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H. 知的所有権の取得状況

1. 特許取得

発明名称：	グレリン及びその誘導体又はGHS-R1aに作用する物質を有効成分とする脊髄神経修復促進治療剤
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発明名称：	デスアシルグレリン及びその誘導体を有効成分とする脊髄神経修復促進治療剤
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発明者（所属）：	村上　昇（宮崎大）、 中原桂子（宮崎大）、 橋本美穂（宮崎大）、 寒川賢治（国立循環器病センター）、 林友二郎（アスビオファーマ株式会社）

発 明 名 称： 成長ホルモン分泌促進因子レセプターに作用する物質または薬物的に許容される塩を有効成分とする糖尿病性神経障害治療剤
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林友二郎（アスビオファーマ株式会社）

発 明 名 称： グレリン及びその誘導体又は成長ホルモン分泌促進因子レセプター1a アゴニストを有効成分とする慢性呼吸器感染症治療剤
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発明者（所属）： 中里雅光（宮崎大学）、
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2. 実用新案登録
なし
3. その他
なし

研究成果の刊行に関する一覧表

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著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
なし							

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Purification and properties of ghrelin from the intestine of the goldfish, *Carassius auratus*

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ABSTRACT

In goldfish, intraperitoneal (IP) or intracerebroventricular (ICV) administration of synthetic ghrelin consisting of 12- or 19-amino-acid residues, deduced from its precursor cDNA, with an octanoic acid modification at the third N-terminal serine residue (Ser^3), stimulates growth hormone release and food intake. However, native ghrelin generated from its precursor has not yet been identified in this species. Therefore, we purified ghrelin from the goldfish intestine using acid extraction, cation-exchange and reverse-phase high-performance liquid chromatography combined with immune-affinity purification. In order to confirm ghrelin activity in the fractions at each purification step, we examined the effect of each fraction on intracellular Ca^{2+} mobilization in rat growth hormone secretagogue-receptor (GHS-R)-expressing cells. We characterized the goldfish ghrelin as 11 molecular forms consisting of 14-, 17-, 18- and 19-amino-acid residues with acylation at Ser^3, and the 17-residue form was predominant. We then synthesized 17-residue forms with octanoic acid modification (octanoyl ghrelin17) and without acylation (des-acyl ghrelin17) at Ser^3, and examined their biological activity. Octanoyl ghrelin17, but not des-acyl ghrelin17, increased the intracellular Ca^{2+} concentration in rat GHS-R-expressing cells with a potency similar to those of synthetic ghrelin consisting of 12 residues (octanoyl ghrelin12) and octanoyl rat ghrelin. IP and ICV administration of octanoyl ghrelin17 and octanoyl ghrelin12, but not des-acyl ghrelin17, increased food intake in goldfish. The present findings indicate that native goldfish ghrelin consists of 11 molecular variants, the major form being a 17-residue peptide. This dominant form with acylation is implicated in the regulation of food intake in goldfish.

1. Introduction

Ghrelin is a 28-amino-acid peptide identified in the rat and human stomach as an endogenous ligand for the growth hormone secretagogue-receptor (GHS-R) [14]. Ghrelin possesses an *n*-octanoic acid modification at the third N-terminal serine residue (Ser^3), and this fatty acid modification of ghrelin has been considered an essential structure for ghrelin's biological activity in mammals [14,24]. In rats, peripheral or central administration of ghrelin results in release of growth hormone (GH) from the pituitary gland [1,27,31]. Administration of ghrelin also increases food intake and body weight gain in rodents [25,31]. GHS-R is a member of the GTP-binding protein-coupled receptor superfamily with seven transmembrane domains. The GHS-R and its mRNA are present in the brain, pituitary, gastrointestinal tract, kidneys, pancreas and heart [4,19,23]. Ghrelin is now recognized as a multifunctional peptide involved in the stimulation of gastric acid secretion and motility, improvement of cardiovascular function and regulation of energy homeostasis in peripheral organs [2,5,10].

In goldfish, a cDNA encoding the ghrelin precursor has been identified [29]. Its deduced primary structure has several putative cleavage sites and amidation signals, and thus it has been anticipated that goldfish ghrelin is a 12- or 19-amino-acid residue amidated peptide with an *n*-octanoic acid modification at Ser^3. The biological activities of these putative goldfish ghrelins [29] have been examined in a goldfish model; their intraperitoneal (IP) or intracerebroventricular (ICV) administration stimulated release of GH and luteinizing hormone, food intake and locomotor activity [15,16,20,21,30]. However, goldfish ghrelin used in these studies was deduced from its precursor, and the native form of goldfish ghrelin has not yet been identified in the goldfish itself. In several species including humans and fish, the existence of ghrelin with different fatty acid modifications has been reported [3,7–9,11,12].

