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SHORT COMMUNICATION

Extraordinary metabolic stability of peptides containing α-aminoxy acids

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Abstract The metabolic stability of peptides containing a mixed sequence of α-aminoxy acids and α-amino acids is significantly improved compared to peptides composed of only natural α-amino acids. The introduction of an α-aminoxy acid into peptide chain dramatically improves the stability of the amide bonds immediately before and after it. These peptides containing α-aminoxy acids represent excellent structural scaffold for the design of metabolically stable and biologically active peptides.

Keywords Metabolism · Peptides · Stability · Aminoxy acid

Introduction

Biological stability and flexibility of peptide backbone are the main obstacles for the utilization of peptides in pharmaceutical industry. A lot of efforts have been made to solve these problems (Giannis 1993; Haldar 2008). For example, β-peptides have been found to form rigid secondary structures (Seebach and Matthews 1997; Gellman 1998; Cheng et al. 2001; Bautista et al. 2007; Hill et al. 2001), and they also exhibit outstanding stability toward proteolytic enzymes (Frackenpohl et al. 2001; Seebach et al. 1998; Hintermann and Seebach 1997; Heck et al. 2006). Our group has found that peptides composed of aminoxy acids can form strong intramolecular hydrogen bonds between adjacent residues. For example, peptides consisting of α-, β- and γ-aminoxy acids form 8-membered-ring bonds (α N–O turns) (Yang et al. 1996, 1999), 9-membered-ring bonds (β N–O turns) (Yang et al. 2002b, 2004) and 10-membered-ring bonds (γ N–O turns) (Chen et al. 2004, 2008), respectively. In addition, oligomers of homochiral α-, β- and γ-aminoxy acids can form helical structures consisting of consecutive N–O turns. Peptides containing α-aminoxy acids have also been found to be good receptors for anions because of the acidity of aminoxy amide protons (Yang et al. 2002a, 2005b). Peptides derived from α-aminoxy acids have been used as effective chemical shift reagents for measuring the *ee* value of carboxylic acids (Yang et al. 2005a), and some form chloride channels to mediate chloride ion transportation across the cell membrane and regulate cell membrane potentials and voltage-gated calcium channels (Li et al. 2007, 2009). To date, there are few studies on the stability of such peptides in biological systems. It was reported that peptides composed of natural α-amino acids were completely cleaved by trypsin within 10 min, while peptides composed of α-aminoxy acids were stable toward the same protease for at least 52 h (Lee et al. 2003; Kisfaludy et al. 1978). However, no systematic investigation of the metabolic stability of peptides that contain both α-aminoxy acids and natural α-amino acids has been conducted. To pave the way for the utilization of peptides containing both α-aminoxy acids and α-amino acids as potential drugs, we investigated the

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metabolic properties of these peptides and the results are reported herein.

Seven representative peptides (peptides **1**–**7**, Fig. 1) were synthesized. The detailed synthetic procedures and all characteristic data of the peptides are provided as Supplementary Materials.

The metabolic stability of the peptides was investigated using rat liver S9 subcellular fraction, which is a major tool for the metabolic study of xenobiotics. The S9 fraction contains a wide variety of phase I and phase II drug metabolizing enzymes, including cytochrome P450 monooxygenases, flavin-containing monooxygenases, carboxylesterases, epoxide hydrolases, UDP-glucuronosyltransferases, glutathione *S*-transferases, sulfotransferases, methyltransferases, and acetyltransferases. Peptides **1**–**7** were incubated with S9 fraction at 37°C for 30 min. Figure 2 shows the HPLC chromatograms of those peptides after incubation. The denatured S9 fraction was used as control in which all the enzymes were deactivated by heat. Phenylacetic acid was used as internal standard (IS) for HPLC analysis. The metabolic stability was determined by comparison of the ratio of peak areas of peptides and IS upon incubation with S9, or denatured S9 fraction. Comparing with the control, the peak of peptide **1** at 9.5 min disappeared, while a new peak M1 was detected at 7.0 min. M1 was identified to be phenylalanine by comparison of the retention time, UV and mass spectra with the authentic compound. These results suggested that peptide **1** consisted of α-amino acids was unstable and rapidly metabolized in the S9 fraction.

