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A novel approach for preparing disulfide-rich peptide-KLH conjugate applicable to the antibody production

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ABSTRACT

To produce the antiserum against a small peptide, the target peptide-keyhole limpet hemocyanine (KLH) conjugate is generally used as an antigen, although the disulfide-rich peptide-KLH conjugate is still difficult to prepare. In our previous study, we have developed a preparation method of the disulfide-rich peptide-KLH conjugate, and this method was applied to produce the antiserum against a relaxin-like peptide. However, this method is limited to the synthetic peptide antigen, and is not applicable to a native or a recombinant peptide. In this study, to expand the applicability of this method to wide variety of peptides, we newly designed a novel thiol probe enabling the conjugation between various peptides and KLH, and applied it to produce the antiserum against relaxin-like peptide of a starfish *Asterias amurensis*. The antiserum obtained here showed high antibody-titer and good specificity, strongly suggesting that the method developed in this study is applicable to various peptides.

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KEYWORDS
Relaxin-like gonad stimulating peptide; disulfide-rich peptide; peptide conjugate

Antibodies against peptides/proteins are widely used in biochemical and histochemical studies. To obtain specific antibodies against proteins, proteins or peptides with partial sequence of the target proteins are generally immunized as an antigen to animals. Since it is difficult to recognize a small peptide by antibodies in immunized animals, the peptide needs to enlarge its molecular structure by the oligomerization [1] or by the conjugation with a large protein, such as bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) [2], for the use as an antigen.

The production of peptide-KLH conjugate is generally performed using a specific reaction between a cysteinyl peptide and KLH modified with maleimide groups. On the other hand, since a sulfanyl group of the Cys side chain has an ability to reduce disulfide bond(s) denaturing the disulfide-containing peptides, it is difficult to apply this method to disulfide-containing peptides. Thus, the preparation of disulfide-rich peptide-KLH conjugate is still challenging issue. Recently, we have developed a method to prepare the disulfide-rich peptide-KLH conjugate, and this method was applied to produce the antisera against relaxin-like gonad-stimulating peptide (RGP) from a starfish *Patiria pectinifera* (Ppe-RGP) (Figure 1) [3,4]. In this method, RGP having Ser residue at the N-terminus of A chain was chemically synthesized with the ordinary solid-phase peptide synthesis (SPPS) and the regioselective disulfide bond formation reactions, and a chemical probe having both sulfanyl and aminooxy groups was finally introduced to the N-terminal aldehyde group which was generated from N-terminal Ser residue with sodium periodate oxidation [4]. To minimize the undesirable side reaction, we thought that the additional sulfanyl group should be taken apart from the antigen peptide, and polyethylene glycol (PEG) chain was inserted between the N-terminal Ser residue and the peptide part. Since the antisera obtained by this method showed high antibody titers and high specificity against Ppe-RGP, these antisera could be used for the development of a specific and sensitive radioimmunoassay (RIA) [5] and enzyme-linked immuno-sorbent assay (ELISA) [6] for the measurement of Ppe-RGP. However, due to the necessity of PEG chain insertion between the reactive N-terminal Ser residue and the antigen peptide portion, our preparation method of peptide-KLH conjugate was not applicable to native peptides or recombinant peptides.

To produce the carbonyl group in the target peptide in our previous study, we used the sodium periodate oxidation of N-terminal Ser residue [4]. In the preparation of recombinant peptides with bacterial expression system, since the N-terminal initial Met residue attached to Ser is automatically cleaved with methionine aminopeptidase in bacterial cells [7], recombinant peptides carrying Ser residue at the N-terminus can be easily prepared by the conventional bacterial expression systems, and therefore the problem on PEG insertion is

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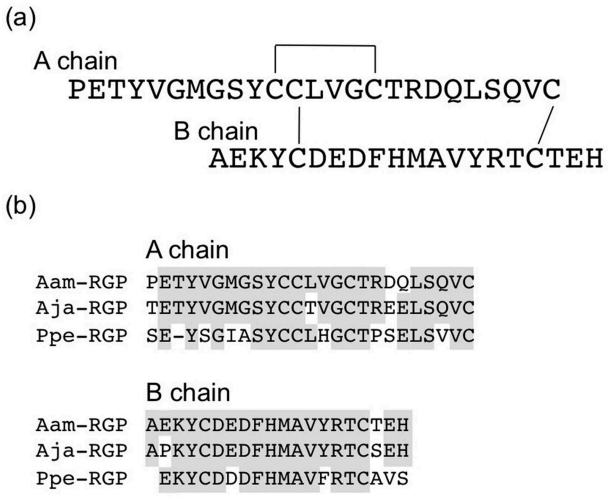


Figure 1. Structures of starfish relaxin-like gonad-stimulating peptides (RGPs). (a) Primary structure of *Asterias amurensis* RGP. Solid lines represent the disulfide bonds. (b) Amino acid sequence alignment of starfish RGPs. Aam-RGP, *Asterias amurensis* RGP; Aja-RGP, *Aphelasterias japonica* RGP; Ppe-RGP, *Patiria pectinifera* RGP. Characters with a gray background represent identical residues.

likely to be overcome by reconsideration of the thiol probe design.

On the other hand, it is well known that the N-terminal amino group can be converted with carbonyl group with transamination reaction, which is performed with glyoxylic acid in the presence of nickel ion (Ni^{2+}) [8,9] or with pyridoxal 5-phosphate [10]. This method has been widely used for site-specific modification reactions, such as fluorescent labeling [11,12] and biotinylation [13]. The combination of transamination reaction and the newly designed thiol probe might also enable the preparation of the peptide-KLH conjugate using native disulfide-rich peptides. In this paper, we describe the design and synthesis of the new thiol probe with PEG chain and its application to produce the antisera against RGP of the starfish *Asterias amurensis* (Aam-RGP) among RGP orthologs (Figure 1) [14].