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Therefore, in the present study, in order to clarify the physiological role of native ghrelin, we purified native ghrelin from goldfish intestine, where ghrelin mRNA is highly expressed [16,29]. Furthermore, we examined the effect of synthetic goldfish ghrelin identified in this study using an intracellular calcium influx assay in cell lines expressing rat GHS-R, and on food intake in the goldfish after IP or ICV administration.

2. Materials and methods

2.1. Animals

Juvenile goldfish (*Carassius auratus*, 3–10 g body weight, BW) of both sexes were obtained commercially, and kept for 2 weeks under controlled light–dark conditions (12 L–12 D) in a temperature-regulated fish tank (20–24 °C) before use in the experiment. The fish were fed a commercially available granule diet (containing 32% protein, 4% dietary fat, 3% dietary fibre, 9% mineral and 8% water, and 44% other components; Tetragold, Tetra GmbH, Melle, Germany,) once a day at noon. All animal experiments were conducted in accordance with the University of Toyama guideline and the Declaration of Helsinki for the care and use of animals. Every effort was made to minimize the number of animals used and their suffering.

2.2. Tissue extraction

Ghrelin mRNA is expressed mainly in the intestine (given that goldfish lack a stomach) [16,28], and a total of 1,540 intestines (approximately 200 g tissue weight) were collected from goldfish of both sexes. Each dissected tissue was immediately rinsed, weighed, frozen in liquid nitrogen and stored at −80 °C until use. A crude tissue extract was prepared as described in previous reports of fish ghrelin purification [7–9,12]. Frozen tissues were boiled for 10 min in 5 volumes of Milli-Q-level water to inactivate intrinsic proteases. After cooling the boiled tissues were treated with 1 M acetic acid, and homogenized with a Polytron mixer. The homogenate was then centrifuged for 30 min at 15,500 × *g*. The supernatant were treated with cold acetone at a final concentration of 66% (v/v), and then the acetone was removed by evaporation. The supernatant was loaded onto a Sep-Pak C18 Vac 35 cc cartridge (Waters, Milford, MA, USA) equilibrated in 0.1% trifluoroacetic acid (TFA). The cartridge was washed with 25% acetonitrile containing 0.1% TFA, and the peptide component including ghrelin was eluted in 60% acetonitrile containing 0.1% TFA. The eluate was evaporated and lyophilized.

2.3. Purification procedure

Lyophilized crude extract was dissolved in 1 M acetic acid and adsorbed on a cation-exchange column of SP-Sephadex C-25 (H^+-form, GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, England) equilibrated in 1 M acetic acid. The adsorbed materials were eluted successively with 2 M pyridine (SP-II) and then with 2 M pyridine-acetic acid (pH 5.0) (SP-III). After Sep-PaK purification, lyophilized SP-III fraction was subjected to carboxymethyl (CM)-ion exchange high performance liquid chromatography (HPLC) on a column of TSK gel CM-2SW (7.8 mm × 300 mm, Tosoh, Tokyo, Japan) using a linear gradient from 10 mM ammonium formate containing 10% acetonitrile (pH 4.8) for 120 min at a flow rate of 2 ml/min. Fractions were collected every 2 min (4 ml/fraction) and aliquots of each fraction were subjected to an intracellular calcium influx assay using GHS-R- -expressing cells (CHO-GHSR62 cells) as described below [6,14].

According the elution profile, fractions containing ghrelin activity in CM-HPLC were divided into eight groups, and desalted using a Sep-Pak C18 cartridge. Each active fraction was dissolved in 0.1 M sodium phosphate buffer (pH 7.4) and applied to an anti-rat-ghrelin immunoglobulin (Ig) G immuno-affinity column. The column-bound materials were eluted with 60% acetonitrile containing 0.1% TFA. The eluate was appropriately evaporated and subjected to reverse-phase (RP)-HPLC on a Symmetry 300 C18 column (3.9 mm × 150 mm, Waters) equilibrated with 10% acetonitrile containing 0.1% TFA at a flow rate of 1 ml/min. A linear gradient elution from 10% to 60% acetonitrile containing 0.1% TFA was then performed for 40 min. Fractions (1 ml/tube) were collected 15 min after injection. Active fractions containing ghrelin activity were evaporated, and further purified with RP-HPLC on a diphenyl column (2.1 mm × 150 mm, 219TP5125, Vydac, Hesperia, CA, USA) equilibrated with 10% acetonitrile containing 0.1% TFA at a flow rate of 0.2 ml/min. A linear gradient elution from 10% to 60% acetonitrile containing 0.1% TFA was then performed for 40 min. Fractions were collected according to absorbance peaks. To analyze the peptide sequences, the isolated peptides were applied to a protein sequencer (model 494HT, Applied Biosystems, Foster City, CA, USA). The molecular weights of the isolated peptides were determined using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) (model 4800 MALDI TOF/TOF Analyzer, Applied Biosystems). Acylation patterns were determined by the difference between detected molecular mass and theoretical molecular mass calculated from the amino-acid sequence of each peptide [7–9,14].