In order to enhance the metabolic stability of peptides, the diethyl amino group was introduced at the N-terminus to investigate whether it could stabilize the structure of the peptide. Based on this rationale, peptides **2** and **3** containing an aminoxy amide group (Fig. 1) were synthesized. Comparing with the control, the amount of the intact peptides **2** and **3** after incubation was reduced by 14.1 and 7.66%, respectively, indicating the metabolic stability of peptides **2** and **3** was significantly better than that of **1**. The results suggested that the aminoxy amide bond was more stable than that of the regular amide bond formed between natural α-amino acids. To further improve the stability of aminoxy acid-containing peptides, peptide **4** consisting of one α-amino acid and two consecutive α-aminoxy acids was synthesized, and incubated with S9 under the above condition. The results demonstrated only 12.1% biotransformation after incubation, suggesting that peptide **4** with two aminoxy amide bonds was also stable in S9 fraction.

The metabolic stability of peptides **2**–**4** with aminoxy amide bond as the linker between the monomers was proved to be better than that of peptides composed of natural α-amino acids. We have previously found that peptides composed of alternating α-aminoxy acids and α-amino acids formed a 7–8 helix structure (Yang et al. 2003). Thus, the stability of a tetrapeptide containing two aminoxy amide bonds and one regular amide bond (peptide **5**) was also studied. Surprisingly, only 16.4% peptide **5** was metabolized after incubation, suggesting that not only the aminoxy amide bonds but also the regular amide bond in peptide **5** becomes stable. A possible reason is that the presence of the preceding α-aminoxy acid leads to stabilization of the middle amide bond through the formation of a special secondary structure, which subsequently affected the recognition of the peptide by the metabolic enzymes. To further study the effect of aminoxy amide bonds on the stability of neighboring regular amide bonds, tripeptides **6** and **7** were synthesized with aminoxy amide bond at the right and left sides of a regular amide bond, respectively. After incubation, peptide **6** was completely metabolized, and a new peak at 7.0 min was detected and identified to be phenylalanine, the same metabolite (M1) of peptide **1**. In