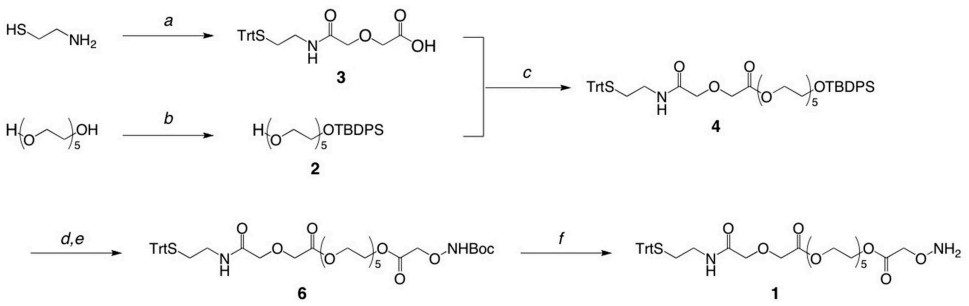
Results and discussion

Design and synthesis of pegylated thiol probe

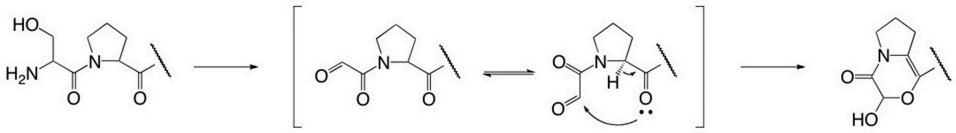
Pentaethylene glycol was inserted into the structure of previously designed thiol probe [4], and the new probe was designed as compound **1** and was synthesized as shown in Scheme 1. One of two hydroxyl groups of pentaethylene glycol was protected with *tert*-butyldiphenylsilyl (TBDPS) group, giving mono-*O*-TBDPS-pentaethylene glycol **2** in 40% yield. On the other hand, the sulfanyl group of 2-aminoethanethiol was protected with trityl (Trt) group, and the product was then condensed with diglycolic anhydride, giving compound **3** in 34% yield. Compounds **2** and **3** was condensed using 1,3-diisopropylcarbodiimide (DIC) and 4-(dimethylamino)pyridine (DMAP) as condensation reagents, giving compound **4** in 70% yield. After TBDPS group was removed with tetra-*n*-butylammonium fluoride (TBAF) to give compound **5**, *tert*-butoxycarbonyl (Boc)-aminooxyacetic acid was condensed with DIC-DMAP, giving compound **6** in 34% yield. Finally, Boc group was removed with trifluoroacetic acid (TFA), affording the desired thiol probe **1** in 69% yield.

Chemical synthesis of the antigen

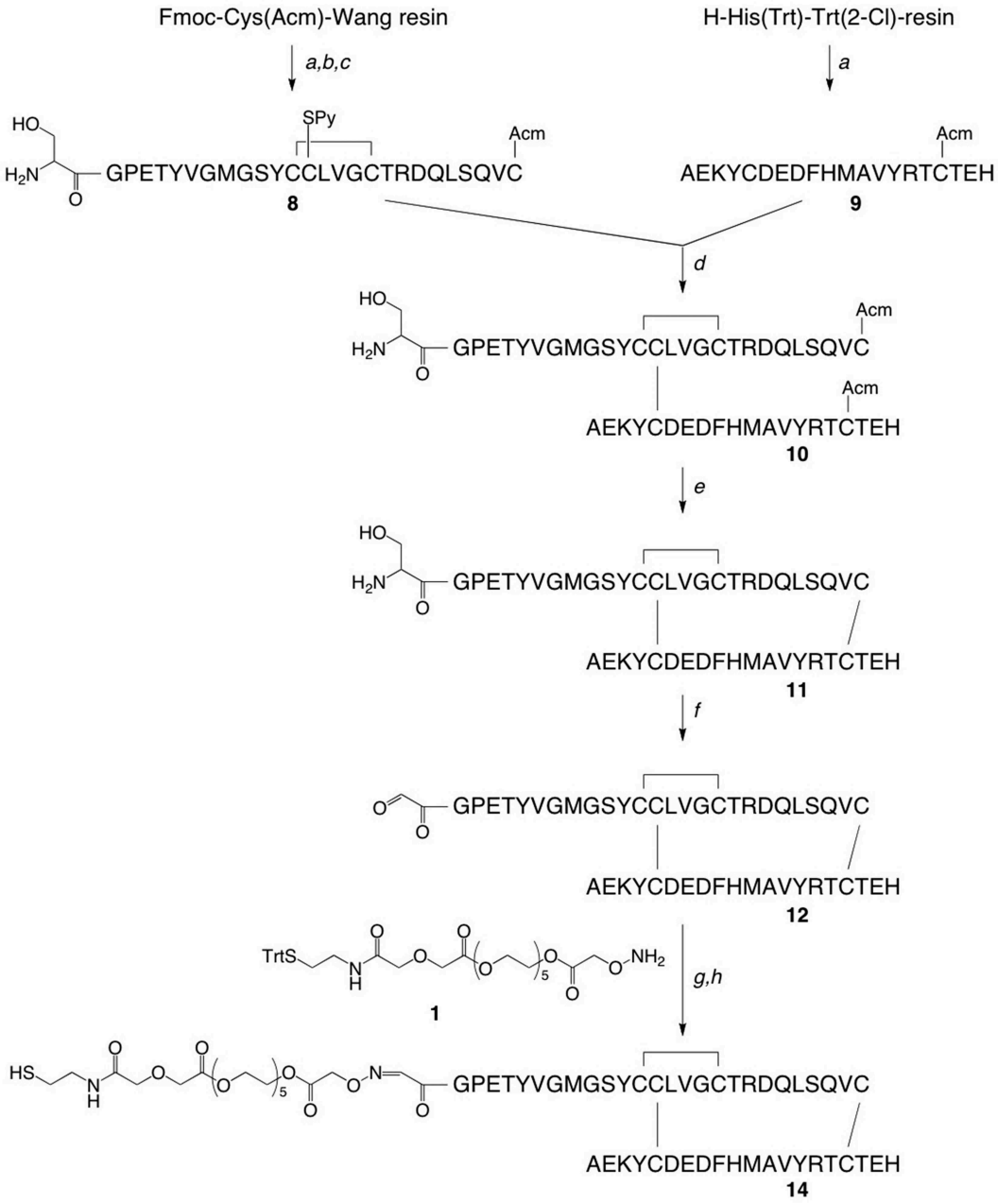
To introduce an aldehyde group using the reaction with the thiol probe **1**, Aam-RGP having an additional Ser residue at the N-terminus of A chain was chemically synthesized at first. The N-terminal Ser residue of this peptide, however, was not converted to aldehyde with $NaIO_4$ oxidation. Since the undesirable side reaction in which the aldehyde group was immediately hindered via the cyclization with the carbonyl group of Pro residue was found (Scheme 2), the introduction of thiol probe **1** did not proceed. This side reaction is likely to occur at the N-terminal aldehyde group directly attached the Pro residue, because the side reaction can proceed when peptide bond has the *cis* configuration as shown in Scheme 2, and only the peptide bond at



