Design and synthesis of a hydrogen peroxide-responsive amino acid that induces peptide bond cleavage after exposure to hydrogen peroxide

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1. Introduction

Reactive oxygen species (ROS) play a wide variety of roles in health and disease. 1 Hydrogen peroxide (H2O2) is one of the ROS and its controlled generation is essential for living organisms, e.g., cellular signalling 2 and removal of invading pathogens. 3 Loss of ROS regulation, however, can cause oxidative damage to cellular components, and thereby H2O2 is thought to be involved in numerous diseases and aging. 4 Cancer is an oxidative stress related disease and production of ROS in most cancer cells is known to be elevated. 5 For example, the intracellular concentration of H2O2 in cancer cells was estimated to be much higher than that in normal cells (in cancer cells: 10 to 100 μM; in normal cells: up to 0.7 μM). 6 Therefore, it is of interest to develop H2O2-responsive fluorophores, 7 prodrugs, 6a,8 and drug carriers 9 to detect and treat cancer. With these in mind, we decided to prepare an H2O2-responsive amino acid that induces peptide bond cleavage in the presence of H2O2, because it would potentially be applicable to the development of oxidative stress-responsive peptidyl prodrugs and drug carriers.

Figure 1. Stimulus-responsive peptide bond cleavage system (PG: a protective group that can be removed by an appropriate stimulus; TBDPS: t-butyldiphenylsilyl).
Previously, we had reported a stimulus-responsive amino acid and its application to control peptide function in living cells (Scheme 1).10 When peptide 1, which possesses the stimulus-responsive amino acid, is exposed to an appropriate stimulus, removal of a PG (a protective group that can be removed by the stimulus) followed by lactonization of the trimethyl lock unit11 will cause peptide bond cleavage. In principle, peptide 1 can respond to any stimuli simply by changing the PG. In this paper, the development of the H2O2-responsive amino acid and its application to the H2O2-responsive peptide are reported.

2. Results and discussion

As the H2O2-responsive protective group, a 4-methylphenylboronic acid was employed because it is widely used as a triggering device that responds to intracellular H2O2. The H2O2-responsive protective group is advantageous because it is easily oxidized to carboxylic acid and activates the protective group via formation of the trimer. Therefore, disodium tetraborate (Na2B4O7), which would competitively prevent the boronic acid-protected boronic acid on the peptide may cause the aggregation induced aggregation, was added to the Fmoc removal cocktail initiated from Boc derivative 9. Removal of the Boc group by protic acids was first examined. However, partial deprotection of the boronate was observed (data not shown). In this study, therefore, aprotic conditions using t-butyldimethylsilyl triflate (TBSOTf) was employed,15 and the obtained amine was protected with an Fmoc group to generate MIDA protected building block 3. Purification of 3 by open column chromatography was more difficult than that of 2, because of its high polarity. Therefore, purification of 3 using preparative TLC was carefully performed, and the product was obtained in moderate yield.

Fmoc-protected H2O2-responsive amino acid derivatives 2 and 3 were prepared as shown in Scheme 3A. Since an oxidation reaction after the introduction of the boronate unit was expected, oxidation-tolerant MIDA protection was employed. Alkylation of the reaction after the introduction of the boronate unit was expected, and the t-butoxycarbonyl (Boc) group was replaced was removed in the presence of sodium bicarbonate in aqueous methanol, and the t-butoxycarbonyl (Boc) group was replaced was removed in the presence of sodium bicarbonate in aqueous methanol, and the t-butoxycarbonyl (Boc) group was replaced with an Fmoc group by the usual manner to generate building block 2. Conversely, preparation of MIDA derivative 3 was also prepared from Boc derivative 9. Removal of the Boc group by protic acids was first examined. However, partial deprotection of the boronate was observed (data not shown). In this study, therefore, aprotic conditions using t-butyldimethylsilyl triflate (TBSOTf) was employed,15 and the obtained amine was protected with an Fmoc group to generate MIDA protected building block 3. Purification of 3 by open column chromatography was more difficult than that of 2, because of its high polarity. Therefore, purification of 3 using preparative TLC was carefully performed, and the product was obtained in moderate yield.

Next, the incorporation of H2O2-responsive units 2 and 3 into a model peptide was attempted (Scheme 3B). Peptide elongation was performed on NovaSyn® TGR resin and the H2O2-responsive unit was incorporated by usual Fmoc SPPS to afford protected peptide resin 10 or 11. Elongation of a peptide on 11 was not problematic; however, the resin became sticky when 10 and its peptide-elongated derivatives were subjected to Fmoc removal with 20% (v/v) piperidine/DMF was used; 1) Fmoc SPPS. Before global deprotection, the resin was treated with sat. NaHCO3 aq./H2O/MeOH = 1/1/2 (v/v). Global deprotection conditions: TFA/triethylsilane/H2O = 95/2.5.2.5 (v/v). (A: alanine; D: aspartic acid; G: glycine; Q: glutamine; R: arginine; S: serine; Y: tyrosine)
and suppression of the aggregation of the resin was successfully achieved to yield model peptide 12. Conversion of peptide resin 11 to peptide 12 was also accomplished by the usual Fmoc SPPS followed by on-resin removal of the MIDA group and subsequent global deprotection. Regardless of which building block was used the chemical yield of peptide 12 was quite similar. Therefore, building block 2, which can be easily purified and obtained in higher yield, was employed in the following experiments.

