

# Comparison of the catalytic activities of 5-mer synthetic peptides derived from Box A region of Tob/BTG family proteins against the amyloid-beta fragment peptides

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## Abstract

We previously reported the catalytic activity of 9-mer peptide, JAL-TA9 (YKGSFRMI), derived from the Box A region of Tob/BTG family of proteins. This was the first report of the catalytic activity of shorter synthetic peptides. Therefore, we used 'Catalytide' (catalytic peptide) as the general term for peptides possessing the hydrolase activity. NMR study of JAL-TA9 suggested that the minimum sequence required for the catalytic activity is a 5-mer peptide (GSGFR). In this study, we examined the catalytic activity of this 5-mer peptide against A $\beta$  fragment peptides, A $\beta$ 1-20 and A $\beta$ 11-29, to find the next Catalytide. Activity of all peptides identified as Catalytides, especially, GQAYR (BTG3) and GQAFR (BTG4), was higher activity than that of GSGFR (Tob1 and 2) and GSGYR (BTG1 and 2). The cleavage mechanism of these Catalytides is still not well understood and needs further investigation. Nonetheless, 5-mer Catalytides are attractive candidates for the development of peptide drugs as a new strategy for treating Alzheimer's disease (AD).

**Abbreviations:** APP: Amyloid Precursor Protein; A $\beta$ : Amyloid Beta; AD: Alzheimer's Disease; HSA: Human Serum Albumin; TFA: Trifluoroacetic Acid; HPLC: High-Performance Liquid Chromatography; NMR: Nuclear Magnetic Resonance.

## Introduction

We recently reported a synthetic nona-peptide, JAL-TA9 (YKGSFRMI), which possesses proteolytic activity. Our study was the first to report peptide enzyme. Therefore, we used the term Catalytide (Catalytic peptide) for the shorter proteolytic peptides [1]. JAL-TA9 is derived from the Box A region of Tob1, a member of the Tob/BTG family comprising BTG1, BTG2, BTG3/ANA, BTG4, and Tob2. The Box A region of Tob/BTG family is a highly conserved homology domain. According to the recent reports, these proteins display antiproliferative activity in a variety of cell types and are involved in the regulation of tumorigenesis. However, the role of Box A region of Tob/BTG family of proteins remains unclear [2-7].

JAL-TA9 (YKGSFRMI) is the first Catalytide reported to have auto-proteolytic and hydrolase activities against A $\beta$  fragment peptides. In addition, the secondary structure of JAL-TA9 predicted using NMR, suggested that the 5-mer peptide (GSGFR) might possess the catalytic activity similar to that of JAL-TA9 [1]. In the current study, we evaluated the proteolytic activity of 4 types of 5-mer synthetic peptides derived from the Box A region in Tob/BTG family of proteins (SFig 1) to find a new Catalytides like JAL-TA9 and understand the hydrolase activity of Tob/BTG family proteins.

## Methods

### Chemical peptide synthesis

The peptides were synthesized from fluorenylmethyloxycarbonyl chloride (Fmoc)-protected L-amino acid derivatives according to the method described by Kojima *et al.* using an automated peptide synthesizer (model 433A, Applied Biosystems, California, USA, 0.1 mmol scale with preloaded resin) [8]. After deprotection according to the manufacturer's protocol, each peptide was purified using reverse-phase high-performance liquid chromatography (HPLC, Capcell Pak C18 column, SG, 10 or 15 mm i.d.  $\times$  250 mm; Shiseido Co., Ltd. Japan) with linear gradient elution from 0.1% trifluoroacetic acid (TFA) to 50% or 70% CH<sub>3</sub>CN containing 0.1% TFA over 30 min. The flow rate was set to 3.0 or 6.0 mL/min. The primary peak was collected and lyophilized. The purity of the synthetic peptides and the progress of the enzymatic reaction were confirmed using an analytical reverse-phase HPLC (Capcell Pak C18 column, MGII, 4.6 mm i.d.  $\times$  150 mm; Shiseido Co., Ltd., Japan), and the flow rate was set to 1.0 mL/min. Linear gradient elution was carried out from 0.1% TFA to 70% CH<sub>3</sub>CN containing