Scheme 1. Synthetic procedure of the PEGylated thiol probe **1**.Reaction conditions: *a*, (1) Trityl chloride, TFA; (2) Diglycolic anhydride, CH_2Cl_2, DIEA; *b*, TBDPS-Cl, imidazole, DMF; *c*, 1,3-diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazole (HOBt), 4-(dimethylamino)pyridine (DMAP), DMF; *d*, TBAF, acetic acid, THF; *e*, Boc-aminooxyacetic acid, DIC, HOBt, DMAP, CH_2Cl_2; *f*, TFA, CH_2Cl_2.



Scheme 2. Possible mechanism of side reaction in $NaIO_4$ oxidation at the N-terminal Ser-Pro sequence.



Scheme 3. Synthetic procedure of the antigen **14**. Reaction conditions: *a*, solid-phase peptide synthesis; *b*, DMSO, 6 M guanidine-HCl/50 mM phosphate buffer (pH 7.0); *c*, Dipyridyl disulfide, thioanisole, TFA, TfOH; *d*, 30% acetonitrile/50 mM sodium bicarbonate; *e*, iodine, HCl, methanol/H_2O; *f*, $NaIO_4$, 50 mM phosphate buffer (pH 7.0); *g*, **1**, 5% acetic acid/DMA; *h*, TIS, H_2O, TFA.

N-terminal side of Pro residue can have *cis* configuration. To take apart the aldehyde from Pro residue, a Gly residue was inserted between Ser-Pro, and we synthesized Ser-Gly-Aam-RGP as shown in Scheme 3.

Ser-Gly-A chain was prepared by the ordinary 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase peptide synthesis (SPPS) method. To enable the regioselective formation of disulfide bonds, *S*-methoxybenzyl (MeOBn)-cysteine and *S*-acetamidomethyl (Acm)-cysteine derivatives were used at Cys^{12} and Cys^{25}, respectively. After the cleavage of crude peptide from the resin with TFA treatment, an intrachain disulfide bond at

Cys^{11}-Cys^{16} was formed with dimethyl sulfoxide (DMSO) oxidation in a neutral buffer solution, giving partially protected Ser-Gly-A chain **7** in 13% yield. MeOBn group at Cys^{12} was then cleaved with triflic acid (TfOH), and simultaneously *S*-pyridylsulfenylated with 2,2′-dipyridyl disulfide (DPDS), giving SPy-attached Ser-Gly-A chain **8** quantitatively.

B chain was also prepared by the ordinary Fmoc-SPPS, and Cys^{17}(Acm)-B chain **9** was obtained in 15% yield. Equimolar amounts of peptides **8** and **9** were mixed in an aqueous solution to form the interchain disulfide bond at Cys^{A12}-Cys^{B5} with thiolysis reaction, giving the heterodimeric peptide **10** in 43% yield. The third disulfide bond was regioselectively formed with iodine oxidation in an acidic solution, giving Ser-Gly-Aam-RGP **11** in 40% yield.

The N-terminal Ser residue at the A-chain was converted to aldehyde group with sodium periodate oxidation, giving aldehyde-Gly-Aam-RGP **12** in 69% yield. Then, peptide **12** was treated with the thiol probe **1** under weakly acidic conditions, giving peptide **13** in 88% yield. Finally, the Trt group was removed in TFA solution containing triisopropylsilane, giving the designed antigen **14** in 45% yield. It is well known that the disulfide exchange reaction is quite slow under acidic conditions. After the removal of Trt group, acidic conditions were kept until the antigen **14** was purified and lyophilized, and no significant disulfide isomerization was observed on the HPLC chromatogram in purification step of peptide **14**.

The physiological conditions in animal are at a neutral pH, although peptide **14** was likely to be labile under these conditions. Therefore, we used peptide **13** instead of peptide **14** for the conformational analysis. The conformation of peptide **13** was confirmed by circular dichroism (CD) spectral measurement. Aam-RGP with native form which was also synthesized by the Fmoc-SPPS and the regioselective disulfide bond formation reactions [15] showed the spectral pattern typical for α-helical peptides (Figure 2). This spectral pattern is similar to those of other insulin-family peptides reported previously [4,16–23], strongly suggesting that Aam-RGP has a tertiary structure similar to the other insulin-family peptides. Peptide **13** showed the CD spectrum essentially the same as that of Aam-RGP (Figure 2). The α-helix contents of peptide **13** and Aam-RGP were calculated based on the α values at 208 nm [24] to be 36.0% and 37.5%, respectively. These results strongly suggest that peptide **13** possess the same conformation as Aam-RGP, and that the attachment of thiol probe **1** does not affect the peptide conformation.

