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Supporting Information

Seleno-relaxin analogues: Effect of internal and external diselenide bonds on the foldability and a fibrosis-related factor of endometriotic stromal cells

Yuri Satoh,[a] Yosuke Ono,[b] Rikana Takahashi,[a] Hidekazu Katayama,[c] Michio Iwaoka,[a,d] Osamu Yoshino[b] and Kenta Arai*[a,d]

[a] Department of Chemistry, School of Science, Tokai University, Kitakaname, Hiratsuka-shi, Kanagawa 259-1292, Japan

[b] Department of Obstetrics and Gynecology, University of Yamanashi, 1110 Shimokato, Chuo-shi, Yamanashi 409-3898, Japan

[c] Department of Bioengineering, School of Engineering Kitakaname, Hiratsuka-shi, Kanagawa 259-1292, Japan

[d] Institute of Advanced Biosciences, Tokai University, Kitakaname, Hiratsuka-shi, Kanagawa 259-1292, Japan

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1. Experimental

1.1 General

Matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF)-mass was recorded using a JMS-S3000 spectrometer (JEOL Ltd., Tokyo, Japan) under a positive mode. A part of syntheses of peptides was conducted by using a Biotage Initiator+Alstra, an automated microwave peptide synthesizer (Biotage Japan, Tokyo, Japan). The synthetic peptides were quantified by amino acid analysis (AAA), which was performed using a LaChromamino acid analyzer (Hitachi, Tokyo, Japan) after hydrolysis with a 6 M HCl solution at 150 °C for 2 h in a vacuum-sealed tube. HPLC analysis of the sample solutions obtained from oxidative chain assembly, reductive unfolding, and stability assessment in human serum were performed with a SHIMADZU HPLC Prominence Series (SHIMADZU, Kyoto, Japan) or HITACHI HPLC Chromaster (HITACHI, Tokyo, Japan) equipped with a 1 mL sample solution loop and a reverse-phase (RP) column (TSKgel ODS-100V φ 4.6 × 150 mm: Tosoh [Tokyo, Japan]) equilibrated with 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile (eluent B) at a flow rate of 1.0 mL min^{-1}. Synthesized peptides were purified with a HITACHI HPLC Chromaster (HITACHI, Tokyo, Japan) equipped with a 3 mL sample solution loop and a RP-column (ODS-HL φ10 × 250 mm: GL science [Tokyo, Japan]) equilibrated with eluent A and eluent B at a flow rate of 4.7 mL min^{-1}. Circular dichroism (CD) spectra were recorded with a J-820 spectropolarimeter (JASCO Corporation, Tokyo, Japan) over a wavelength range of 260–190 nm, using a scan speed of 50 nm min^{-1} and bandwidth of 1 nm. Glutathione (GSH and GSSG) and DL-dithiothreitol (DTT^{red}) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Recombinant human relaxin-2 (rH2 relaxin) as a standard sample was purchased from Merck Japan (Tokyo, Japan) and used without purification. Pooled human serum was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). ELISA assay kit of H2 relaxin was purchased from abcam (Cambridge, UK) and R&D Systems, Inc. (Minneapolis, USA). Peptide **1**,[1] 2-aminoethyl methanethiosulfonate (AEMTS),[2] Fmoc-Sec(MPM)-OH,[3] and Boc-Thr(Fmoc-Ser(^{t}Bu))-OH[4] were synthesized by the literature methods. Human endometriotic stromal cells (ESCs) were purified from surgical specimens and cultured as described previously.[5] Dulbecco's Modified Eagle Medium (DMEM)/F12 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Life Technologies (Minato-ku, Tokyo, Japan). All other general reagents were commercially available and used without further purification. All experiments were performed in accordance with relevant guidelines and regulations.

*1.2 SPPS of peptide **2***

A standard solid-phase peptide synthesis (SPPS) protocol using *N*,*N*'-dicyclohexylcarbodiimide

(DCC) as a condensing reagent with 1-hydroxybenzotriazole (HOBt) was employed. Fmoc-Ser(^{t}Bu)-Alko resin (63.3 mg, 0.05 mmol) having 0.79 mmol g^{-1} of substituent was swelled with *N*,*N*-dimethyl formamide (DMF) over 15 h at 4 °C. After removing the solvent, the resin was treated with 20% piperidine in DMF (2 mL) for 5 min with vortex mixing. After the Fmoc-deprotection, the reaction was repeated with fresh 20% piperidine in DMF (2 mL) for 15 min, and then the resin was fully washed with DMF (×5). Fmoc-Trp(Boc)-OBt solution (600 μL), which was prepared by mixing Fmoc-Trp(Boc)-OH (105 mg, 0.2 mmol), 0.5 M HOBt/DMF (300 μL), and 0.5 M DCC/DMF (300 μL) for 30 min, was added to the resin. The mixture was vortexed for 60 min at 50 °C. After the coupling, the resin was washed with 50% MeOH/DCM (2 mL × 3). The reaction progression was monitored by a Kaiser test. After the coupling, the resin was washed with DMF (2 mL × 3). The unreacted amino groups on the resin were acetylated by treating a DMF solution (3 mL) containing 10% Ac_2O and 5% diisopropylethylamine (DIPEA) for 5 min, and then the resin was fully washed with DMF (2 mL × 5). Similarly, at Ser26-Thr27, Boc-Thr(Fmoc-Ser(tBu))-OH was then conjugated with the resin instead of normal amino acid derivatives. Applying the same protocol, the peptide chain was elongated on the resin, and the N-terminal Fmoc group was finally deprotected by 20% piperidine in DMF. The obtained resin was fully washed with 50% MeOH/DCM (2 mL × 3) and DCM (2 mL × 3) and dried in vacuo to yield Asp(O^{t}Bu)-Ser(^{t}Bu)-Trp(Boc)-Met-Glu(O^{t}Bu)-Glu(O^{t}Bu)-Val-Ile-Lys(Boc)-Leu-Cys(Trt)-Gly-Arg(Pbf)-Glu(OtBu)-Leu-Val-Arg(Pbf)-Ala-Gln(Trt)-Ile-Ala-Ile-Cys(Trt)-Gly-Met-Ser-Thr-Trp(Boc)-Ser(^{t}Bu)-Alko resin (266 mg) as a yellow material. Ser-Thr represents *O*-AIP unit having *O*^{t}Bu- and *N*Boc-protecting groups. A portion of the obtained resin (50 mg, 9.40 μmol) was treated with a TFA cocktail (trifluoroacetic acid (TFA) : H_2O : phenol : thioanisole (TA) : 1,2-ethanedithiol (EDT) : dimethyl sulfide (DMS) : NH_4I = 83.5 : 3 : 5 : 5 : 2 : 1.5, v/v/v/v/v/v/v, 1.8 mL), and the mixture was stirred for 2 h at room temperature. After the removal of TFA by N_2 stream, the deprotected peptide was precipitated with an excess of Et_2O, washed with Et_2O (×3) and dried in vacuo. The resulting crude peptide was purified by using HPLC, which was equipped a 3 mL sample solution loop and a RP column (ODS-HL φ10 × 250 mm [GL science, Tokyo, Japan]), at a flow rate of 4.7 mL min^{-1}. After injecting the sample solution, the ratio of eluent B was increased linearly from 30% to 50% in 0–10 min, 50% to 60% in 10–20 min, and 60% to 80% in 20–23 min. The corrected fraction containing the target peptide was lyophilized to yield peptide **2** (see Fig. 2 in the main text) as a white powder (329 nmol, 3.5%). MALDI-TOF-MS (m/z) found: 3314.5, calcd for $[M+H]^+$: 3313.9. AAA: $Asp_{0.94}Thr_{0.82}Ser_{2.42}Glu_{4.06}Gly_{2}Ala_{2.12}Val_{1.66}Met_{1.72}Ile_{2.30}Leu_{1.94}Lys_{1.06}Arg_{1.86}$.

