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Molecular evolution of *Streptomyces* serine peptidase for the synthesis of β-alanyl-histidine

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Research aims

Anserine (β-Ala-π-methyl-L-His), carnosine (β-Ala-L-His; Car), and balenine (β-Ala-τ-methyl-L-His) have been known to exist in excitable tissues such as skeletal muscle and brain. Moreover, β-Ala-branched chain amino acids (β-Ala-BCAA) also induced hyperactivity behavior as observed with carnosine.[1] Therefore, β-Ala containing dipeptides received special attention as a research target for functional analyses because of the availability of the reagent.

Some serine peptidases exhibit peptide or amino acid ligation (*i.e.* aminolysis) in accordance with their hydrolytic activity. Moreover, mutation of catalytic Ser to Cys to engineer the serine peptidase into "transpeptidase" for the peptide ligation has been well characterized.[2,3] Our recent approach demonstrates that wide distribution of family S9 aminopeptidases was confirmed.[4] Among enzymes from actinomycetes, we obtained S9 aminopeptidase from *Streptomyces thermocyaneoviolaceus* (S9AP-St) that can hydrolyze β-Ala derivatives. In this study, peptide synthetic activity of S9AP-St and its engineered variant, S502C S9AP-St was evaluated.

Methods

The engineered S9AP-St variant, S502C S9AP-St, was constructed on the basis of mutagenesis technique for QuickChange site directed mutagenesis kit (Stratagene). Wild-type and S502C S9AP-Sts were expressed in *E. coli* BL21 (DE3) harboring the expression vector for wild-type or S502C S9AP-St production. The enzymes were purified with anion exchange chromatography.

Peptide synthesis by aminolysis reaction was performed as follows: 10 μl of acyl donor substrate and 10 μl of acyl accepter substrate (they are amino acid derivative solution (in DMSO) at an appropriate concentration (~0.5 M)) were added into 75 μl of 0.25 M Tris-HCl (pH 8.0). The reaction was initiated by adding 5 μl of enzyme solution (2 mg/mL). The reaction was then continued at 25°C for an appropriate time (20 min to 50 h). After the reaction was terminated by adding 0.1 ml of 3% formic acid, the reaction mixture was analyzed by UPLC-ESI-TOF-MS or amino acid analyzer.

Results

S9AP-St displayed efficient hydrolytic activity toward L-Phe-pNA and slight hydrolytic activity toward β-Ala-pNA. Its hydrolytic activity was abolished by the site-directed mutagenesis of catalytic Ser^{502} into Cys. We evaluated the aminolytic activity of wild-type and S502C S9AP-Sts using various aminoacyl derivatives. When hydrophobic aminoacyl derivatives were used as substrates, S502C S9AP-St exhibit high peptide synthetic activity, as expected. However, the enzyme showed no aminolytic activity toward β-Ala-derivatives. In contrast, β-alanyl homopeptides were synthesized by wild-type S9AP-St whereas the enzyme showed no aminolytic activity toward hydrophobic aminoacyl derivatives (Table 1). We next evaluated the synthetic ability of heteropeptides containing β-Ala by using β-Ala-OBzl and various aminoacyl-derivatives (−OMe or OEt) as substrates. Wild-type S9AP-St could synthesize a wide variety of β-Ala peptides (Table 1). Interestingly, S9AP-St preferentially synthesized β-Ala-Xaa derivatives. In contrast, S502C S9AP-St could synthesize only Xaa-β-Ala derivatives with the specificity toward hydrophobic aminoacyl derivatives. The results indicated that the substrate specificity of aminolysis reaction was changed by the substitution of catalytic Ser^{502} with Cys.

Because S9AP-St had synthetic ability of β-Ala-L-His-OMe (Car-OMe) and β-Ala-BCAA-OMes, we next investigated the optimization of production of such biologically active peptides using S9AP-St. For this investivation, Car-OMe was selected as a main product. As shown in Fig. 1A, the Car-OMe was steadily produced at the β-Ala-OBzl concentration of 10~40 mM when L-His-OMe concentration was maintained at a steady level of 20 mM. In contrast, the productivity of β-Ala-β-Ala-OBzl was increased following the increase of β-Ala-OBzl concentration. On the other hand, the productivity of Car-OMe was increased and that of β-Ala-β-Ala-OBzl was decreased following the increase of L-His-OMe concentration (Fig. 1A). As a result of the investigation on the effect of pH, the pH dependence curve for Car-OMe production was similar with that for the β-Ala-pNA hydrolytic activity (Fig. 1B). The Car-OMe was synthesized at this pH range with maximum production at pH 8.5. However, the productivity of byproduct, β-Ala-β-Ala-OBzl, was increased following the increase of pH (Fig. 1B). The production of another undesirable product, β-Ala-β-Ala-β-Ala-OBzl, hardly observed at these investigations.

