide bank as summarized in Table 1.

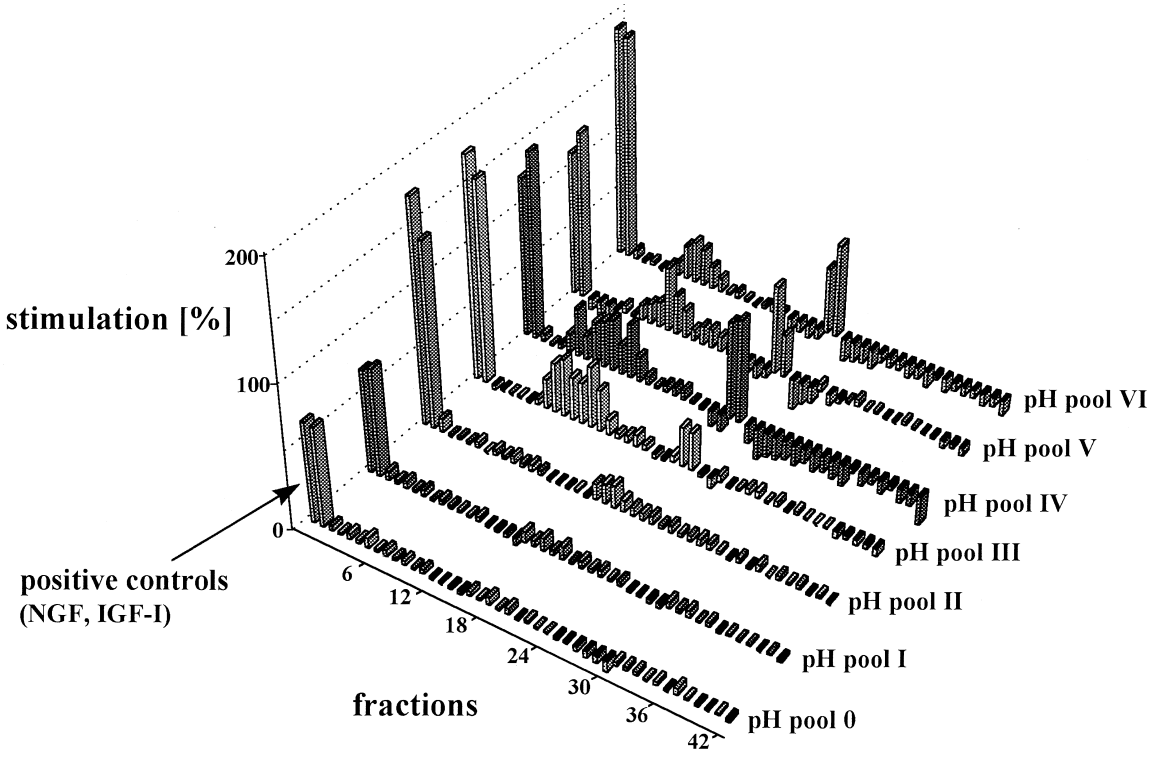

Fig. 5. Primary screen of the peptide bank fractions in a survival assay of a neuronal cell line (PC-12). This figure shows the activity (stimulatory and inhibitory) pattern of the RP fractions (1–42) of the respective pH pools (0–VI) tested. 100% represents the stimulation induced by 10% FCS (fetal calf serum) minus blank (cells treated with serum-free medium only). Abbreviations used: NGF: nerve growth factor; IGF-1: insulin-like growth factor-1.

3.5. Characterization of the biological activity of the brain peptide bank

In order to demonstrate the biological activity profile of the brain peptide bank, aliquots of the about 300 fractions were tested in the PC-12 survival assay. Fig. 5 shows the activity pattern of the RP fractions (1–42) of the respective pH pools (0–VI) tested. In summary, two main activity peaks are found. The first one starts with fraction 5 and lasts up to fraction 14 while the second activity peak comprises fractions 22 and 23. Both activities start basically with pH pool III up to VI. In contrast to the widespread activity pattern of the first peak, a sharp pattern is seen for the second one. Furthermore, not only stimulatory but also inhibitory activities are demonstrated in the primary screen as indicated e.g., in fractions 24–28 of pH pool IV. These results demonstrate that the fractions of the brain peptide bank contain bioactive peptides which modulate the survival capacity of PC-12 cells. Therefore, we conclude that the extraction procedure and the subsequent combination of cation-exchange and RP chromatography preserve the biological activity of the brain peptides. Meanwhile, the bioactive fractions are further purified in order to isolate and characterize the bioactive peptides.

4. Conclusions

We demonstrate the preparation and the characterization of the porcine brain peptide bank. Using a three-step purification procedure by means of ultrafiltration and cation-exchange combined with RP chromatography, several kilograms of brain tissue are fractionated following extraction. These fractions represent the brain peptide bank covering the specific peptide composition of the brain. Screening of the peptide bank by a survival assay reveals stimulatory as well as inhibitory effects, suggesting the biological activity of the extracted peptides. Furthermore, the strategy of peptide trapping using RP chromatography assisted by MALDI-MS results in the isolation of several peptides in two further purification steps.

From these data we conclude that this peptide bank can be used for the screening of biological activities in a variety of assay systems such as orphan receptor screening, a method by which orphanin FQ/nociceptin, orexin and prolactin-releasing peptide have been isolated in recent years.

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