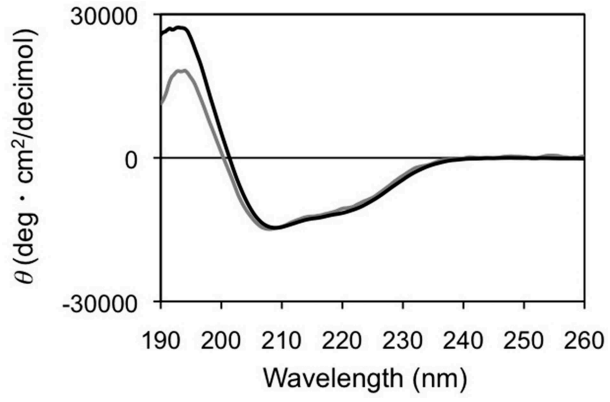


Figure 2. Circular dichroism spectra of Aam-RGP (black line) and TrtS-PEG-Gly-Aam-RGP **13** (gray line).

Production and evaluation of anti-Aam-RGP antiserum

Using antigen **14**, the conjugation with KLH was carried out with the maleimide method, which was performed in Eurofins Genomics (Tokyo, Japan). The conjugate was immunized against a rabbit. After four weeks, the serum was collected, and the antibody titer against Aam-RGP was measured by enzyme-linked immuno-sorbent assay (ELISA). As shown in Figure 3, Aam-RGP was recognized with the antiserum in the dilution level of 10^{-5}. This antibody titer was comparable to that of the anti-Ppe-RGP antisera obtained in our previous study [4].

To confirm the specificity of antiserum against Aam-RGP, Western blotting analysis using several insulin/relaxin-family peptides was performed, and the result was shown in Figure 4. Aam-RGP gave a band on the membrane (lane 2), indicating that the antisera obtained in this study recognized Aam-RGP properly. RGP from *Aphelasterias japonica* (Aja-RGP) (Figure 1(b)) [25] also gave a faint band on the membrane (lane 3), whereas RGP from *Patiria pectinifera* (Ppe-RGP) [3] did not (lane 1). These results are coincided well with that Aam-RGP shows the higher sequence identity to Aja-RGP (87%) than to Ppe-RGP (67%) (Figure 1) [25,26]. In the lanes of

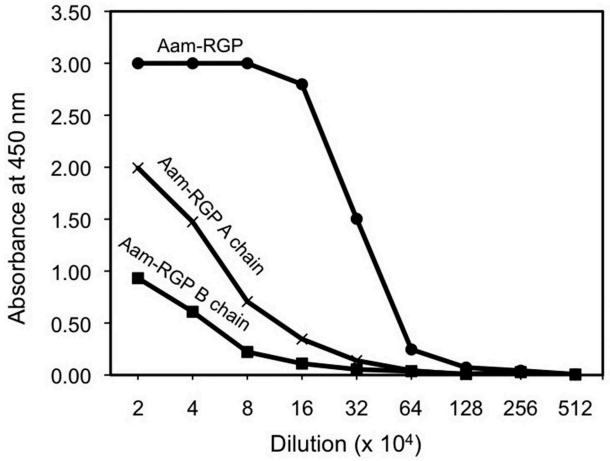


Figure 3. Titer-curves of anti-serum against Aam-RGP and its chains. Peptides were used for immobilized antigens at a concentration of 1 mM. Circles, crosses and squares indicate Aam-RGP, Aam-RGP A-chain and Aam-RGP B-chain, respectively.

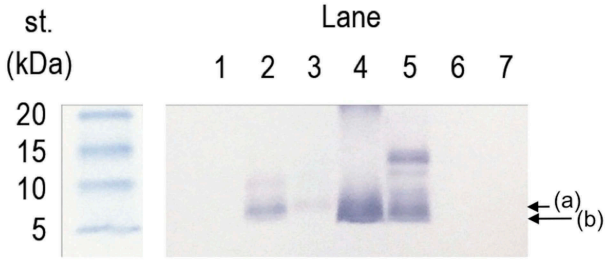


Figure 4. Western blotting analysis using an anti-Aam-RGP antiserum against various insulin/relaxin family peptides. Ppe-RGP (lane 1), Aam-RGP (lane 2), Aja-RGP (lane 3), Aam-RGP A-chain (lane 4), Aam-RGP B-chain (lane 5), bovine insulin (lane 6), and human relaxin-3 (lane 7) at the concentrations of 0.1 nmol/well were separated by SDS-PAGE and used for Western blotting. Arrows (a) and (b) indicate the positions of RGPs and A and B chains, respectively.

A and B chains of Aam-RGP (lanes 4 and 5), thick bands were found at the proper molecular weight position, indicating that the antibodies also recognized each chain as an antigen. A chain of Aam-RGP gave signals at high molecular weight region. It may due in part to a low solubility of A chain in an aqueous solution, and aggregate of A chain may gave some signals. B chain also gave a several bands with high molecular weights. It was likely that these were oligomers of B chain connected with disulfide bond(s). Since B chain easily underwent oxidation under air atmosphere and a reducing reagent could not be used in this experiment, these bands could not be disappeared. Neither human relaxin-3 (lane 6) nor bovine insulin (lane 7) gave a band in the Western blotting analysis, illuminating that the antiserum obtained here had a high specificity against Aam-RGP.

These results indicated that the preparation method of antigen developed in this study is useful at the same level as previously developed one in which only the synthetic peptides could be applicable.

Conclusion

In this study, we designed and synthesized the novel thiol probe for utilizing the antibody production against Aam-RGP. The synthetic thiol probe might be applicable not only to the synthetic peptides but also to native and recombinant peptides. The anti-Aam-RGP antiserum obtained here was confirmed to have high antibody titer with high specificity, and therefore it must be useful for the studies on starfish reproduction.