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**Key words:** catalytide, hydrolase activity, Tob/BTG, Box A region, 5-mer synthetic peptide, amyloid- $\beta$  peptide, alzheimer's disease

**Received:** May 27, 2019; **Accepted:** June 13, 2019; **Published:** June 17, 2019

0.1% TFA. The column eluate was monitored with a photodiode-array detector (SPD-M20A; Shimadzu, Japan). Each purified peptide was characterized by electrospray ionization (ESI)-mass spectrometry (MS) using a Qstar Elite Hybrid LC-MS/MS system [8].

### Analysis of proteolytic activity and determination of cleavage sites

5-mer peptides (final concentration of 0.2 mM) were individually incubated with the peptide fragment derived from the A $\beta$ 42 (final concentration of 0.05 mM) in the presence of human serum albumin (HSA) (final concentration of 0.025% w/v) in phosphate buffered saline (PBS, pH 7.4) at 37 °C. Ten microliters of each reaction mixture was analyzed in a time-dependent manner using the analytical HPLC system described above. For peak collection, 20  $\mu$ L of the reaction mixture was loaded into the same HPLC system, and the peak fractions monitored at 220 nm were collected into microtubes (Eppendorf Safe-Lock Tubes, 1.5 mL).

After lyophilization, an appropriate quantity, as determined from the chromatographic peak heights, of 36% CH<sub>3</sub>CN containing 0.1% HCOOH was added with stirring by an automatic mixer. The cleavage sites were determined by ESI-MS using the flow injection method with 70% CH<sub>3</sub>CN containing 0.1% HCOOH on a Qstar Hybrid LC-MS/MS system (ABI), and the flow rate was set to 0.1 mL/min [12].

### Results

We synthesized 4 types of 5-mer synthetic peptides, GSGFR, GSGYR, GQAYR, and GQAFR which were derived from the Box A region of Tob/BTG family proteins (SFig. 1). Each peptide was purified using a reversed-phase preparative HPLC and the purity of each peptide was checked using an analytical HPLC system using a photodiode array detector. The molecular weights of purified peptides were confirmed with MS (SFig. 2). On the basis of these data, we judged that all peptides were of adequate purity for carrying out the following experiments.

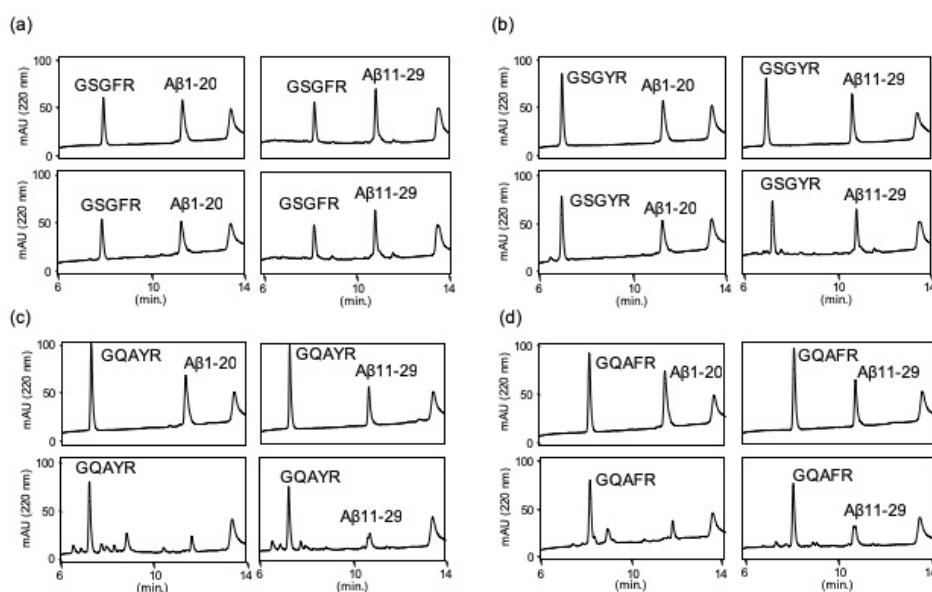
The proteolytic activity was analyzed as reported previously [1]. Each peptide (final concentration 0.2 mM) was co-incubated with A $\beta$ 1-