*1.3 SPPS of selenopeptide **3***

Selenopeptide **3** was prepared by a method similar to the synthesis of peptide **2**. Using H-Cys(Trt)-Trt(2-Cl)-resin (169 mg, 0.12 mmol) having 0.71 mmol g^{-1} of substituent as a starting material, Fmoc-His(Trt)-Val-Gly-Cys(Trt)-Thr(tBu)-Lys(Boc)-Arg(Pbf)-Ser(tBu)-Leu-Ala-Arg(Pbf)-Phe-Cys(Trt)-Trt(2-Cl)-resin was yielded by using an automated microwave peptide synthesizer. Subsequently, the obtained resin was manually treated with 20% piperidine in DMF to remove the N-terminal Fmoc group and then fully washed with DMF. Fmoc-Sec(MPM)-OBt solution (360 μL), which was prepared by mixing Fmoc-Sec(MPM)-OH (122 mg, 240 μmol), DIC (55.7 μL, 360 μmol) and 1 M HOBt in DMF (360 μL) for 30 min at room temperature, was added to the resin, and the mixture was vortexed for 30 min at 50 °C. DIC (55.7 μL, 360 μmol) was added to the mixture solution again, and the resulting mixture was further incubated with mixing for 30 min at 50 °C. The unreacted amino groups on the resin were acetylated by treating a DMF solution (3 mL) containing 10% Ac_2O and 5% DIPEA for 5 min, and then the resin was fully washed with DMF (2 mL × 5). For obtained resin, applying the DCC-HOBt system in DMF as described in section 1.2, amino acid derivatives were sequentially conjugated. The resulting resin was treated with 20% piperidine in DMF, fully washed with 50% MeOH/DCM and DCM, and dried in vacuo to yield Gln(Trt)-Leu-Tyr(^{t}Bu)-Ser(^{t}Bu)-Ala-Leu-Ala-Asn(Trt)-Lys(Boc)-Cys(Trt)-Sec(MPM)-His(Trt)-Val-Gly-Cys(Trt)-Thr(^{t}Bu)-Lys(Boc)-Arg(Pbf)-Ser(^{t}Bu)-Leu-Ala-Arg(Pbf)-Phe-Cys(Trt)-Trt(2-Cl)-resin (1386 mg). A portion of the obtained resin (50 mg, 4.33 μmol) was treated with a TFA cocktail (TFA : H_2O : triisopropylsilane (TIS): 2,2'-dipyridyl disulfide (DPDS) = 90 : 2.5 : 2.5 : 5, v/v/v/v, 0.82 mL), and the mixture was stirred for 2 h at room temperature. After the removal of TFA by N_2 stream, the deprotected peptide was precipitated with an excess of Et_2O, washed with Et_2O (×3), and dried in vacuo. The obtained crude product was dissolved in 25 mM sodium bicarbonate buffer solution containing 1 mM DL-dithiothreitol (DTT^{red}) and 2 M urea at pH 10.0 (2.0 mL), and the solution was incubated at 25 °C for 24 h. The mixture was diluted to 20 mL with 0.1% TFA in water. The resulting crude peptide was purified by using RP HPLC, which was equipped a 3 mL sample solution loop and a RP-column (ODS-HL φ10 × 250 mm [GL science, Tokyo, Japan]), at a flow rate of 4.7 mL/min. After injecting the sample solution, the ratio of eluent B was increased linearly from 20% to 43% in 0–20 min. The corrected fraction containing the target peptide was lyophilized to yield selenopeptide **3** (see Fig. 2 in the main text) as a white powder (350 nmol, 8.1%). MALDI-TOF-MS (m/z) found: 2718.1, calcd for $[M+H]^+$: 2718.2. AAA: $Asp_{1.05}Thr_{1.00}Ser_{1.84}Glu_{1.07}Gly_{1}Ala_{3.28}Val_{0.97}Leu_{3.16}Tyr_{1.04}Phe_{1.10}Lys_{2.32}His_{1.07}Arg_{2.15}$.