Experimental procedure

General

Aam-RGP was chemically synthesized as described [15]. NMR spectra were recorded by a Bruker AVANCE III HD spectrometer (500 MHz in ^{1}H-NMR, Bruker, Germany). MALDI-TOF mass spectra were recorded using an Autoflex spectrometer (Brucker). High-resolution ESI mass spectra were measured with The AccuTOF-plus JMS-T100LP spectrometer (JEOL, Tokyo, Japan). Amino acid composition was determined using a LaChrom amino acid analyzer (Hitachi, Tokyo, Japan) after hydrolysis with a 6 M HCl solution at 150°C for 2 h in a vacuum-sealed tube. Circular dichroism (CD) spectra were measured with a Jasco J-820 spectropolarimeter (JASCO, Tokyo, Japan) at room temperature with a 2-mm path length cell using a phosphate buffer (50 mM, pH 7.0) as a solvent. Helix contents were calculated by the method as described previously [24].

Mono-o-(tert-butyldiphenylsilyl)pentaethylene glycol 2

Pentaethylene glycol (0.53 g, 2.2 mmol) and imidazole (0.30 g, 4.4 mmol) were dissolved in DMF (10 mL), and *tert*-butylchlorodiphenylsilane (0.58 mL, 2.2 mmol) was added dropwisely under Ar atmosphere. The reaction mixture was stirred at room temperature for 2.5 h. The solution was diluted with EtOAc, washed with 1 M HCl, distilled water and brine, and dried over Na_2SO_4. After filtration and evaporation, the residue was chromatographed on silica gel with toluene/methanol (19:1) to give the desired compound **2** (0.42 g, 0.88 mmol, 40%) as a colorless oil: R_f 0.55 (toluene/methanol, 9/1); ^{1}H-NMR ($CDCl_3$, 500 MHz): d 7.73–7.33 (m, 10H, Ar), 3.81–3.60 (m, 20H, O-CH_2-CH_2-), 1.49 (s, 9H, Bu^t); ^{13}C-NMR: δ 135.6, 133.7, 129.6, 127.7 (Ar), 72.6, 72.4, 70.8, 70.7, 70.6, 70.6, 70.3, 63.4, 61.3 (OCH_2), 26.8 [C(CH_3)$_3$], 19.2 [C(CH_3)$_3$]; ESI mass, found: *m/z* 499.248, calcd: 499.248 for (M+ Na)$^+$.

O-[n-(tritylthioethyl)carbamoyl]methoxyacetic acid 3

Trityl chloride (0.93 g, 3.3 mmol) and 2-aminoathanethiol hydrochloride (0.36 g, 3.2 mmol) were dissolved in trifluoroacetic acid (TFA, 10 mL), and the solution was stirred at room temperature for 10 min. After the evaporation, the residue was dissolved in dichloromethane (DCM, 10 mL), and diglycolic anhydride (0.43 g, 3.7 mmol) and *N*, *N*-diisopropylethylamine (DIEA, 1.1 mL, 6.3 mmol) were added. The reaction mixture was stirred at room temperature for 20 h. The solution was diluted with EtOAc, washed with 1 M HCl, distilled water and brine, and dried over Na_2SO_4. After filtration and evaporation, the residue was chromatographed on silica gel with toluene/methanol/acetic acid (90:10:1) to give the desired compound **3** (0.49 g, 1.1 mmol, 34%) as a colorless solid: R_f 0.37 (toluene/methanol/

acetic acid, 90/10/1); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 7.96 (t, 1H, J = 6.0 Hz, N*H*), 7.38–7.23 (m, 15H, Ar), 4.10 (s, 2H, -O-CH_2-CO-), 3.94 (s, 2H, -O-CH_2-CO-), 3.08 (q, 2H, J = 6.7 Hz, -NH-CH_2-), 2.24 (t, 2H, J = 7.0 Hz, -S-CH_2-); ^{13}C-NMR: d 171.9, 169.1 (*C* = O), 145.1, 129.5, 128.5, 127.2 (Ar), 70.4, 68.2 (OCH_2), 66.4 (*C*Ph_3), 49.1 (-*C*H_2-NH-), 40.1 (-*C*H_2-S-); ESI mass, found: *m/z* 458.142, calcd: 458.140 for (M+ Na)$^+$.

Mono(tert-butyldiphenylsilyl)pentaethylene glycolyl O-[N-(Tritylthioethyl)carbamoyl] methoxyacetate 4

Compound **3** (0.31 g, 0.72 mmol) was dissolved in DMF (3 mL), and 1-hydroxybenzotriazole (HOBt, 0.12 g, 0.86 mmol) and 1,3-diisopropylcarbodiimide (DIC, 0.13 mL, 0.86 mmol) were added. The solution was stirred at room temperature for 30 min. Compound **2** (0.34 g, 0.72 mmol) and 4-(dimethylamino)pyridine (DMAP, 18 mg, 0.14 mmol) were added to the solution, and the mixture was stirred for further 20 h. The solution was diluted with EtOAc, washed with 1 M HCl, distilled water and brine, and dried over Na_2SO_4. After filtration and evaporation, the residue was chromatographed on silica gel with toluene/EtOAc (1:1) to give the desired compound **4** (0.43 g, 0.51 mmol, 70%) as a colorless oil: R_f 0.56 (toluene/EtOAc, 1/1); ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.70–7.20 (m, 25H, Ar), 6.93 (brs, 1H, N*H*), 4.28 (t, 2H, J = 4.8 Hz, -COO-CH_2-), 4.15 (s, 2H, -O-CH_2-CO-), 4.01 (s, 2H, -O-CH_2-CO-), 3.81–3.59 (m, 18H, O-CH_2-CH_2-), 3.13 (q, 2H, J = 6.5 Hz, -NH-CH_2-), 2.41 (t, 2H, J = 6.7 Hz, -S-CH_2-), 1.05 (s, 9H, Bu^t); ^{13}C-NMR: d 169.8, 168.2 (*C* = O), 144.7, 135.6, 133.7, 129.7, 129.6, 128.0, 127.7, 126.8 (Ar), 70.6, 66.8 (OCH_2CO-), 72.5, 71.1, 70.8, 70.7, 68.9, 68.6, 64.2, 63.5 (OCH_2, Ph_3*C*-), 37.7 (-*C*H_2-NH-), 31.9 (-*C*H_2-S-), 26.9 [C(*C*H_3)$_3$], 19.2 [*C*(CH_3)$_3$]; ESI mass, found: *m/z* 916.387, calcd: 916.389 for (M+ Na)$^+$.