As shown in Fig. 1C, when 20 mM β-Ala-OBzl and 20 mM L-His-OMe were used as substrates, Car-OMe was efficiently synthesized until 5 h, and the quantity of Car-OMe

Table 1. Tested chemicals and peptides synthesized by both wild-type and S502C S9AP-Sts.

Substrate	Products					
	wild-type S9AP-St			S502C S9AP-St		
	w/βA-OBzl				w/βA-OBzl	
		βA-Xaa, Xaa-βA	Byproducts		βA-Xaa, Xaa-βA	Byproducts
G-OMe	n.d.	n.d.	(βA)$_2$-OBzl	G$_3$-OMe	G-βA-OBzl	G$_3$-OMe, G$_2$-βA-OBzl
L-V-OMe	(L-V)$_2$-OMe	βA-L-V-OMe	(βA)$_2$-OBzl, (βA)$_2$-L-V-OMe	n.d.	n.d.	n.d.
L-L-OEt	n.d.	βA-L-L-OEt	(βA)$_2$-OBzl	(L-L)$_2$-OMe, (L-L)$_3$-OMe etc	L-L-βA-OBzl	(L-L)$_2$-OMe, (L-L)$_3$-OMe etc
L-I-OMe	n.d.	βA-L-I-OMe	(βA)$_2$-OBzl	n.d.	n.d.	n.d.
L-M-OMe	n.d.	βA-L-M-OMe	(βA)$_2$-OBzl, (βA)$_2$-L-M-OMe etc	(L-M)$_2$-OMe	L-M-βA-OBzl	(L-M)$_2$-OMe, (L-M)$_2$-βA-OBzl
L-F-OEt	n.d.	βA-L-F-OEt	(βA)$_2$-OBzl, (βA, L-F)-βA-OBzl etc	(L-F)$_2$-OMe, (L-F)$_3$-OMe etc	L-F-βA-OBzl	(L-F)$_2$-OMe, (L-F)$_3$-OMe etc
L-W-OMe	n.d.	βA-L-W-OMe	(βA)$_2$-OBzl	(L-W)$_2$-OMe	L-W-βA-OBzl	(L-W)$_2$-OMe
L-Y-OMe	n.d.	βA-L-Y-OMe	(βA)$_2$-OBzl, (βA)$_2$-L-Y-OMe etc	(L-Y)$_2$-OMe	L-Y-βA-OBzl	(L-Y)$_2$-OMe, (βA)$_2$-L-Y-OMe
L-P-OMe	n.d.	L-P-βA-OBzl	(βA)$_2$-OBzl, L-P-(βA)$_2$-OBzl etc	n.d.	n.d.	n.d.
L-S-OMe	n.d.	n.d.	(βA)$_2$-OBzl	n.d.	n.d.	n.d.
L-T-OMe	(L-T)$_2$-OMe	βA-L-T-OMe	(βA)$_2$-OBzl, (L-T)$_2$-OMe	n.d.	n.d.	n.d.
L-N-OMe	n.d.	βA-L-N-OMe	(βA)$_2$-OBzl	n.d.	n.d.	n.d.
L-H-OMe	n.d.	βA-L-H-OMe	(βA)$_2$-OBzl	n.d.	n.d.	n.d.
L-K-OMe	n.d.	βA-L-K-OMe	(βA)$_2$-OBzl	n.d.	n.d.	n.d.
L-R-OMe	n.d.	βA-L-R-OMe	(βA)$_2$-OBzl	n.d.	n.d.	n.d.
L-D-OMe	n.d.	n.d.	(βA)$_2$-OBzl	n.d.	n.d.	n.d.
L-E-OMe	n.d.	n.d.	(βA)$_2$-OBzl	n.d.	n.d.	n.d.
bA-OMe	n.d.	—	—	n.d.	—	—
bA-OBzl	(βA)$_2$-OBzl	—	—	n.d.	—	—
bA-OtBu	(βA)$_2$-OtBu	—	—	n.d.	—	—