*1.4 SPPS of selenopeptide **4***

By applying synthetic protocol for peptide **2**, Fmoc- Gly-Arg(Pbf)-Glu(OtBu)-Leu-Val-Arg(Pbf)-Ala-Gln(Trt)-Ile-Ala-Ile-Cys(Trt)-Gly-Met-Ser-Thr-Trp(Boc)-Ser(^{t}Bu)-Alko resin was first obtained from Fmoc-Ser(*t*Bu)-Alko resin (63.5 mg, 0.05 mmol) having 0.79 mmol g^{-1} of substituent as a starting material. To couple Sec residue to the peptide on the resin, Fmoc-Sec(MPM)-OH (51 mg, 100 μmol) was reacted with the resin in a same manner applied to preparation of peptide **3**. For obtained resin, applying the DCC-HOBt system in DMF described as section 1.2, amino acid derivatives were sequentially conjugated. The resulting resin was treated with 20% piperidine in DMF, fully washed with 50% MeOH/DCM and DCM, and dried in vacuo to yield Asp(O^{t}Bu)-Ser(^{t}Bu)-Trp(Boc)-Met-Glu(O^{t}Bu)-Glu(O^{t}Bu)-Val-Ile-Lys(Boc)-Leu-Sec(MPM)-Gly-Arg(Pbf)-Glu(OtBu)-Leu-Val-Arg(Pbf)-Ala-Gln(Trt)-Ile-Ala-Ile-Cys(Trt)-Gly-Met-Ser-Thr-Trp(Boc)-Ser(^{t}Bu)-Alko resin (236 mg) as a yellow material. A portion of the obtained resin (50 mg, 10.6 μmol) was treated with a TFA cocktail (TFA : H_2O : phenol : TIS : DPDS = 90 : 2.5 : 5 : 2.5 : 5, v/v/v/v, 2.7 mL), and the mixture was stirred for 2 h at room temperature. After the removal of TFA by N_2 stream, the deprotected peptide was precipitated with an excess of Et_2O, washed with Et_2O (×3) and dried in vacuo. The resulting crude peptide was purified by using RP-HPLC. HPLC conditions were same as those for purification of peptide **2**. The corrected fraction containing the target peptide was lyophilized to yield selenopeptide **4** (see Fig. 2 in the main text) as a white powder (307 nmol, 2.9%). MALDI-TOF-MS (m/z) found: 3578.6, calcd for [M+H]$^+$: 3578.5. AAA: Asp$_{0.93}$Thr$_{0.76}$Ser$_{2.30}$Glu$_{3.92}$Gly$_{2}$Ala$_{2.06}$Val$_{1.66}$Met$_{1.86}$Ile$_{2.16}$Leu$_{1.77}$Lys$_{1.41}$Arg$_{1.81}$.

1.5 SPPS of selenopeptide ***5***

By applying synthetic protocol for selenopeptide **3**, Gln(Trt)-Leu-Tyr(^{t}Bu)-Ser(^{t}Bu)-Ala-Leu-Ala-Asn(Trt)-Lys(Boc)-Cys(Trt)-Sec(MPM)-His(Trt)-Val-Gly-Cys(Trt)-Thr(^{t}Bu)-Lys(Boc)-Arg(Pbf)-Ser(^{t}Bu)-Leu-Ala-Arg(Pbf)-Phe-Cys(Trt)-Trt(2-Cl)-resin (144 mg) as a yellow material was yielded from H-Cys(Trt)-Trt(2-Cl)-resin (141 mg, 0.10 mmol) having 0.71 mmol g^{-1} of substituent as a starting material. A portion of the obtained resin (50 mg, 34.7 μmol) was treated with a TFA cocktail (TFA : H_2O : TIS : DPDS = 90 : 2.5 : 2.5 : 5, v/v/v/v, 5 mL), and the mixture was stirred for 2 h at room temperature. After the removal of TFA by N_2 stream, the deprotected peptide was precipitated with an excess of Et_2O, washed with Et_2O (×3) and dried in vacuo. The resulting crude peptide was purified by using RP HPLC. HPLC conditions were same as those for purification of selenopeptide **3**. The corrected fraction containing the target peptide was lyophilized to yield selenopeptide **5** (see Fig. 2 in the main text) as a white powder (451 nmol, 1.3%). MALDI-TOF-MS (m/z) found: 2985.1, calcd for [M+H]$^+$: 2986.2. AAA: Asp$_{0.91}$Thr$_{0.88}$Ser$_{1.64}$Glu$_{0.89}$Gly$_{1}$Ala$_{2.74}$Val$_{0.91}$Leu$_{2.76}$Tyr$_{0.89}$Phe$_{0.99}$Lys$_{2.58}$His$_{0.99}$Arg$_{1.99}$.

1.6. Oxidative chain assembly of relaxin analogues

Synthetic A-chain analogue (peptide **1**, **3**, or **5**; 160 nmol) was dissolved in a sodium bicarbonate buffer (25 mM, pH 10.0, 150 μL) containing urea (1.5 M) and EDTA (1 mM). Similarly, B-chain analogue (peptide **2** or **4**; 160 nmol) was also dissolved in the same buffer solution (150 μL). For preparation of H2 relaxin, the solution of peptide **1** was added to the solution of peptide **2**, and the resulting mixture (300 μL) was mixed with 2.86 mM GSH (350 μL) and 0.57 mM GSSG (350 μL), which were prepared by a sodium bicarbonate buffer solution (25 mM, pH 10.0) without urea. For preparation of SeRlx-α, the solution of peptide **3** was added to the solution of peptide **4**, and the resulting mixture (300 μL) was mixed with 6.86 mM DTT^{red} (700 μL), which were prepared by a sodium bicarbonate buffer solution (25 mM, pH 10.0) without urea. For preparation of SeRlx-β, the solution of peptide **5** was added to the solution of peptide **2**, and the resulting mixture (300 μL) was mixed with 6.86 mM DTT^{red} (700 μL), which were prepared by a sodium bicarbonate buffer solution (25 mM, pH 10.0) without urea. The obtained sample solution (1000 μL) was incubated at 4 °C. To monitor the reaction progress, small aliquots (5 μL) were taken from the mixture, quenched by addition of aqueous AEMTS solution (7 mg mL^{-1}, 200 μL), diluted with a 20 % acetonitrile in water containing 0.1 % TFA (835 μL), and analyzed by RP HPLC system equipped with a sample solution loop (1 mL) and a Tosoh TSKgel ODS-100V φ 4.6 ×150 mm RP column (Tosoh, Japan), which was equilibrated with a 80:20 (v/v) mixture of eluents A and B at a flow rate of 1 mL min^{-1}. After injection of the sample solution (1.0 mL) into the HPLC system, a solvent gradient was applied: a ratio of eluent B linearly increased from 20 to 30% in 0–10 min, from 30 to 53% in 10–17.5 min, and from 53 to 90% in 17.5–19 min. The products during the folding were detected by the absorbance at 220 nm. After completion of the reaction, generated relaxin analogues were isolated by the same HPLC system and lyophilized to obtain as white powders.