2.4. Intracellular calcium influx assay in cell lines expressing rat GHS-R

In order to measure ghrelin activity in fractions at each purification step, we used the calcium-imaging method on CHO-GHSR62 cells as described previously [6,14]. Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) were measured using a fluorometric imaging plate reader (FLIPR^{tetra}; Molecular Devices, Sunnyvale, CA, USA). CHO-GHSR62 cells were plated at 5×10^4 cells/well in flat-bottom, black-wall 96-well plates (Corning Corstar Corp., Cambridge, MA, USA) and cultured for at least 18 h prior to the assay. Cells were loaded for 1 h with 4.4 μM Fluo-4-AM fluorescent indicator dye (Invitrogen Corp., Carlsbad, CA, USA) in an assay buffer [1× Hank's balanced salt solution, 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 250 μM probenecid (Sigma–Aldrich, St. Louis, MO, USA), 1% fetal calf serum (FCS)], and then washed 4 times with the assay buffer without FCS. Samples were dissolved in the assay buffer containing 0.001% Triton X-100 and subjected to FLIPR assay. Maximum fluorescence change over the baseline was used to determine agonist responses.

2.5. Peptides

A 17-residue peptide was found to account for the vast majority obtained during the purification. Accordingly, this 17-residue peptide possessing or lacking *n*-octanoic acid, respectively, was synthesized at the Peptide Institute Inc. (Osaka, Japan). A 12-residue peptide possessing *n*-octanoic acid was synthesized at the Biopharma Center of Daiichi Asubio Pharmaceuticals Inc. (Gunma, Japan) as previously described [17]. The synthetic peptides were purified by RP-HPLC on a Zorbax 300SB-C18 column (Agilent Technologies, Palo Alto, CA, USA) and the quality of the synthetic peptides was analyzed by mass spectrometry (HP 1100 series LC/MSD, Agilent Technologies). Rat ghrelin was purchased commercially (Peptide Institute Inc., Osaka, Japan).

2.6. Effects of synthetic ghrelin on food intake in a goldfish model

Detailed methods for evaluating the feeding behavior of goldfish have been reported elsewhere [15,16,20,21]. Briefly, 2 h

prior to starting the experiments at noon, each fish was supplied with food equivalent at 1% of its BW. For IP administration of goldfish ghrelin, fish were injected with synthetic goldfish ghrelin at 10 pmol/g BW. Fish in the control group were given injections of the same volume of saline. For ICV administration of ghrelin, each fish was placed in a stereotaxic apparatus under anesthesia with 2 mM MS-222 (3-aminobenzoic acid ethyl ester, Sigma–Aldrich). A small part of the parietal bone was carefully removed using a surgical blade (No. 19, Futaba, Tokyo, Japan), and 1 μl of ghrelin at 1 pmol/g BW was injected into the third ventricle of the brain using a small Hamilton syringe. The bone gap was then filled with a surgical bonding agent (Aron Alpha, Sankyo, Japan). The accuracy of the injection site was confirmed after the experiment by examining the localization of Evans blue dye present in the ventricle. Control fish were injected with the same volume of saline in the same way as in the experimental group. Each fish that had received IP or ICV injection of ghrelin or saline was also placed in a small experimental tank (diameter 24 cm) filled with 3.0 l of tap water, and supplied with food equivalent to 3% BW. Food intake was then measured every 15 min during the 60-min period following treatment.

2.7. Data analysis

Cumulative food intake was represented as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Bonferroni's method. Statistical significance was determined at the 5% level.