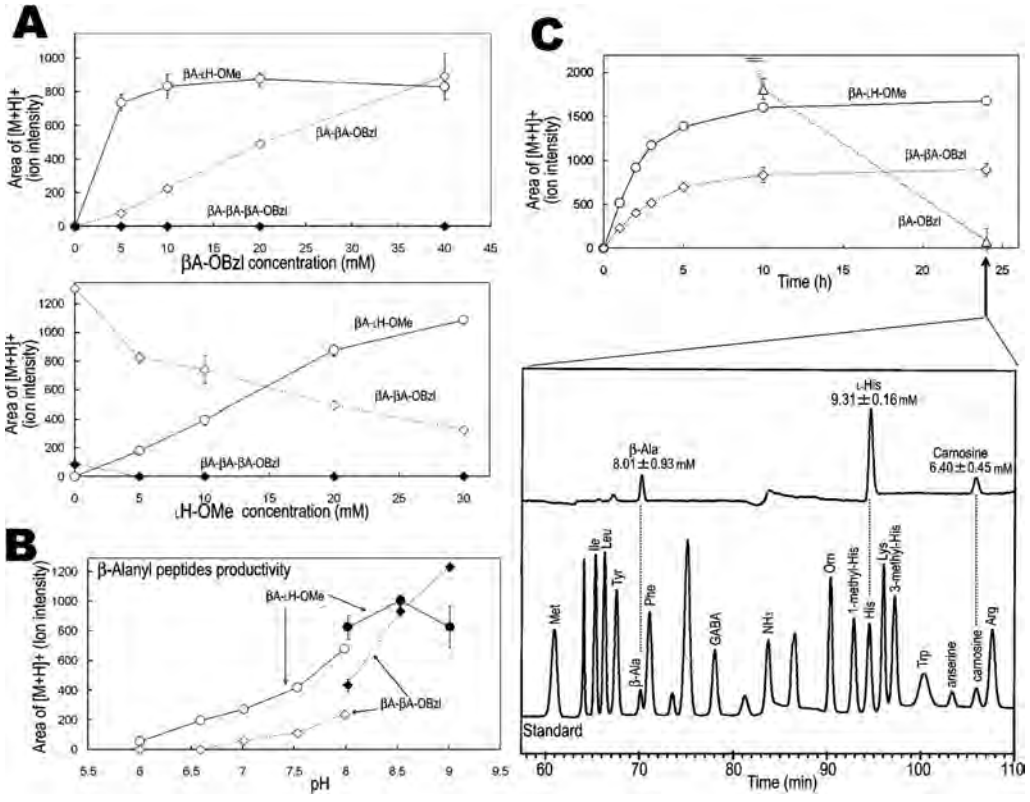

Fig. 1. (A) Effect of the substrate concentration on the production of Car-OMe and β-Ala-β-Ala-OBzl. Upper panel: Effect of β-Ala-OBzl concentration. L-His-OMe at 20 mM and β-Ala-OBzl at 0 to 40 mM were used as the acyl acceptor and donor, respectively. Lower panel: Effect of L-His-OMe concentration. L-His-OMe at 0 to 30 mM and β-Ala-OBzl at 20 mM were used as the acyl acceptor and donor, respectively. (B) Effect of pH on production of Car-OMe and β-Ala-β-Ala-OBzl. (C) Time dependence and yield evaluation of Car-OMe synthesis. The upper panel shows the time dependence in the production of Car-OMe and β-Ala-β-Ala-OBzl. L-His-OMe at 20 mM and β-Ala-OBzl at 20 mM were used as the acyl acceptor and donor, respectively. The lower panel shows a chromatogram of amino acid analyzer. L-His-OMe at 20 mM and β-Ala-OBzl at 20 mM were used as the acyl acceptor and donor, respectively.

hardly increased after that. Similarly, byproduct, β-Ala-β-Ala-OBzl, was also produced until 5 h. After 24 h reaction, 20 mM β-Ala-OBzl was completely consumed and converted into Car-OMe, β-Ala-β-Ala-OBzl, and free β-Ala. To evaluate the conversion rate of 20 mM β-Ala-OBzl to Car-OMe and the synthesis of undesirable products such as

β-Ala-β-Ala-OBzl and free β-Ala, we quantified Car, free β-Ala, and L-His in the alkaline-treated reaction mixture of 24 h reaction by amino acid analyzer. The concentration of Car in the reaction mixture is approximately 6.4 mM, and those of β-Ala and L-His were respectively approximately 8.0 mM and 9.3 mM (Fig. 1C). From these values, the conversion rate of β-Ala-OBzl to Car-OMe was estimated to be over 30%.

Conclusion

We have found that Car-OMe could be produced from β-Ala-OBzl and L-His-OMe by aminolysis reaction of S9AP-St. To engineer the serine peptidase into "transpeptidase" for tailoring highly capable biocatalyst, we constructed S9AP-St variant, S502C S9AP-St. However, the substrate specificity of aminolysis reaction was changed by the substitution of catalytic Ser^{502} with Cys. Instead, it has successfully produced Car-OMe with a conversion rate of β-Ala-OBzl to Car-OMe being over 30% by the optimization of production of Car-OMe using S9AP-St.

References

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