Characterization of H2 relaxin. HPLC yield: 47%; Isolated yield: 30.4 nmol (19%); MALDI-TOF-MS (m/z) found: 5981.1, calcd for $[M+H]^+$: 5981.8. AAA: $Asp_{2.00}Thr_{1.77}Ser_{4.26}Glu_{5.01}Gly_{3}Ala_{5.24}Val_{2.61}Met_{1.70}Ile_{2.50}Leu_{5.00}Tyr_{1.08}Phe_{1.09}Lys_{2.86}His_{0.91}Arg_{3.94}$.

Characterization of SeRlx-α. HPLC yield: 73%; Isolated yield: 44.9 nmol (28%); MALDI-TOF-MS (m/z) found: 6074.9, calcd for $[M+H]^+$: 6075.9. AAA: $Asp_{1.97}Thr_{1.72}Ser_{4.16}Glu_{5.12}Gly_{3}Ala_{5.22}Val_{2.57}Met_{1.99}Ile_{2.48}Leu_{5.01}Tyr_{1.04}Phe_{1.00}Lys_{3.28}His_{0.96}Arg_{4.44}$.

Characterization of SeRlx-β: HPLC yield: 34%; Isolated yield: 27.1 nmol (17%); MALDI-TOF-MS (m/z) found: 6074.9, calcd for $[M+H]^+$: 6076.0. AAA: $Asp_{1.96}Thr_{1.70}Ser_{4.24}Glu_{5.09}Gly_{3}Ala_{4.98}Val_{2.81}Met_{1.83}Ile_{2.41}Leu_{4.85}Tyr_{0.96}Phe_{0.96}Lys_{3.66}His_{0.98}Arg_{3.84}$.

1.7. Tryptic digestion of relaxin analogues

A folded relaxin analogue (4 nmol) was dissolved in 100 mM Tris-HCl buffer solution (25 μL) containing 1 mM EDTA at pH 8.0. To the peptide solution was added with trypsin solution (1.0 mg mL^{-1}, 2.5 μL) in 10 mM HCl, and the reaction was progressed at 37 °C for 30 min. An aliquot (5 μL) from the sample solution was acidified by addition of 0.1% TFA in water (1045 μL) to quench the reaction. The obtained sample solution was analyzed by RP HPLC. The analytical conditions were same as those applied for the oxidative chain assembly of relaxin analogues (see section 1.6.). Eluted solution containing peptide fragments during the HPLC analysis was collected, lyophilized, and analyzed by MALDI-TOF mass spectrometry.

1.8. CD measurement of relaxin analogues

Circular dichroism (CD) spectra of relaxins were measured with a JASCO J-820 spectrophotometer using a quartz cuvette with a 0.1 cm path length. Samples were prepared using 10 μM of relaxins in 10 mM Tris-HCl buffer sodium (pH 7.5). CD signals between 190 and 260 nm were recorded at 25 °C, and 8 scans of individual samples were accumulated and averaged. The bandwidth and scan rate were 1 nm and 50 nm min^{-1}, respectively.

1.9. Reductive unfolding of relaxin analogues

Reductive unfolding was initiated by mixing the solutions of a relaxin analogue (9 nmol) and GSH (300 nmol) in 100 mM Tris-HCl buffer solution (300 μL) at pH 7.5 and 25 °C. After a certain period of time, an aliquot (30 μL) was transferred into an aqueous AEMTS solution (8 mg mL^{-1}, 200 μL) in a micro-centrifuge tube at 25 °C. After 5 min, the mixture was diluted with an aqueous TFA solution (0.1%, 810 μL) and stored at −30 °C. The sample solutions were analyzed by RP HPLC. The analytical conditions were same as those applied for the oxidative chain assembly of relaxin analogues (see section 1.6.). Peptide peak areas were integrated, and the % of remaining peptide compared to the initial was graphed against the incubation time. The experiments were repeated 3 times for each analogue.

1.10. Stability assessment of relaxin analogues in human serum.

Each sample of relaxin analogues was dissolved in water (100 nmol/100 μL), and 5 μL (i.e., 5 nmol of relaxin) was transferred to a 1.5 mL microcentrifuge tube. The sample solution was diluted with water (20 μL) and then mixed with a pooled human serum (475 μL; COSMO BIO Co., LTD.), which was precentrifuged for 10 min at 13000 rpm and room temperature to remove lipid and preincubated at 37 °C for 10 min. The resulting mixture (500 μL) was incubated at 37 °C. After specific time points (0, 2, 4, 8, 24 h), a potion (60 μL) of the sample solution was taken up, and to the aliquot was added with cooled solution of 1% formic acid in 90% aqueous CH_3CN (300 μL).

The resulting solution was immediately and vigorously stirred for 10 sec at room temperature by vortexing, and incubated on ice for 30 min. The suspension was centrifuged at 13,000 rpm for 10 min at room temperature. Supernatant (200 μL) was taken up and diluted with water containing 0.1 TFA (850 μL) and analyzed by RP HPLC. The analytical conditions were same as those applied for the oxidative chain assembly of relaxin analogues. (see section 1.6.) Peptide peak areas were integrated, and the % of remaining peptide compared to the initial was graphed against the incubation time. The experiments were repeated 3 times for each analogue.

1.11. Isolation and Culture of Human Endometriotic Stromal Cells (ESCs)

The experimental procedures were approved by the institutional review board of University of Yamanashi (approved number: R03211), and signed informed consent for the use of samples was obtained from each patient. Primary ESC culture was conducted as described previously[6]. Briefly, endometriotic tissue was dissected free of underlying parenchyma, minced into small pieces, incubated in DMEM F12 with type I collagenase (2.5 mg mL^{-1}) and DNase I (15 U mL^{-1}) for 1–2 h at 37 °C, and separated using serial filtration. Debris was removed using a 100 nm nylon cell strainer (Becton Dickinson, Lincoln Park, NJ, USA), and dispersed epithelial glands were eliminated with a 70 μm nylon cell strainer. Stromal cells remaining in the filtrate were collected by centrifugation, resuspended in DMEM/F12 with 10% charcoal-stripped FBS, penicillin (100 U/mL), streptomycin (100 ng/mL), and amphotericin B (250 ng/mL), plated onto 100 mm dishes (Iwaki Co, Chiba, Japan). When the cells became confluent after two days, they were dissociated with 0.25% trypsin, harvested by centrifugation, and replanted in six-well plates at 2×10^5 cells/well. They were kept at 37 °C in a humidified 5% CO_2/95% air environment until they were grown to confluence. Purification of the stromal cell population was confirmed by immunocytochemical staining for the following antibodies: vimentin (stromal cells), cytokeratin (epithelial cells), and CD45 (monocytes and other leukocytes). The purity of the stromal cell was more than 98%, as judged by positive cellular staining for vimentin and negative cellular staining for cytokeratin and CD45.