With H$_2$O$_2$-responsive peptide 12 in hand, we examined the H$_2$O$_2$-responsive cleavage of the peptide (Figure 2A). In this experiment, guanidine hydrochloride was added to dissolve the peptide in an aqueous sodium phosphate buffer (pH 7.4). Glycine and benzamide were also added to scavenge for an electrophilic quinone methide via 1,6-addition of an amino group$^{9}$ and as an internal standard, respectively. H$_2$O$_2$ was added to the solution of peptide 12 and the obtained mixture was incubated at 37 °C (concentration of the components just after the addition of H$_2$O$_2$ is as follow: 10 μM peptide 12, 6 M guanidine hydrochloride, 100 mM glycine, 25 ppm benzamide, 20 mM phosphate, 100 μM H$_2$O$_2$). The reaction progress was monitored by HPLC, and the peptides were characterized by ESI-TOF MS (Figure 2B). A compound eluted at 9.8 min was identified as quinone methide-glycine conjugate 17 by coinjection with an authentic sample prepared according to literature.$^{9}$ Within 1 h of the incubation, substrate 12 had disappeared completely (half-life = 11.4 min) (Figure 2C). Intermediate 13, but not 14, was observed; therefore, removal of the 4-hydroxyphenylmethyl group was estimated as a rate-limiting step. After the complete consumption of substrate 12, disappearance of intermediate 13 showed first-order dependence on the relative amount of 13 and the half-life was determined as 231 min. The cleavage products showed first-order dependence on the relative amount of $\mu$M H$_2$O$_2$ (Figure 2C). Intermediate 13, but not 12, had disappeared completely (half-life = 11.4 min) (Figure 2C). Therefore, building block 2, which can be easily purified and obtained in higher yield, was employed in the following experiments.  


References and notes
12. As the hydrogen peroxide-responsive amino acid, compound 18 was also designed and we first attempted to prepare it. However, mass of the product was identical to that of 19 but not 18 (ESI-Ion Trap MS, m/z calcd for [M + Li, Na, or K]+ 360.2, 376.2, or 392.2, found 359.7, 375.8, or 392.1, respectively). In a previous study, it was also observed that introduction of a sterically demanding substituent on the phenol of the trimethyl lock unit was problematic than that of benzyl derivatives.10b In this study, therefore, we attempted to synthesize 2 and 3 that possesses the benzyl type protection on the phenol.


17. To perform the reaction without guanidine, concentration of peptide 12 was reduced. However, the peptide was not dissolved in an aqueous solvent even if the concentration of 12 was 1 μM. Therefore, the use of guanidine is essential for the reaction of peptide 12. For application of the H₂O₂-responsive peptide in cells, in our opinion, incorporation of a hydrophilic sequence such as cell penetrating peptides represented by an octaarginine would help to dissolve the peptide in an aqueous solvent without guanidine. About the octaarginine, see: Nakase, I.; Takeuchi, T.; Tanaka, G.; Futaki, S. Adv. Drug Delivery Rev. 2008, 60, 598–607 and references cited therein.

18. Whereas extracellular pH is acidified, intracellular pH is normally maintained above pH 7 in a certain tumors such as solid tumors. See: Vaupel, P.; Kallinowski, F.; Okunieff, P. Cancer Res. 1989, 49, 6449–6465, and references therein.

19. When concentration of glycine was reduced to 10 mM, addition of peptides to a quinone methide was observed (data not shown). Therefore, 100 mM of glycine was employed to completely suppress the reaction of peptides and a quinone methide for clarity of HPLC charts.


Supplemental materials

Details for preparation of the hydrogen peroxide-responsive peptides and the oxidation induced peptide bond cleavage can be found in the Supporting Information.
Figure 2. Hydrogen peroxide-responsive peptide bond cleavage. A) Reagents and conditions: a) 100, 10, or 0 μM H$_2$O$_2$, 10 μM 12, 20 mM sodium phosphate buffer with 100 mM glycine and 6 M guanidine hydrochloride (pH 7.4), 25 ppm benzamide as an internal standard, 37 °C. B) HPLC profiles of the reaction using 100 μM H$_2$O$_2$. *Non-peptidyl peaks. **Benzamide as an internal standard. C) Time course of the disappearance of the substrate 12, intermediate 13 and products 15 + 16. The relative amount was calculated using the following equation in which PA$_x$ means peak area of $x$ in the HPLC chromatogram. Relative amount of 13 or 15 + 16 = $(\text{PA}_{13} \text{ or } \text{PA}_{15} + \text{PA}_{16}) \times \text{PA}_{12}(t = 0) \div (\text{PA}_{12}(t = 0) + \text{PA}_{13} \times \text{PA}_{12}(t = 0))$. The average and standard deviation of three measurements is shown at each time point.