20 or A $\beta$ 11-29 as substrates (final concentration 0.05 mM) in PBS at 37 °C in the presence of HSA (human serum albumin), and 10  $\mu$ L of the reaction mixture was subsequently analyzed by an analytical HPLC on day 0, 2, 4, and 5 (SFig. 3 and 4). A $\beta$ 1-20 and A $\beta$ 11-29, fragment peptides of A $\beta$  peptide, were stable in PBS containing HSA (SFig 3). The proteolytic activity was evaluated by the decrease in substrate concentration. For GSGFR and GSGYR, the peak heights of A $\beta$ 1-20 and A $\beta$ 11-29 slightly decreased; however, no new peak was observed on the chromatogram even in day 2 sample, indicating that the proteolytic activities of GSGFR and GSGYR were very weak (Figures 1a & 1b). On the other hand, GQAYR and GQAFR completely cleaved A $\beta$ 1-20 in 2 days, and several new peaks were observed (Figures 1c & 1d).

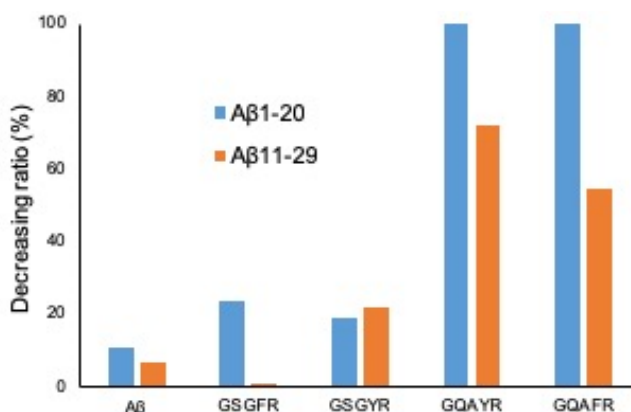
The decreasing ratio of 4 peptides against A $\beta$ 1-20 and A $\beta$ 11-29 is shown in Figure 2. These data indicate that the proteolytic activities of GQAYR and GQAFR against A $\beta$ 1-20 and A $\beta$ 11-29 are stronger than those of GSGFR and GSGYR.

We next identified the cleavage sites on A $\beta$ 1-20 and A $\beta$ 11-29 when using GQAYR or GQAFR. The peak heights of both 5-mer peptides and substrates decreased in a time dependent manner. When A $\beta$ 1-20 was used as a substrate, it almost disappeared on day 2; however, GQAYR and GQAFR were still remained. Therefore, we continued the incubation (SFig. 4) and collected every peak that appeared on day 5 by monitoring at 220 nm (Figure 3). Additional 12 (A1-10) and 4 peptides (C1-4) were identified as fragment peptides derived from A $\beta$ 1-20 and GQAYR, respectively (Figure 3a). For GQAFR, 12 (A1-7) and 5 peptides (C1-5) were also identified as fragment peptides derived from A $\beta$ 1-20 and GQAFR, respectively (Figure 3b). The cleavage sites on A $\beta$ 1-20 corresponding to GQAYR and GQAFR are shown in Fig. 3c. These data indicate that GQAYR and GQAFR possess the auto-proteolytic activity as well as can cleave the A $\beta$ 1-20 similar to JAL-TA9 [1].