After 8 h stimulation with the recombinant or synthetic *H2 relaxin* and SeRlx analogues, total RNA was extracted from human ESCs using RNeasy Kits (QIAGEN, Tokyo, Japan). Reverse transcription (RT) was performed using Rever Tra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Tokyo, Japan). One μg of total RNA was reverse-transcribed in a 20-μL volume. For the quantification of various mRNA levels, real-time PCR was performed using the Mx3000P Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions[7]. The PCR primers were selected from different exons of the corresponding genes to

discriminate PCR products that might arise from possible chromosomal DNA contaminants. The expression of each mRNA was normalized by GAPDH mRNA. The following PCR primers were used: PAI-1 primer (NM_002438-4; 307-287 and 212-229). Amplification was performed with 30 cycles of denaturing (98 °C, 10 s), annealing (60 °C, 10 s), and extension (72 °C, 10 s)

2. Supplementally data

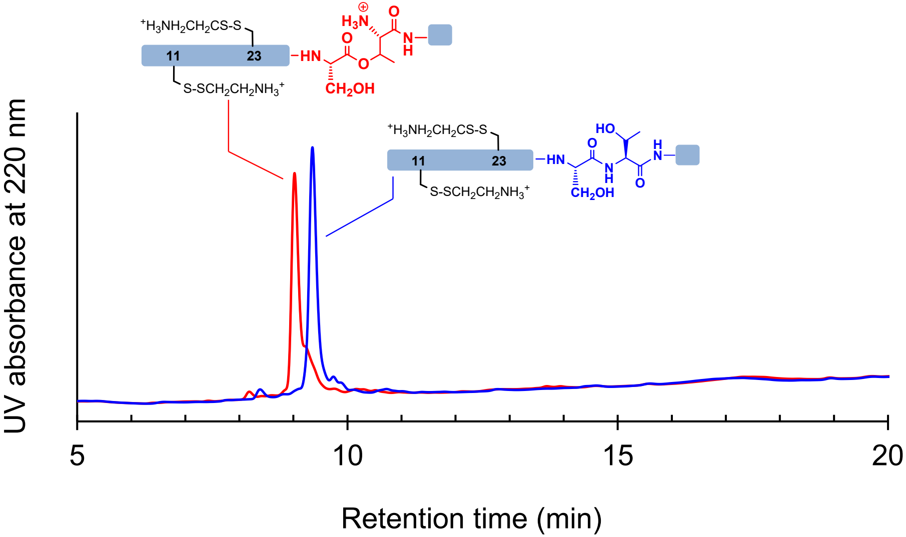

Fig. S1: Conversion of *O*-AIP moiety in peptide **2** into native peptide bond. Reaction conditions: [**2**] = 20 μM at pH 4.0 or pH 10.0 and 25 °C for 20 min. To completely dissolve the peptides in an acidic solution, free SH groups of the peptides were modified to $-SSCH_2CH_2NH_3^+$ by AEMTS.

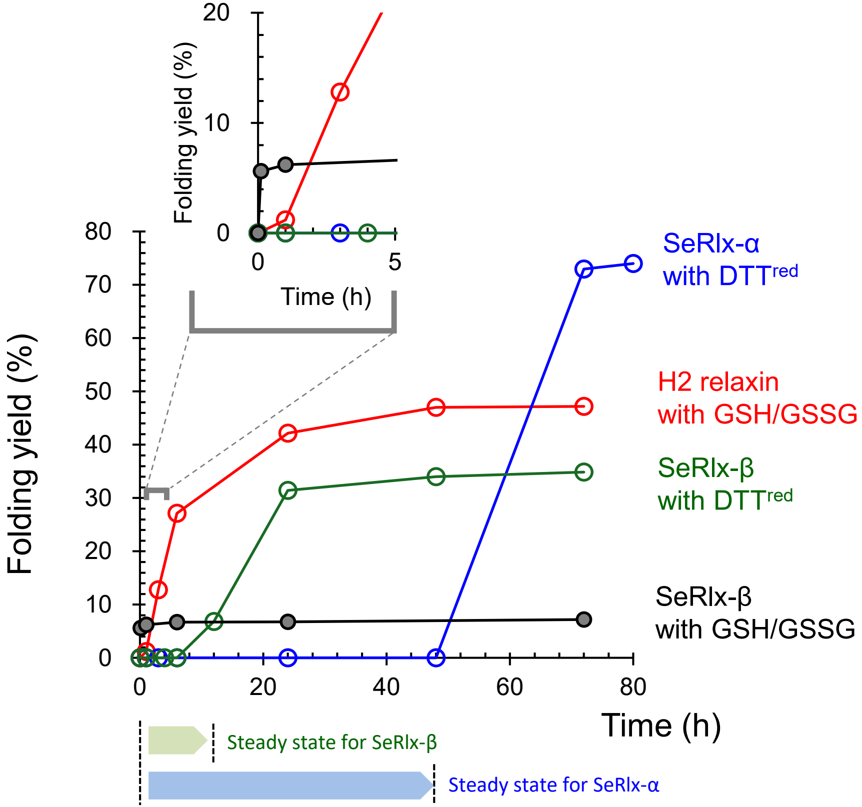

Fig. S2: Time course of generated relaxin analogues during the oxidative chain assembly. The folding yields were estimated from the HPLC chromatograms obtained from the oxidative chain assembly experiments shown in Figs. 3b, c, d and S4.