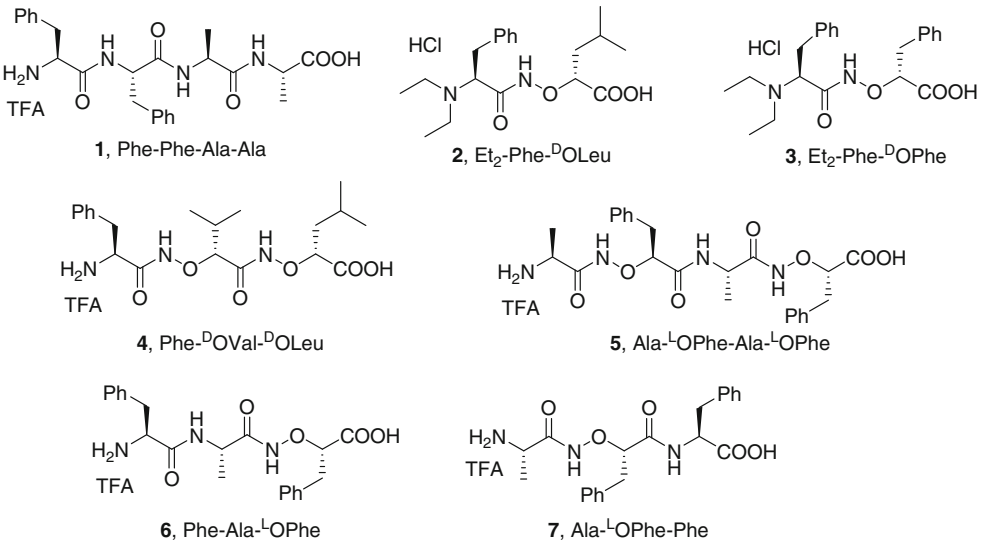

Fig. 1 Structures of the seven peptides

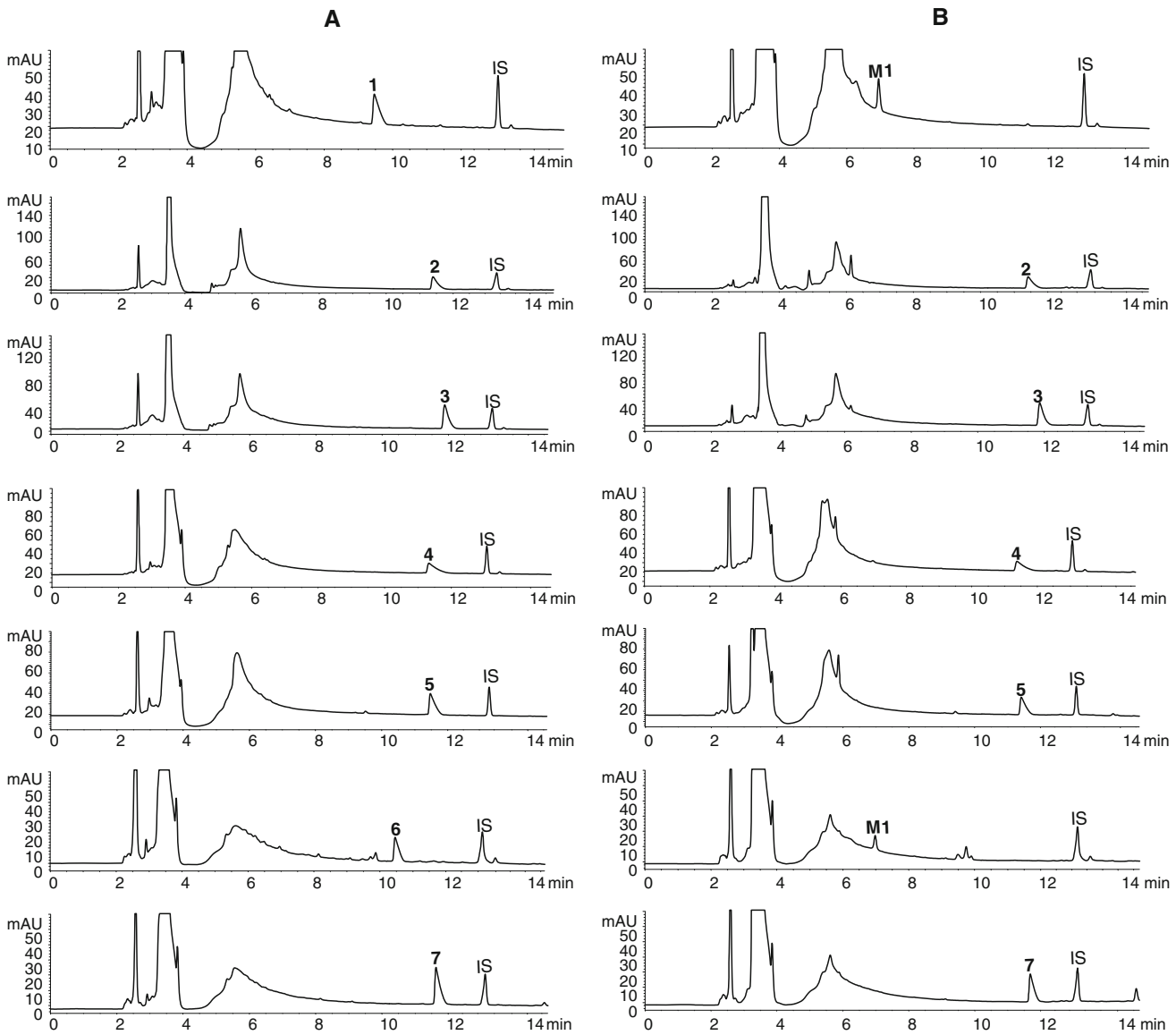

Fig. 2 HPLC chromatograms of peptides incubated with denatured rat S9 (**a**) and S9 (**b**). The peaks with retention time at 9.50, 11.4, 11.9, 11.3, 11.4, 10.5, 11.6, 7.0 and 13.1 min correspond to peptides **1**, **2**, **3**, **4**, **5**, **6**, **7**, metabolite M1 and the internal standard (IS) phenylacetic acid, respectively