3. Results

3.1. Purification of goldfish ghrelin

Fig. 1 shows the CM-HPLC profile and the distribution of ghrelin activity in its fractions. Ghrelin activity, evaluated by intracellular calcium assay using CHO-GHSR62 cells, was present in the fraction indicated by the black columns. In accordance with the ghrelin activity, fractions in CM-HPLC were divided into eight groups on the basis of retention time. Each active group was loaded onto an anti-rat-ghrelin IgG immuno-affinity column, and separated by a Symmetry 300 C18 column (Fig. 2). Each active fraction was next subjected to successive RP-HPLC on a diphenyl column to test its uniformity and to isolate it as a single peak (Fig. 3). A representative fraction showing ghrelin activity was analyzed using a protein sequencer, but the amino-acid residue at the N-terminal third position was not determined, due to acyl modification as seen in other fish ghrelins [7–9,12]. All the sequence thus determined were identical to the ghrelin sequences deduced from a goldfish cDNA encoding the ghrelin precursor [29]. Therefore we concluded that the unknown amino-acid residue is the serine residue. Next, the molecular masses of all isolated peptides were measured by MALDI-TOF mass spectrometry, and the molecular forms including the fatty acid modifications were predicted by the differences in the molecular weights deduced from the expected amino-acid sequence. The isolated ghrelins were composed of 14-, 17-, 18- and 19-amino-acid residues (Fig. 4), and it was presumed that these peptides were modified by saturated *n*-octanoic acid, *n*-nonanoic acid or *n*-decanoic acid, and their unsaturated forms (Table 1). In total, we were able to obtain 380 pmol of the 17-residue form, 178 pmol of the 18-residue form, 88 pmol of the 14-residue form, and 78 pmol of the 19-residue form.

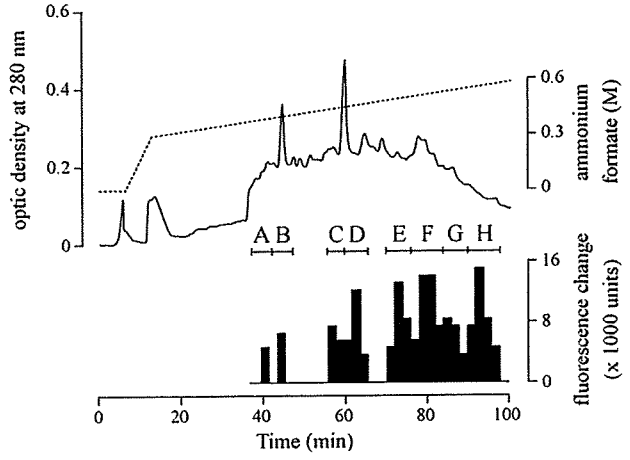

Fig. 1. Elution profile of SP-Sephadex C-25-bound substances (SP-III) on a cation-exchange column (TSK gel CM-2SW) with a linear gradient of ammonium formate (10 mM–0.6 M). Ghrelin activities in the fractions were measured using the calcium-imaging method on growth hormone secretagogue receptor-expressing cells, and are indicated as black columns in the panel. Active fractions containing ghrelin activities were divided into eight groups (A–H) according to their retention times. Each group was then subjected to anti-rat-ghrelin IgG immuno-affinity chromatography.

3.2. Comparison of retention time between synthetic and native ghrelins

The 17-residue form accounted for the overwhelming majority of the ghrelins isolated. We synthesized the 17-residue form with octanoic acid modification, or without acylation at Ser^3 (octanoyl ghrelin17 and des-acyl ghrelin17, respectively), to examine their biological activity. Fig. 5 shows the RP-HPLC profiles and a comparison of the retention times between native and synthetic ghrelin. Synthetic and native *n*-octanoyl ghrelin17 s were eluted at the same retention time (Fig. 5).

3.3. Effects of synthetic goldfish ghrelin determined by intracellular calcium influx assay in cell lines expressing rat GHS-R

We examined the ability of synthesized goldfish ghrelin to stimulate the rat GHS-R using CHO-GHSR62 cells. Octanoyl ghrelin17 over a concentration rage of 10^{-9} to 10^{-6} M increased $[Ca^{2+}]_i$ in a dose-dependent manner with a potency similar to ghrelin12 and rat ghrelin (Fig. 6). On the other hand, under the same conditions, des-acyl ghrelin17 had no effect at any dose on these cells.

3.4. Effects of IP and ICV administrations of synthetic goldfish ghrelin on food intake in goldfish

IP injection of octanoyl ghrelin17 or octanoyl ghrelin12 (10 pmol/g BW) induced a significant increase in food intake for 60 min (Fig. 7A). On the other hand, no changes were observed after i.p. injection of des-acyl ghrelin17. ICV injection of octanoyl ghrelin17 or octanoyl ghrelin12 (1 pmol/g BW) also induced a significant increase in food intake for 60 min (Fig. 7B). On the other hand, no changes were observed after i.c.v. injection of des-acyl ghrelin17.