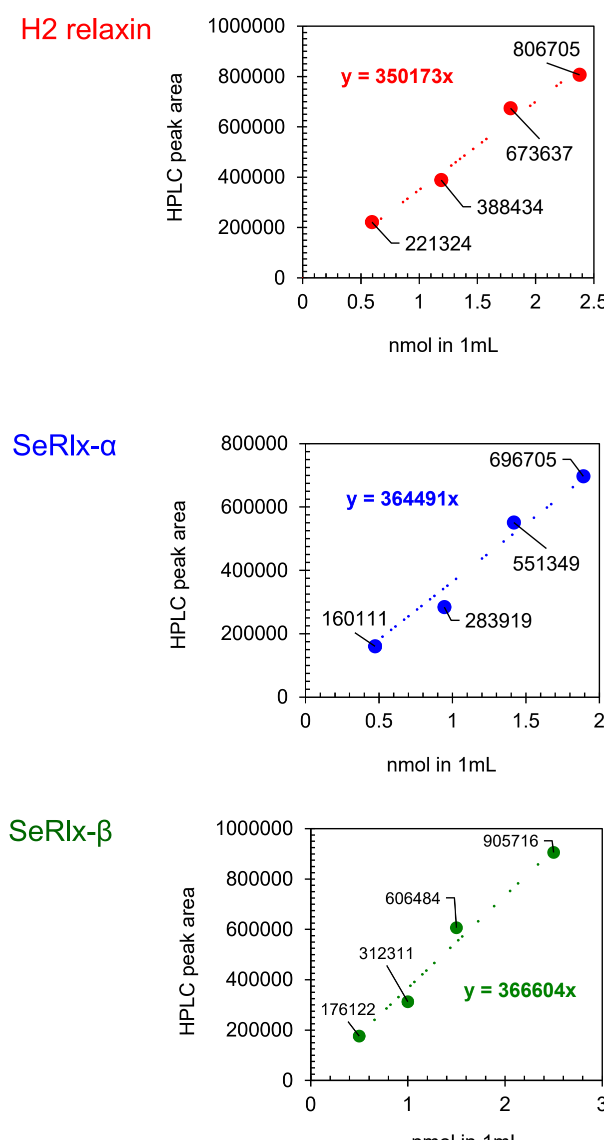

Fig. S3: Calibration curves of relaxin analogues. Purified relaxins (0.5–2.5 nmol) were dissolved in water containing 0.1% TFA (1000 μL), and the samples were analyzed by HPLC under the same conditions as those adopted in the analysis of the samples obtained from the oxidative chain assembly experiments.

Note: Good linearities were observed. As shown in Section 1.6, Small portion (5 μL) taken from the sample solution obtained from the chain assembly experiments was quenched by AEMTS solution (7 mg mL^{-1}, 200 μL), diluted with a 20 % acetonitrile in water containing 0.1 % TFA (835 μL), and analyzed by RP HPLC system equipped with a sample solution loop (1 mL). Therefore, using the observed peak area (*y*) of folded relaxin and the slope in the calibration curve (*a*), the HPLC yield can be estimated from the following equation (eq 1.)

$$HPLC\ yield\ (\%) = \frac{\left[\frac{y}{a} \times \frac{1040}{1000}\right] \times \frac{1000}{5}}{160} \times 100 \qquad (1)$$

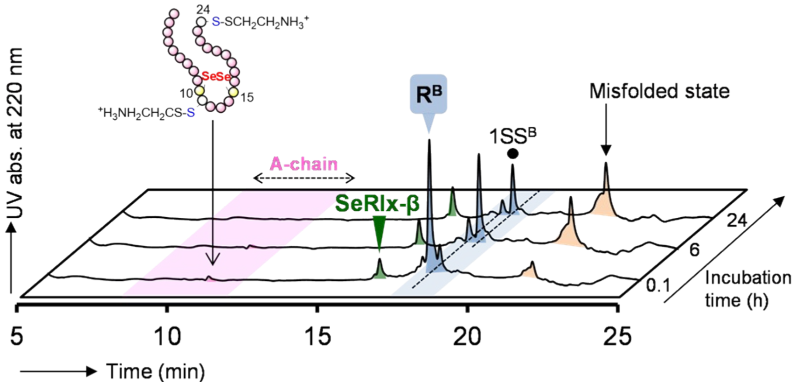

Fig. S4: HPLC chromatograms of samples obtained from oxidative chain assembly of peptides **2** and **5** in the GSH/GSSG redox system. Reaction conditions: $[\mathbf{2}]_0 = [\mathbf{5}]_0 = 160$ µM, $[\mathrm{GSH}]_0 = 1.0$ mM, $[\mathrm{GSSG}]_0 = 0.20$ mM, 4 °C, and pH 10.0 in the presence of 0.9 M urea. The reaction was repeated more than four times, and good reproducibility was confirmed.

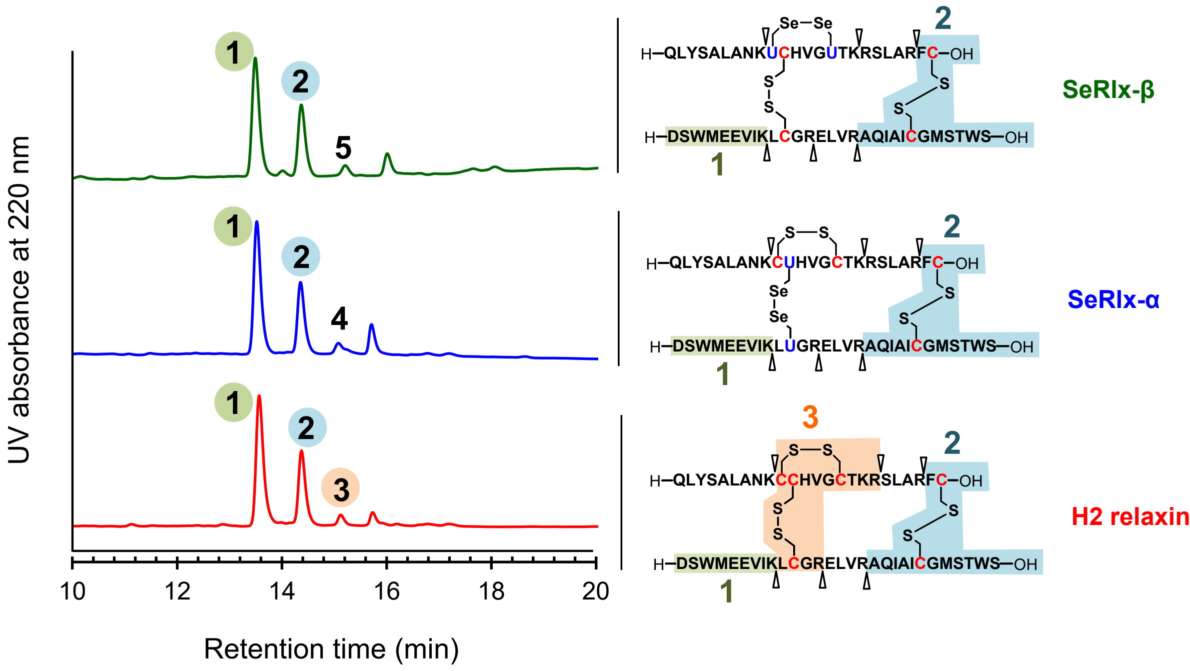

Fig. S5: HPLC chromatograms of sample solutions obtained from tryptic digestion of H2 relaxin (red), SeRlx-α (blue), and SeRlx-β (green). Reaction conditions: $[\mathrm{relaxins}]_0 = 145$ µM and $[\mathrm{trypsin}]_0 = 90$ µg/mL in 100 mM Tris-HCl buffer solution at pH 8.0 and 37 °C for 30 min.

