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Communication

Enzymatic clickable functionalization of peptides *via* computationally engineered peptide amidase

Tong Zhu[a,b,1], Lu Song[a,c,1], Ruifeng Li[a,b], Bian Wu[a,*]

[a] CAS Key Laboratory of Microbial Physiological and Metabolic Engineering, State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China
[b] University of Chinese Academy of Sciences, Beijing 100101, China
[c] State Key Laboratory of Transducer Technology, Chinese Academy of Sciences, Beijing 100101, China

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ABSTRACT

Directed peptides C-terminal modification enabled by the engineered biomolecular catalyst – peptide amidase 12B has been achieved *via* computational protein engineering. The engineered enzyme exhibits great promising potential in the C-terminal modification of opioid peptides using prop-2-yn-1-amine (PYA) or prop-2-en-1-amine (PEA) as the nucleophile. A variety of opioid peptides could be readily functionalized at the C-terminal chain in high yield in a mild and selective manner. Notably, modified opioid peptides bearing alkynyl moiety could be further functionalized through well-established click reaction.

In recent decades, the notable expansion of peptide therapeutics development led to an unprecedented number of marketing approvals and provided a robust pipeline for innovative applications in the near future [1]. As one of the most important strategies in this field, modification of peptides could improve their pharmacological properties, biological efficacies and physicochemical properties [2–12]. Opioid peptides that served as classical peptides have attracted keen attention as promising pharmaceutical agents for pain alleviation owing to their elevated potency and centrally mediated actions of pain process [13–16]. However, their low protease-resistance, inefficiency to penetrate the blood-brain barrier (BBB) and toxic effects severely impede their therapeutic applications [17,18]. Peptide modification has emerged as an invaluable tool to improve peptide stability and enhance immunogenicity, but there remains a need for additional and complementary reactions with improved kinetics and selectivities [19,20]. In particular, opioid peptides such as endomorphin-1 (EM1) and endomorphin-2 (EM2) have a biologically indispensable N-terminal tripeptide fragment, and the modification of this fragment would usually influence their affinity to opioid receptors [21]. Therefore, generally applicable methods for highly chemo- and regioselective C-terminal functionalization are still urgently demanded [22–24].

C-terminal modification of opioid peptides holds great promise to ameliorate their stability and BBB permeability [25,26]. More importantly, it could further facilitate the introduction of functional groups, such as fluorescent reporters or synthetic polymers, allowing peptide biophysical studies and applications [27]. Recently, we demonstrated that the computationally engineered (framework for rapid enzyme stabilization by computation, FRESCO strategy) peptide amidase (PAM) could be well-applied as a versatile catalyst for diverse C-terminal peptide modification reactions [28]. By decreasing the steric hindrance at residue 171, PAM 12B was obtained with enhanced catalytic performance to tolerate bulky nucleophiles such as benzylamine. Herein, we would like to report the application of PAM 12B, which exhibited exciting potentials in the C-terminal modification of opioid peptides by employing prop-2-yn-1-amine (PYA) or prop-2-en-1-amine (PEA) as the nucleophile. A wide range of opioid peptides could be readily functionalized at the C-terminus in a mild and selective fashion (Scheme 1).

Initially, we set out to evaluate the catalytic efficiency of PAM 12B in the aminolysis of amide between a commercially available model peptide Cbz-G-Y-NH_2 and PYA in organic solvents. Such reaction will provide a platform for the installation of functional groups into the target peptide. As shown in Table 1, in the presence of 9.0 equiv. of PYA, the reaction proceeded efficiently to afford the desired product **a1** in 99% yield in 5 h, and only trace amount of

* Corresponding author.
E-mail address: wub@im.ac.cn (B. Wu).
[1] These authors contributed equally to this work.

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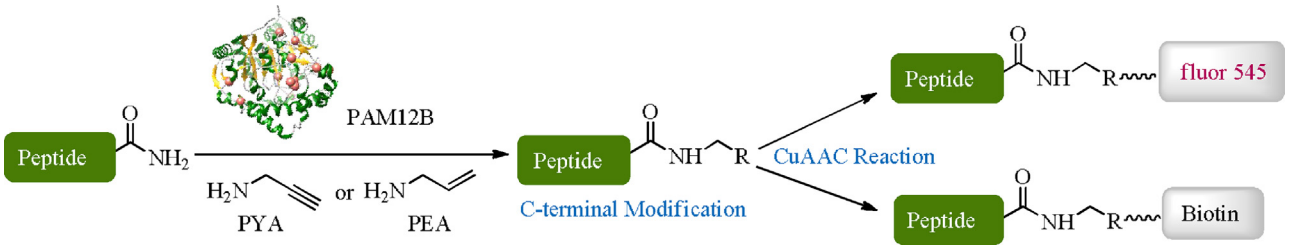
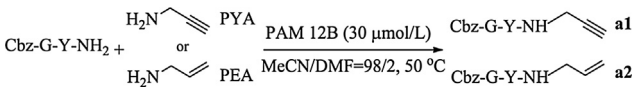


Scheme 1. PAM 12B-catalyzed peptide C-terminal click-functionalization.

Table 1
Optimization for PAM 12B-catalyzed aminolysis.