contrast, about 30% of peptide **7** was metabolized. Although it was relatively susceptible to metabolism, peptide **7** was still much more stable than peptides **1** and **6**. The N-terminal amide bond of peptide **6** was between two α-amino acid units, whereas it was between one α-amino acid and one α-aminoxy acid for peptide **7**. During the hydrolysis of a peptide, the carbonyl group of a target amide bond is susceptible to be attacked by proteases. The N-terminal carbonyl group in peptide **6** belongs to an α-amino acid, whereas it belongs to an α-aminoxy acid in peptide **7**. It is expected that with the addition of an oxygen atom on the backbone of an α-amino acid, the environment of the amino acid is changed. Consequently, the carbonyl group of α-aminoxy acid was not easily reached by proteases, resulting in better metabolic stability of peptide **7** than that of **6**. As peptide **7** has a natural α-amino acid at its C-terminus, it is more susceptible to carboxylesterases, which recognize the carboxylate group, than that of peptide **5** with an α-aminoxy acid residue at its C-terminus. These results indicate that peptides can be stabilized not only by the presence of aminoxy amide bonds, but also by the preceding α-aminoxy acid residue of regular amide bonds.

In summary, our study has further demonstrated that peptides containing a mixed sequence of α-aminoxy acids and α-amino acids have better metabolic stability than that of peptides consisting of only natural α-amino acids. The introduction of an α-aminoxy acid into peptide chain significantly improved the stability of the amide bonds

immediately before and after it. With the strong ability to form rigid secondary structures, peptides containing a mixed sequence of α-aminoxy acids and α-amino acids have a potential to become an excellent scaffold for the de novo design of metabolically stable and biologically active peptides.

Experimental section

The incubation system consisted of rat liver S9 at 2 mg/mL, the aminoxy peptides at concentration of 1 mM, NADPH-regenerating system (1 mM $NADP^+$, 1 mM NAD^+, 10 mM glucose 6-phosphate, 2 U/mL glucose-6-phosphate dehydrogenase, 4 mM $MgCl_2$), and 50 mM Tris buffer containing 150 mM KCl, pH 7.4, in a total volume of 500 μL. In the control group, peptides were incubated with denatured rat liver S9, which was heated at 100°C for 30 min. Reactions were initiated by the addition of NADPH-regenerating system. The mixture was incubated for 30 min at 37°C with gentle shaking. Each test was conducted in triplicate. The reaction was terminated by the addition of an equal volume of acetonitrile containing IS (phenylacetic acid, 10 mM). The mixture was centrifuged at 13,000*g* (Eppendorf 5415D, Hamburg, Germany) for 5 min and the supernatant was filtered (4 mm, 0.45 μm PTFE filter) and subjected to HPLC analysis. The chromatograms were recorded at a wavelength of 250 nm. The HPLC separation was achieved using an Alltima 4.6 × 250 mm reverse-phase C_{18} column using the following gradient: 2% acetonitrile and 98% water containing 0.5% acetic acid at 0 min, and the percentage of acetonitrile gradually increased to 70% within 15 min. The flow rate of the mobile phase was set at 1 mL/min.

Metabolites of peptides were identified using a Thermo HPLC–MS instrument containing a P4000 series HPLC system coupled with an MAT Navigator mass detector (Thermo, San Jose, CA) using an ESI ionization source with the following settings: capillary temperature, 180°C; sheath gas flow rate, 60; auxiliary gas flow rate, 10; source voltage, 4.2 kV; capillary voltage, 3.0 V; tube lens offset, 15 V. Chromatographic separation was achieved using an Alltima 2.1 × 150 mm reverse-phase C_{18} column under the following gradient: 2% acetonitrile containing 0.1% trifluoroacetic acid and 98% water containing 0.1% trifluoroacetic acid at 0 min, and the percentage of acetonitrile gradually increased to 70% within 15 min. The flow rate of the mobile phase was set at 0.2 mL/min.

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