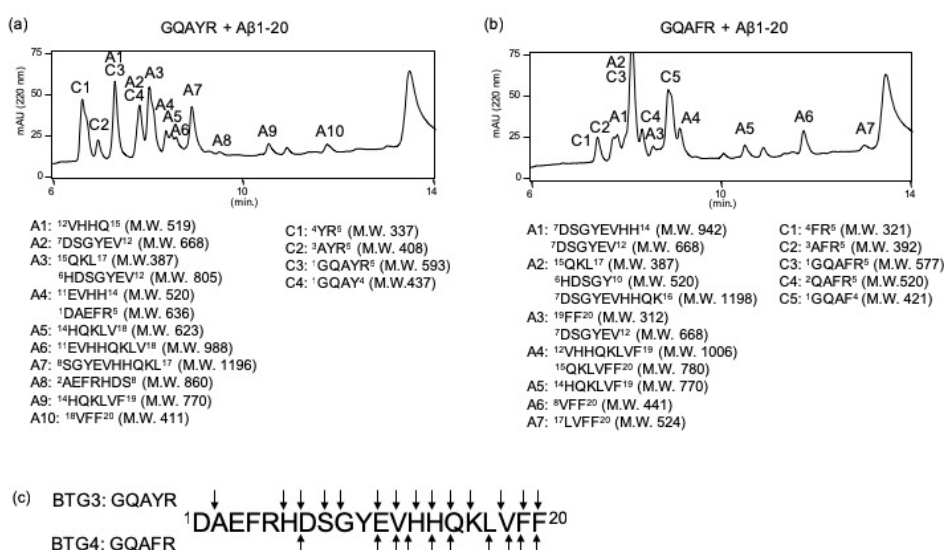
We next identified the cleavage sites on A $\beta$ 11-29. Eight (A1-7) and four peptides (C1-4) were identified as fragment peptides derived from A $\beta$ 11-29 and GQAYR, respectively (Figure 4a). For GQAFR, 12 (A1-7) and 4 peptides (C1-4) were also identified as fragment peptides derived from A $\beta$ 11-29 and GQAFR, respectively (Figure 4b).



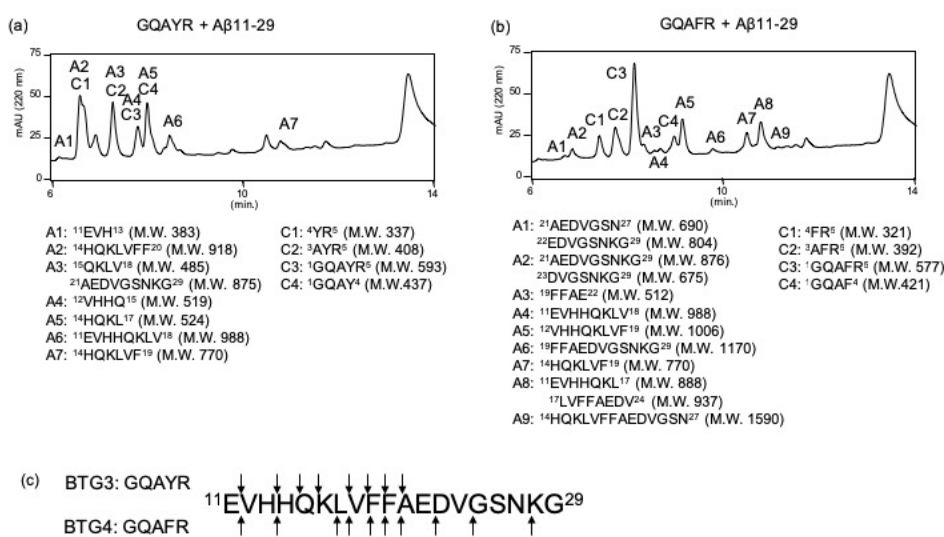
**Figure 1.** Chromatogram of reaction mixtures. (a) GSGFR, (b) GSGYR, (c) GQAYR or (d) GQAFR were co-incubated with (left) A $\beta$ 1-20 or (right) A $\beta$ 11-29 in PBS containing HSA, and 10  $\mu$ L of the reaction mixture was analyzed at 0 (upper) and 2 days (lower)