Entry	PYA/PEA (mmol/L)	t (h)	Yield (%)[a]
1[b]	45	5	99
2[b]	15	8	99
3[b]	90	5	99
4[c]	45	6	99
5[d]	45	12	97

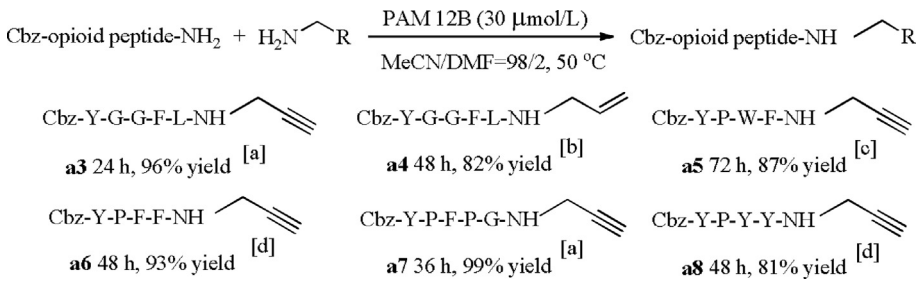
[a] Yields were determined by HPLC.
[b] The substrates were PYA and Cbz-G-Y-NH_2 (5 mmol/L). The product was **a1**.
[c] The substrates were PYA and Cbz-G-Y-OH (5 mmol/L). The product was **a1**.
[d] The substrates were PEA and Cbz-G-Y-NH_2 (5 mmol/L). The product was **a2**.

hydrolytic product was detected (Table 1, entry 1). Reduction of PYA loading was proven to be less efficient, and longer reaction time was needed to reach the same yield (Table 1, entry 2). Meanwhile, increasing the concentration of PYA to 90 mmol/L had no obvious effect on the reaction efficiency, which indicated saturation kinetics with respect to the nucleophile (Table 1, entry 3). The use of the C-terminal non-protected peptide Cbz-G-Y-OH as the acyl donor was also feasible, and the corresponding product was produced in 99% yield in 6 h (Table 1, entry 4). In the case of PEA as the nucleophile, the reaction somehow turned out to be sluggish, but still afforded the desired product **a2** in excellent yield in 12 h (Table 1, entry 5).

We then sought to explore the modification of opioid peptides (Scheme 2). Enkephalins are one of the most important groups of endogenous opioid peptides [29,30], which bind to opioid peptide receptors (OPr), including μ opioid peptide receptor (MOPr) and δ opioid peptide receptor (DOPr) [31], and play significant roles in emotions, attachment behaviors, and responses to stress and pain [32,33]. Since enkephalins share a common 'opioid motif' constituted by the amino acid sequence YGGF, we chose Cbz-YGGFL-NH_2 as the prototypical substrate. The reaction of Cbz-YGGFL-NH_2 with PYA in the presence of PAM 12 B can deliver the corresponding products **a3** in 96% yield (Scheme 2). To investigate the practical applicability of PAM 12B-catalyzed peptide C-terminal alkynylation, we also performed this reaction on a 35 mg semi-preparative scale. After work-up and purification the desired product was collected in 70% isolated yield (Scheme S1 in Supporting information).

Besides PYA, PEA was also a suitable nucleophile for the modification of opioid peptides. As displayed in Scheme 2, Cbz-YGGFL-NH_2 could be well applied in the reaction to give the corresponding products **a4** in 82% yield. Encouraged by the initial success, we moved forward to examine the applicability of this approach for miscellaneous opioid peptides. Endomorphins (EMs) were next examined, which are widely distributed in the central nervous system [34,35]. With Cbz-YPWF-NH_2 (EM1) and Cbz-YPFF-NH_2 (EM2) as the substrates, both reactions proceeded well and expected products **a5** and **a6** were obtained with 87% yield and 93% yield, respectively. Besides the endogenous opioids, exogenous opioids, such as casomorphins, may be released during the digestion of proteins by proteolytic enzymes [36]. In this context, we explored the use of PAM 12B for the modification of Cbz-YPFPG-NH_2 (β-casomorphin). This peptide was efficiently converted to the corresponding aminolytic product **a7** in 99% yield. Furthermore, we investigated the feasibility to modify the antagonist of opioid receptors. The employment of Cbz-YPYY-NH_2 (casoxin B [37]) in this reaction directly afforded the product **a8** in 81% yield. This chemistry thus provides a straightforward and novel access to incorporating terminal alkyne and alkene groups into opioid peptides, implying broad applications in their downstream transformations.

Next, we examined the viability of the opioid peptides functionalization *via* bioorthogonal chemistry, which plays a significant role in chemical biology research by creating the means to carry out selective chemical transformations in complex biological samples. The aminolytic products were subjected to copper(I)-catalyzed azide-alkyne cycloaddition reaction (CuAAC reaction). Initially, benzyl azide, an inexpensive and model azide reagent, was chosen to optimize the reaction conditions. Cbz-G-Y-NH_2 reacted with PYA catalyzed by PAM 12B, then MeCN and residual PYA were removed by heating in boiling water. The



Scheme 2. PAM 12B-catalyzed peptide C-terminal aminolysis. Yields were determined by HPLC. Reaction conditions: [a] peptide (5 mmol/L), PYA (45 mmol/L). [b] peptide (5 mmol/L), PEA (45 mmol/L). [c] peptide (1 mmol/L), PYA (9 mmol/L). [d] peptide (2 mmol/L), PYA (18 mmol/L).

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