4. Discussion

To our knowledge, this is the first report to have documented the purification and characterization of ghrelin from the intestine of the goldfish. Purification of goldfish ghrelin was carried out in accordance with procedures that have already been applied for the isolation of ghrelin from various other teleosts, including the Japanese eel, rainbow trout, tilapia and channel catfish [7–9,12]. During the course of purification, ghrelin activity was monitored by reference to changes in $[Ca^{2+}]_i$ in rat GHS-R-expressing mammalian

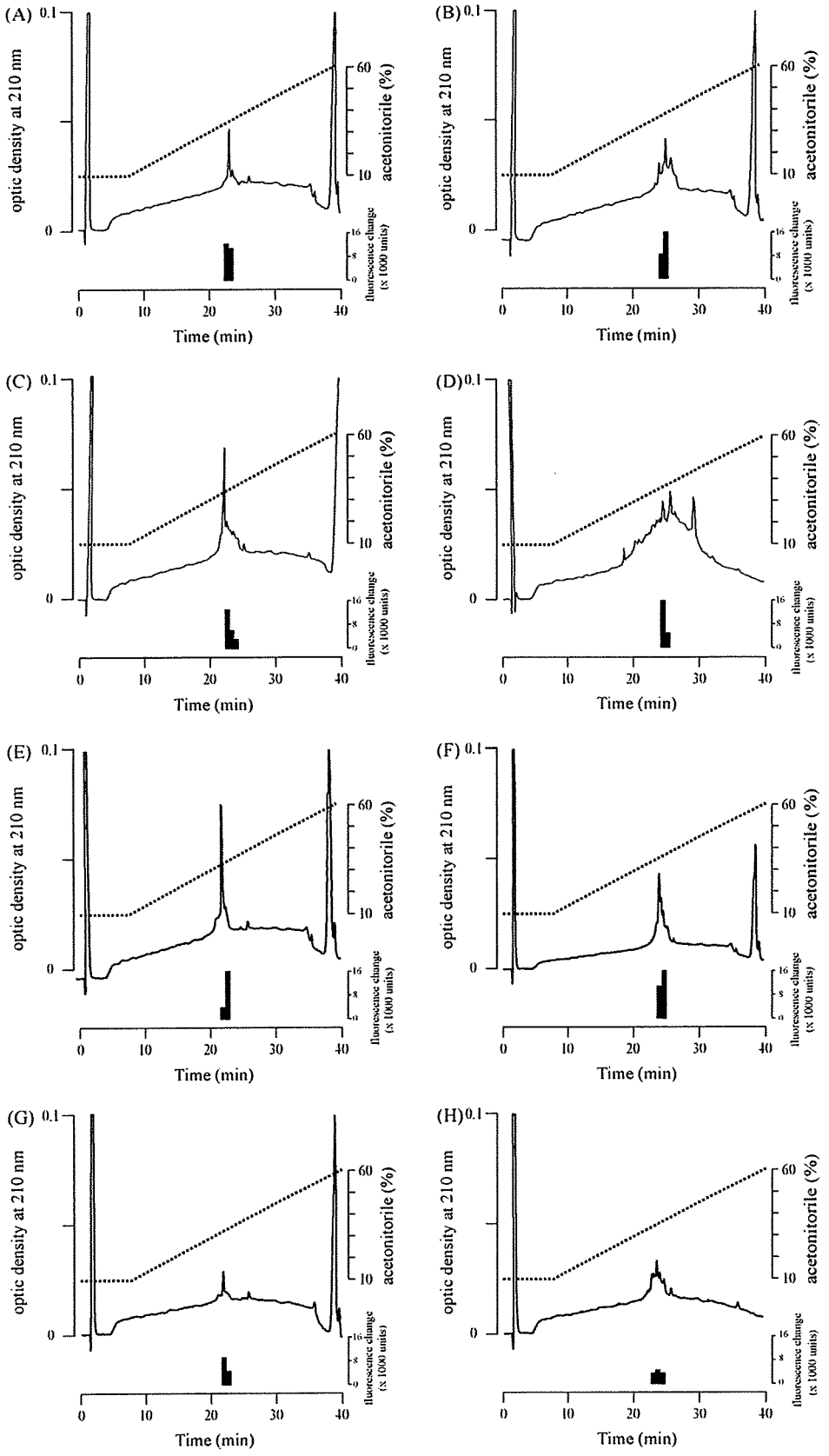

Fig. 2. Elution patterns of eight groups (A–H) obtained by immuno-affinity chromatography on a reverse-phase HPLC column (Symmetry 300 C18) with a linear gradient of acetonitrile (10–60%) containing 0.1% TFA. Ghrelin activities in the fractions were measured, and are indicated as black columns in the panel.