Pentaethylene glycolyl o-[n-(tritylthioethyl) carbamoyl]methoxyacetate 5

Compound **4** (0.50 g, 0.56 mmol) was dissolved in tetrahydrofuran (THF, 2 mL), and 1.0 M tetrabutylammonium fluoride (TBAF)/THF (0.64 mL) was added. The solution was stirred at room temperature for 20 h. After concentration under vacuum, the residue was chromatographed on silica gel with $CHCl_3$/methanol (19:1) to give the desired compound **5** (0.20 g, 0.30 mmol, 55%) as a colorless oil: R_f 0.52 ($CHCl_3$/methanol, 9/1); ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.42–7.19 (m, 15H, Ar), 6.97 (brs, 1H, N*H*), 4.30 (t, 2H, J = 2.3 Hz, -COO-CH_2-), 4.18 (s, 2H, -O-CH_2-CO-), 4.01 (s, 2H, -O-CH_2-CO-), 3.71–3.58 (m, 18H, O-CH_2-CH_2-), 3.13 (q, 2H, J = 6.5 Hz, -NH-CH_2-), 2.41 (t, 2H, J = 6.8 Hz, -S-CH_2-); ^{13}C-NMR: d 169.8, 168.8 (*C* = O), 144.4, 129.6, 128.0, 126.8 (Ar), 70.5, 68.8 (OCH_2CO-), 72.6, 71.0, 70.6, 70.6, 70.5, 70.2, 64.2, 61.7 (OCH_2, Ph_3*C*-), 37.7 (-*C*H_2-NH-), 31.8 (-*C*H_2-S-); ESI mass, found: *m/z* 678.268, calcd: 678.271 for (M+ Na)$^+$.

O-(tert-Butoxycarbonylaminooxyacetyl) pentaethylene glycolyl O-[N-(Tritylthioethyl) carbamoyl]methoxyacetate 6

Compound **5** (0.20 g, 0.30 mmol) was dissolved in DCM (1 mL), and HOBt (62 mg, 0.46 mmol) and DIC (70 μL, 0.46 mmol) were added. The solution was stirred at room temperature for 30 min. Boc-aminooxyacetic acid (87 mg, 0.46 mmol) and DMAP (5.6 mg, 46 μmol) were added to the solution, and the mixture was stirred for further 20 h. The solution was diluted with EtOAc, washed with 1 M HCl, distilled water and brine, and dried over Na_2SO_4. After filtration and evaporation, the residue was chromatographed on silica gel with $CHCl_3$/methanol (19:1) to give the desired compound **6** (0.16 g, 0.18 mmol, 61%) as a colorless oil: R_f 0.49 ($CHCl_3$/methanol, 9/1); ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.18 (brs, 1H, ON*H*), 7.41–7.19 (m, 15H, Ar), 7.01 (brs, 1H, CH_2N*H*), 4.46 (s, 2H, -NHO-CH_2-CO-), 4.33 (t, 2H, J = 2.7 Hz, -COO-CH_2-), 4.29 (t, 2H, J = 3.0 Hz, -COO-CH_2-), 4.18 (s, 2H, -O-CH_2-CO-), 4.04 (s, 2H, -O-CH_2-CO-), 3.72–3.58 (m, 16H, O-CH_2-CH_2-), 3.13 (q, 2H, J = 6.5 Hz, -NH-CH_2-), 2.42 (t, 2H, J = 6.8 Hz, -S-CH_2-), 1.47 (s, 9H, Bu^t); ^{13}C-NMR: d 169.8, 169.7, 169.1, 156.4 (*C* = O), 144.6, 129.5, 128.0, 126.8 (Ar), 82.1 [*C*(CH_3)$_3$], 70.5, 66.8 (OCH_2 CO-), 72.6, 70.9, 70.5, 68.8, 68.8, 68.5, 64.2, 64.1, 61.6 (OCH_2, Ph_3*C*-), 37.8 (-*C*H_2-NH-), 31.2 (-*C*H_2-S-), 28.2 [C(*C*H_3)$_3$]; ESI mass, found: *m/z* 851.345, calcd: 851.340 for (M+ Na)$^+$.

O-(aminooxyacetyl)pentaethylene glycolyl o-[n-(tritylthioethyl)carbamoyl]methoxyacetate 1

Compound **6** (0.16 g, 0.18 mmol) was dissolved in DCM (5 mL) and TFA (5 mL), and the solution was stirred at room temperature for 10 min. After concentration under vacuum, the residue was chromatographed on silica gel with toluene/methanol (17:3) to give the desired compound **1** (0.090 g, 0.12 mmol, 69%) as a colorless oil: R_f 0.19 ($CHCl_3$/methanol, 97/3); ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.42–7.10 (m, 15H, Ar), 6.92 (brs, 1H, CH_2N*H*), 4.33–4.27 (m, 6H, H_2NO-CH_2-CO-, -COO-CH_2-), 4.18 (s, 2H, -O-CH_2-CO-), 4.02 (s, 2H, -O-CH_2-CO-), 3.72–3.58 (m, 16H, O-CH_2-CH_2-), 3.13 (q, 2H, J = 6.4 Hz, -NH-CH_2-), 2.41 (t, 2H, J = 6.7 Hz, -S-CH_2-); ^{13}C-NMR: d 170.7, 169.8, 168.8 (*C* = O), 144.6, 129.6, 128.0, 126.8 (Ar),

70.5, 66.8 (OCH_2CO-), 70.6, 70.6, 70.5, 70.2, 68.9, 68.8, 68.7, 68.5, 64.2, 64.1, 63.8, 61.6 (OCH_2, Ph_3 *C*-), 37.7 (-CH_2-NH-), 31.9 (-CH_2-S-); ESI mass, found: *m/z* 729.310, calcd: 729.305 for $(M + H)^+$.