**Figure 2.** Decreasing ratio of each substrate co-incubate with 5-mer peptide. The decreasing ratio was calculated by the peak height obtained on day 0 and day 2 in HPLC analysis



**Figure 3.** Determination of cleavage site against Aβ1-20. On day 5, 20 μL of reaction mixture was injected and all peaks were collected. Aβ1-20 was co-incubated with (a) GQAYR or (b) GQAFR. (c) Cleavage sites



**Figure 4.** Determination of cleavage site against Aβ11-29. On day 5, 20 μL of reaction mixture was injected and all peaks were collected. Aβ1-20 was co-incubated with (a) GQAYR or (b) GQAFR. (c) Cleavage sites

The cleavage sites on A $\beta$ 11-29 corresponding to GQAYR and GQAFR are shown in Figure 4c. Interestingly, no fragment peak derived from HSA was identified in any of the above cases. The other peptides GSGFR and GSGYR, also showed the proteolytic activity (SFig. 5).

## Discussion

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder caused by the aggregation and accumulation of A $\beta$ 42. Many trials have been conducted to develop drugs for the treatment of AD, however, the results have not been encouraging [9-14]. Thus, new effective drugs are urgently required for treating AD. Among 4 peptides tested in this study, GQAYR and GQAFR strongly cleaved A $\beta$  fragment peptides, especially A $\beta$ 1-20, with many cleavage sites, (Figures 3 & 4) suggesting that these 5-mer peptides can cleave A $\beta$ 42.

The antiproliferative ability of the Tob/BTG family of proteins is due to their association with the target protein in the cell. The highly conserved region in N-terminal region of the Tob/BTG, Box A, mediates the majority of the Tob/BTG interactions. However, no study has reported the hydrolase activity of Tob/BTG family proteins themselves or their fragment peptides. In this study, we found the proteolytic activity of the synthetic 5-mer peptides derived from the Box A region of Tob/BTG family proteins; especially GQAYR from BTG3 and GQAFR from BTG4 fragment peptide showed potent activity (Figure 2) with many cleavage sites (Figures 3 & 4). These results suggest that Tob/BTG family proteins may also possess previously unknown proteolytic activity.

The mechanism underlying the proteolytic activity remains unexplored. To elucidate this mechanism, stereo-structure analyses are required. Therefore, we attempted to predict the secondary structure *in silico* on a personal computer using MM2 and MMFF94 programs. The stereo-structures of GSGFR and GSGYR were similar to each other. In addition, the location of three amino acids, Arg-Phe/Tyr-Ser, seemed to be close (SFig. 6a and b). On the other hand, the stereo-structure and the location of Arg, Phe/Tyr-Gln were also similar to each other but were different from those in GSGFR and GSGYR (SFig. 6c and d). These results may relate to the proteolytic activity (Figure 2). We are currently investing the stereo-structures of 5-mer peptides using NMR analysis.

## Conclusion

We conclude that 5-mer peptides derived from the Box A region of Tob/BTG family of proteins cleave A $\beta$  fragment peptides and are categorized as Catalytides. Therefore, GQAYR and GQAFR can also be categorized as the Catalytides. Along with JAL-TA9, these Catalytides can prove as attractive drug candidates for the prevention and treatment of AD.

## Declarations

Availability of data and material

All data generated or analysed during this study are included in this published article.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

T. A. and R. N. were responsible for the experimental design and data interpretation. T. A., R. N., Y. H., and M. S. mainly contributed to

writing and reviewing the manuscript. R. N. and M. K. conducted all of the experiments, especially the HPLC analyses and the determination of cleavage sites. R. N. and M. K. contributed to the MS analyses. M. K., and R. N. contributed to the structure analyses using NMR and the computer modeling.

## Acknowledgements

This work was partially supported by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (KAKENHI) Program, Grant Number 15K07908, and the Okinawa Institute of Science and Technology (OIST) Proof-of-Concept (POC) Program.

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