Table S1: Assignments of the peptide fragments of relaxin analogues digested by trypsin.

Peak No. [a] **(Retention time [min])**	**Mass observed as [*M*+H]$^{+}$** [b]	**Mass calculated as [*M*+H]$^{+}$**	**Assigned sequence**	**SS linkage or SeSe linkage**
1 (13.6)	1136.9	1136.3	^{1}DSWMEEVIK^{9}	None
2 (14.4)	1447.1	1448.6	^{23}FC^{24} (S–S) $_{18}$AQIAICGMSTWS$_{29}$	C24^{A}–C23^{B}
3 (15.1)	1534.2	1533.7	^{10}CCHVGCTKR^{18} (S—S) (S–S) $_{10}$LCGR$_{13}$	C10^{A}–C15^{A}, C11^{A}–C11^{B}
4 (15.1)	ND[c]	1548.6	^{10}CUHVGCTKR^{18} (S—S) (Se–Se) $_{10}$LUGR$_{13}$	C10^{A}–C15^{A}, U11^{A}–U11^{B}
5 (15.2)	ND[c]	1548.6	^{10}UCHVGUTKR^{18} (Se—Se) (S–S) $_{10}$LCGR$_{13}$	U10^{A}–U15^{A}, C11^{A}–C11^{B}

[a] Peak number corresponds to that in Fig. S2. [b] Recorded by a MALDI-TOF-MS with 4-chloro-α-cyanocinnamic acid (ClCCA) as a matrix. Values are shown as [*M*+H]$^{+}$. [c] Peptide fragments containing diselenide bond was *not detected*, presumably because these were decomposed due to their instability under the conditions for MALDI-TOF-MS analysis.

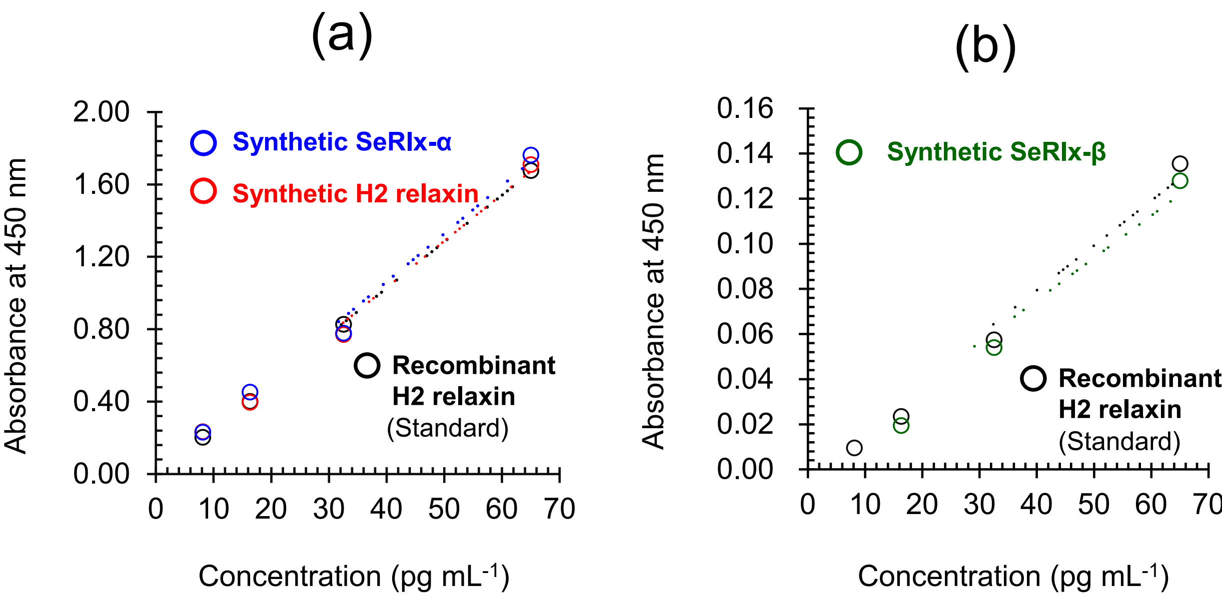

Fig. S6: Affinity assessment of relaxin analogues with a specific antibody at various concentrations by using a human relaxin-2 quantikine enzyme-linked immunosorbent assay (ELISA) kits, ab243688 (**a**) and DRL200 (**b**) purchased from abcam® and R & D systems®, respectively. The experiments were repeated more than two times, and data are shown as mean.