Cys[12](meobn), cys[25](acm)-ser-gly-a chain 7

Fmoc-Cys(Acm)-Wang resin (0.64 mmol/g, 0.234 g, 0.15 mmol) was swelled in 1-methyl-2-pyrrolidinone (NMP) for 30 min, and was treated with 20% piperidine/NMP for 5 and 15 min. After washing with NMP, Fmoc-Val-OBt, which was prepared by mixing Fmoc-Val-OH (0.60 mmol), 1 M *N,N'*-dicyclohexylcarbodiimide (DCC)/NMP (0.9 mL) and 1 M 1-hydroxybenzotriazole (HOBt)/NMP (0.9 mL) at room temperature for 30 min, was added and the reaction mixture was mixed with vortex at 50°C for 1 h. The resin was washed with NMP and 50% dichloromethane/methanol, treated with 10% acetic anhydride (Ac_2O)/5% *N,N*-diisopropylethylamine (DIEA)/NMP for 5 min, and washed with NMP. The peptide chain was elongated in essentially the same manner as described above, and H-Ser(Bu^t)-Gly-Pro-Glu(OBu^t)-Thr(Bu^t)-Tyr(Bu^t)-Val-Gly-Met-Gly-Ser(Bu^t)-Tyr(Bu^t)-Cys(Trt)-Cys(MeOBn)-Leu-Val-Gly-Cys(Trt)-Thr(Bu^t)-Arg(Pbf)-Asp(OBu^t)-Gln(Trt)-Leu-Ser(Bu^t)-Gln(Trt)-Val-Cys(Acm)-resin (0.839 g) was obtained. A part of the resin (104 mg) was treated with TFA cocktail (1.5 mL) at room temperature for 2 h. TFA was removed under an Ar stream and the peptide was precipitated with diethyl ether. After washing twice with ether, the precipitate was dried in vacuo. The crude peptide was dissolved in 6 M guanidine-HCl/50 mM phosphate buffer (pH 7.0, 9 mL), and dimethyl sulfoxide (DMSO, 1 mL) was added. The reaction mixture was gently stirred at room temperature for 2 d, and the crude peptide was purified by RP-HPLC on a Mightysil RP-18 column (Kanto Kagaku, Japan) with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **7** (2.41 μmol, 13% yield). MALDI-TOF mass, found: m/z 3047.0, calcd: 3047.5 for $(M + H)^+$ (average). Amino acid analysis: $Asp_{1.08}Thr_{1.77}Ser_{2.46}Glu_{3.02}Pro_{0.83}Gly_4$ $Val_{3.08}Met_{0.80}Leu_{2.18}Tyr_{1.89}$ $Arg_{1.09}$.

Cys[12](spy), cys[25](acm)-ser-gly-a chain 8

The peptide **7** (2.41 μmol) was dissolved in TFA (2.0 mL) and thioanisole (0.2 mL) containing 2,2'-dipyridyl disulfide (DPDS, 22 mg), and cooled to −10°C. Trifluoromethanesulfonic acid (TfOH, 100 μL) was added to the solution, and the mixture was kept at −10°C for 5 min. The crude peptide was precipitated with diethyl ether, washed twice with ether, and dried in vacuo. The residue was applied to the gel filtration HPLC using a TSKgel G3000PW_{XL} column (7.8φ × 300 mm, Tosoh, Japan) with 0.1% TFA/50% acetonitrile aqueous solution as a solvent at a flow rate of 0.5 mL/min, to give peptide **8** (2.41 μmol, quant.). MALDI-TOF mass, found: m/z 3036.0, calcd: 3036.5 for $(M + H)^+$ (average). Amino acid analysis: $Asp_{1.08}Thr_{1.76}Ser_{2.49}$ $Glu_{3.03}$ $Pro_{0.91}Gly_4Val_{3.04}Met_{0.89}Leu_{2.11}Tyr_{1.84}Arg_{1.09}$.

Cys[17](acm)-b chain 9

Starting from H-His(Trt)-Trt(2-Cl) resin (0.68 mmol/g, 0.368 g, 0.25 mmol), the peptide chain corresponding to the B chain of RGP was elongated essentially according to the method for the A chain **7** described above, and the protected peptide resin, H-Ala-Glu(OBu^t)-Lys(Boc)-Tyr(Bu^t)-Cys(Trt)-Asp(OBu^t)-Glu(OBu^t)-Asp(OBu^t)-Phe-His(Trt)-Met-Ala-Val-Tyr(Bu^t)-Arg(Pbf)-Thr(Bu^t)-Cys(Acm)-Thr(Bu^t)-Glu(OBu^t)-His(Trt)-resin (1.11 g) was obtained. A part of the resin (102 mg) was treated with TFA cocktail (1.5 mL) at room temperature for 2 h. TFA was removed under an Ar stream and the peptide was precipitated with diethyl ether. After washing twice with ether, the precipitate was dried in vacuo. The crude peptide was purified by RP-HPLC on a Mightysil RP-18 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **9** (3.11 μmol, 15% yield). MALDI-TOF mass, found: m/z 2519.2, calcd: 2519.0 for $(M + H)^+$. Amino acid analysis: $Asp_{1.96}Thr_{1.52}Ser_{2.78}Ala_{2.12}Val_{0.98}Met_{0.90}$ $Tyr_{2.03}$ $Phe_1Lys_{0.99}His_{2.00}Arg_{1.01}$.

Cys[a25, b17](acm)-ser-gly-rgp 10

Peptides **8** (2.56 μmol) and **9** (2.56 μmol) were dissolved in 30% acetonitrile/50 mM sodium bicarbonate aqueous solution (12.5 mL) and the solution was gently stirred at room temperature for 1 h. The reaction was quenched by adding acetic acid (240 μL), and the mixture was purified by RP-HPLC on a Mightysil RP-18 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **10** (1.10 μmol, 43% yield). MALDI-TOF mass, found: *m/z* 5444.9, calcd: 5445.0 for $(M + H)^+$ (average). Amino acid analysis: $Asp_{3.09}Thr_{3.56}Ser_{2.62}Glu_{6.04}Pro_{0.94}Gly_4Ala_{2.12}Val_{4.01}$ $Met_{1.65}Leu_{2.06}Tyr_{4.03}$ $Phe_{1.10}Lys_{1.10}His_{2.09}Arg_{2.11}$.

Aldehyde-gly-rgp 12

Peptide **11** (371 nmol) was dissolved in 50 mM phosphate buffer (pH 7.0, 370 μL) containing 1.3 equivalents of sodium periodate (480 nmol), and the mixture was mixed with vortex at room temperature for 1 h. Then, the mixture was purified by RP-HPLC on a Mightysil RP-18 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **12** (257 nmol, 69% yield). MALDI-TOF mass, found: *m/z* 5270.3, calcd: 5269.9 for $(M + H)^+$ (average). Amino acid analysis: $Asp_{3.04}Thr_{3.54}Ser_{1.68}Glu_{6.04}$

$Pro_{0.95}Gly_4Ala_{2.05}Val_{3.96}Met_{1.58}Leu_{2.02}Tyr_{3.90}Phe_{1.09}$ $Lys_{1.14}His_{2.02}Arg_{2.08}$.

Trts-peg-gly-rgp 13

Peptide **12** (78.7 nmol) was dissolved in 5% AcOH/*N*, *N*-dimethylacetamide (100 µL) containing 1% (w/v) of compound **1**, and the solution was mixed with vortex at room temperature for 5 h. The mixture was applied to the gel filtration HPLC using a TSKgel G3000PWXL column (7.8φ x 300 mm) with 0.1% TFA/50% acetonitrile aqueous solution as a solvent at a flow rate of 0.5 mL/min, to give peptide **13** (69.5 nmol, 88% yield). MALDI-TOF mass, found: *m/z* 5979.7, calcd: 5980.6 for $(M + H)^+$ (average). Amino acid analysis: $Asp_{2.96}Thr_{3.49}Ser_{1.90}Glu_{5.71}$ $Pro_{0.94}Gly_4Ala_{2.05}Val_{3.79}Met_{1.63}Leu_{2.17}$ $Tyr_{3.86}Phe_{1.08}$ $Lys_{1.12}His_{2.08}Arg_{1.98}$.

Hs-peg-gly-rgp 14

Peptide **13** (69.2 nmol) was dissolved in TFA/H_2O/triisopropylsilane (96/2/2, 100 µL), and the solution was mixed with vortex at room temperature for 30 min. The crude peptide was precipitated with diethyl ether, washed twice with ether, and dried *in vacuo*. The crude peptide was purified by RP-HPLC on a Mightysil RP-18 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **14** (31.4 nmol, 45% yield). MALDI-TOF mass, found: *m/z* 5738.3, calcd: 5738.3 for $(M + H)^+$ (average). Amino acid analysis: $Asp_{2.85}Thr_{3.33}Ser_{1.54}Glu_{5.61}$ $Pro_{1.37}Gly_4Ala_{2.06}Val_{3.92}Met_{1.64}Leu_{2.07}$ $Tyr_{3.82}Phe_{1.04}$ $Lys_{1.07}His_{2.04}Arg_{1.98}$.

Evaluation of antibodies by ELISA

To obtain titer curves of anti-serum against Aam-RGP, ELISA was conducted. The wells of PVC microtiter plates were coated with 1 µM Aam-RGP, Aam-RGP A-chain, or Aam-RGP B-chain in PBS (100 µL) and incubated overnight at 4°C. After washing and blocking, the plates were dried at 4°C. The anti-serum diluted in PBST (100 µL) was added to the wells and incubated overnight at 4°C. After washing, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (IgG) (Zymed Laboratories Inc., CA) diluted in PBST (100 µL) was added to the wells and incubated for 2 h at room temperature. After washing, tetramethylbenzine (TMB)/hydrogen peroxidase solution (100 µL) was added to the wells and incubated for 30 min at room temperature. The reaction was stopped by adding 1 M sulfuric acid (100 µL), and absorbance at 450 nm was measured by a microplate reader Model 680 (Bio-Rad Laboratories, Inc., Hercules, CA).

Immunoblotting analysis

Ppe-RGP, Aam-RGP, Aja-RGP, Aam-RGP A-chain, Aam-RGP B-chain, bovine insulin (Sigma), and human relaxin-3 (PeproTech, NJ) were dissolved in gel sample buffer without 2-mercaptoethanol. Aliquots (0.1 nmoles/well) were loaded into the lanes of a sodium dodecylsulfate–polyacrylamide (SDS–PAGE) mini slab (15% gel) (Cosmobio, Tokyo, Japan) and resolved by electrophoresis. Peptides separated by SDS–PAGE were transferred to an Immobilon membrane (Millipore, Billerica, MA, USA) by electroblotting, as described previously [27]. The membrane was rinsed in Tris-buffered saline (TBS) consisting of 20 mM Tris–HCl (pH 7.5) and 150 mM NaCl, blocked with 5% nonfat dry milk in TBS containing 0.1% Tween-20 (TTBS), and incubated with a 1:20,000 dilution of anti-serum against Aam-RGP in TTBS overnight at 4°C. After three washes with TTBS, the membrane was incubated with a 1:30,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma-Aldrich, MO). After three further washes with TTBS, phosphatase activity was visualized by treating the membrane with 0.2 mM 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and nitroblue tetrazolium in 100 mM diethanolamine buffer (pH 9.5) containing 5 mM $MgCl_2$.

Author contributions

H. Katayama and M. Mita conceived and designed the study, and wrote the manuscript. H. Katayama, R. Mizuno, and M. Mita performed experiments.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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