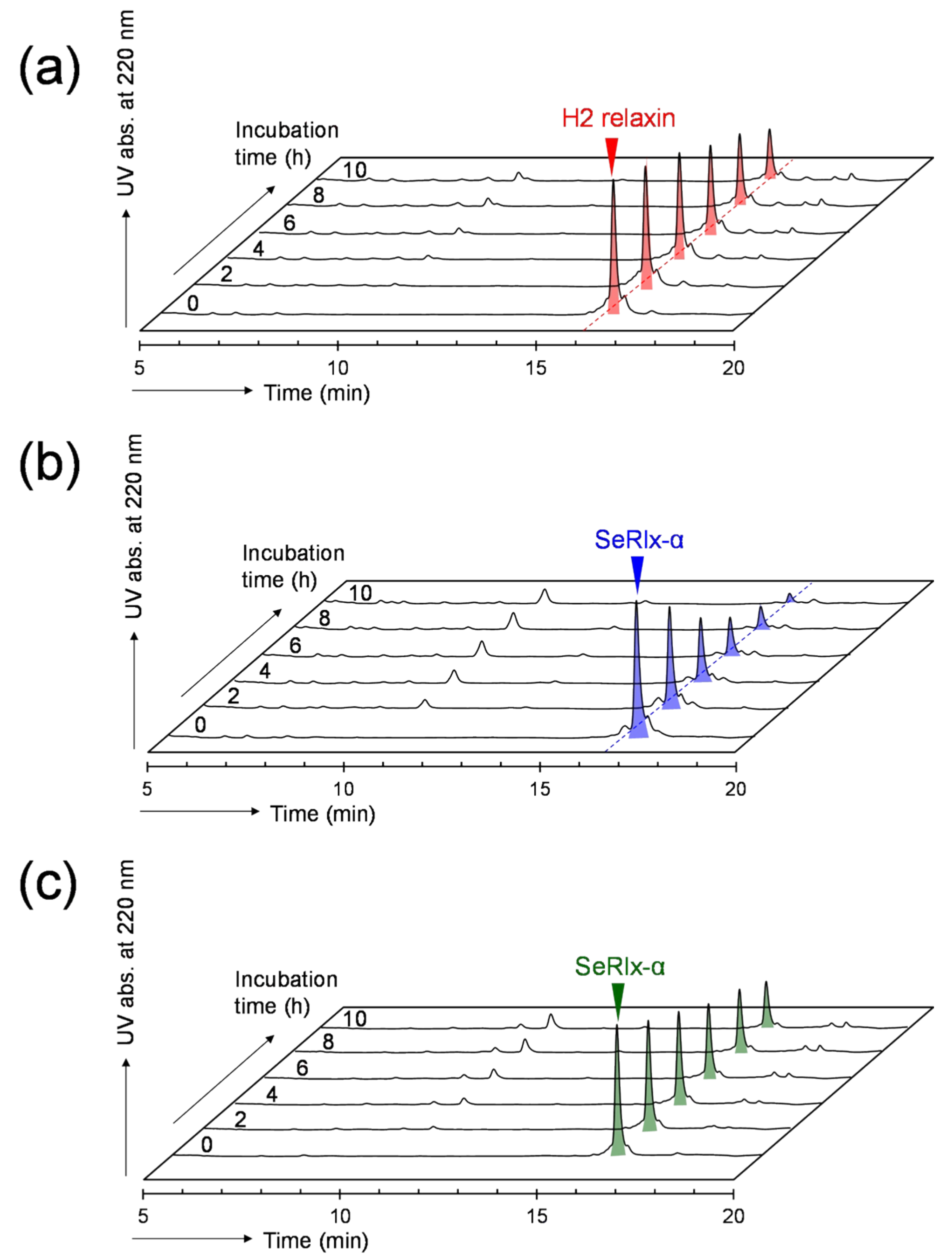

Fig. S7: HPLC chromatograms obtained from the reductive unfolding of H2 relaxin (**a**), SeRlx-α (**b**), and SeRlx-β (**c**). Reaction conditions: $[\text{relaxins}]_0$ = 30 μM and $[\text{GSH}]_0$ = 1.0 mM in 100 mM Tris-HCl buffer solution at pH 7.5 and 37 °C.

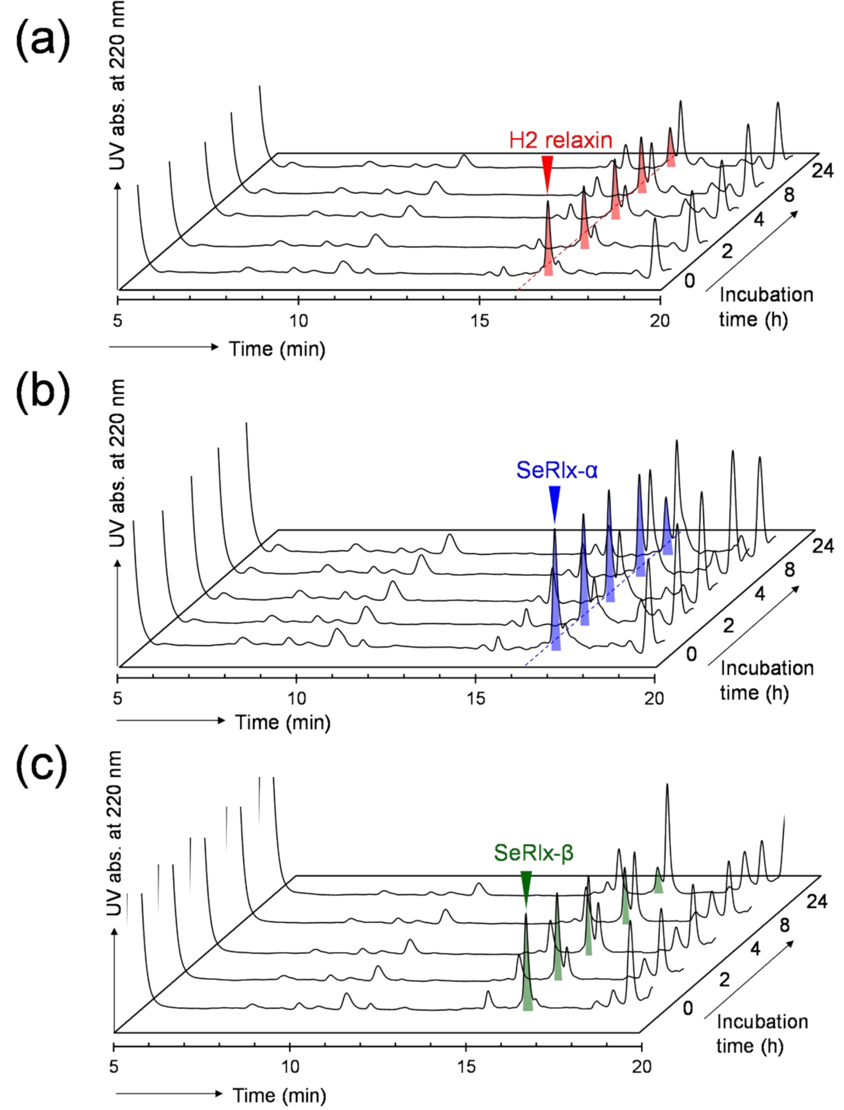

Fig. S8: HPLC chromatograms obtained from the degradation experiments of H2 relaxin (**a**), SeRlx-α (**b**), and SeRlx-β (**c**) in human serum. Reaction conditions: $[\text{relaxins}]_0$ = 10 μM in a pooled human serum at 37 °C.

3. References

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