Enzymatic Stability of Myostatin Inhibitory 16-mer Peptides

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Inhibition of myostatin is a promising strategy for treatment of muscle atrophic disorders. A 16-mer myostatin inhibitory linear peptide, MIPE-1686, administered intramuscularly, significantly increases muscle mass and hindlimb grip strength in Duchenne muscular dystrophy model mice. In this paper, we describe our examination of the enzymatic stabilities of this peptide with recombinant human prostates, aminopeptidase N, chymotrypsin C, and trypsin 3. MIPE-1686 was found to be stable in the presence of these enzymes, in contrast to a peptide (1), from which MIPE-1686 was developed. Modification of the peptides at a position distant from the protease cleavage site altered their enzymatic stability. These results suggest the possibility that the stability to proteases of 16-mer myostatin inhibitory peptides is associated with an increase in their known β-sheet formation properties. This study suggests that MIPE-1686 has a potential to serve as a long-lasting agent in vivo.

Key words protease resistance; linear peptide; myostatin inhibition; secondary structure; long-lasting agent

Introduction

Myostatin, an inhibitor of muscle growth, is a promising target for treatment of atrophic muscle disorders such as muscular dystrophy and cancer cachexia. Several anti-myostatin therapeutic agents, mostly focused on protein-based inhibitors have been examined. For example, a mouse antibody (mRK35) and a humanized analogue, domagrozumab increased muscle mass in both mice and cynomolgus monkeys, and an Fc-protein-fused decoy receptor (ActRIIB-Fc) of myostatin assisted tumor-bearing mice with the cachexia symptom. Several anti-myostatin agents, including MYO-009, BMS-986089, ACE-083/-031, BYM338, FS-344, and domagrozumab, have advanced into clinical trials; however, still not launched as a clinical drug yet. Therefore, alternative anti-myostatin approaches are an important challenge to expand drug diversity and supply drugs for future therapies.

As one such approach, a myostatin precursor-derived prodomain protein, an endogenous inactivator of mature myostatin, has attracted attention, and we recently identified a 23-mer peptide myostatin inhibitor with IC$_{50}$ = 3.56 µM through an exploration of the prodomain sequence of mouse myostatin. Subsequent structure–activity relationship (SAR) studies to identify important residues for the activity led to a potent 16-mer myostatin inhibitor, MIPE-1686 (Table 1) with IC$_{50}$ = 0.13 µM although the first synthesized 16-mer peptide (1, Table 1), a fragment of the 23-mer peptide, was inactive. Intramuscular injection of MIPE-1686 to target muscles leads to a significant increase in muscle mass and grip strength in mice. It was unclear if MIPE-1686, a linear peptide with an unprotected N-terminus, has adequate biological stability necessary for a practical muscle-building agent. Therefore, the enzymatic stability of MIPE-1686 was investigated using human recombinant proteases.

Results and Discussion

All protease solutions were prepared according to the manufacturer’s instruction and stock solutions of myostatin inhibitory peptides in water (1 mM) were prepared. To understand the enzymatic stabilities of MIPE-1686 and related peptides, firstly, their stabilities against aminopeptidase N (APN, 1 µg/mL), a widely distributed exopeptidase in animals, was examined using reversed-phase HPLC. Peptide 1, the lead peptide of MIPE-1686, was susceptible to APN, which degraded 78% of the peptide in 45 min incubation (Table 1, Fig. S1A). On the other hand, MIPE-1686 remained 84.0% intact after 90 min incubation despite having unprotected N-terminus (Fig. 1). To understand this difference, we investigated the sequence-dependency in APN recognition by focusing on the structurally related 16-mer peptides 2–4 (Table 1) which possess myostatin inhibitory activity. Peptide 2, with two tryptophan (Trp) substitutions in the central region, showed slightly higher resistance (32.1%) to APN than peptide 1. MS analyses of new peaks appearing during the incubation identified three 13–15-mer N-terminal truncated fragments 2aa–2ac as expected (Fig. S2A) because APN is known to cleave N-terminal residues. Peptide 3, bearing Trp-tyrosine (Tyr)- after the N-terminus was more resistant to APN degradation than peptide 2 with 57.9% of the peptide remaining intact after 45 min. N-terminal 14–15-mer hydrolyzed fragments 3aa–3ab were similarly identified. The 15-mer (3ab) is a major hydrolyzate (Fig. S2B). Peptide 4, with additional substitutions to peptides 2 and 3, displayed strong resistance to APN with no degradation after 45 min incubation and only slight degradation with 84% remaining after 270 min incubation (Fig. S1B). MIPE-1686, however, remained 84% intact at 90 min and after 270 min, no further degradation was observed, and with no hydrolyzed fragment peak was observed (Fig. 1). Therefore, the non-specific binding (NSB) of peptides (peptide 4 and

Table 1. Sequences and Remaining Rates after APN Treatment of Peptide Derivatives

| Peptide          | Sequence$^a$ (residue 1 = N-terminal Ser) | % Peptide remaining after 45 min$^b$ |
|------------------|------------------------------------------|-------------------------------------|
| 1                | SRIEA IKIQI LSKLR L-NH$_2$               | 22.5 ± 2.2                          |
| 2                | SRIEW IKIQI WSKLR L-NH$_2$               | 32.1 ± 4.6                          |
| 3                | WYIEA IKIQI LSKLR L-NH$_2$               | 57.9 ± 6.0                          |
| 4                | WYIEW IKIQI WSKLR L-NH$_2$               | 100 ± 5.7                           |
| MIPE-1686        | WYIRw IKXQI WSKXR L-NH$_2$               | 84.0 ± 3.4$^c$                      |

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| 4                | WYIEW IKIQI WSKLR L-NH$_2$               | 100 ± 5.7                           |
| MIPE-1686        | WYIRw IKXQI WSKXR L-NH$_2$               | 84.0 ± 3.4$^c$                      |

$^a$ Space is inserted every five residues from N-terminus. Substituted residues are underlined. $^b$ Data are presented as mean values ± standard deviation (S.D.) (n = 3). $^c$ w = d-Trp, X = cyclohexylglycine (Chg). $^d$ Remaining rate (%) at 90 min. $^e$ This value includes non-specific binding (NSB) loss.
MIPE-1686) to experimental instruments was examined in the absence of APN. In these tests, the peak area of peptide 4 remained at almost 100% during incubation, while that of MIPE-1686 decreased to 85–95%, which is similar to that observed in the presence of APN. Essentially no NSB was observed with peptides 2 and 3. These results suggest that this reduction of MIPE-1686 is due to NSB and that MIPE-1686 is extremely stable against exopeptidases like APN.

Since these peptides were modified in their central regions, the observed difference in their stability against exopeptidase APN is probably due to the different secondary structure in each peptide as well as the substrate preference of APN at the N-terminal region. We recently reported that circular dichroic analysis shows that these peptides have different secondary structures.7 Peptide 1 has an α-helix/random coil propensity, while as the peptides are enlarged towards MIPE-1686, their β-sheet propensity increases.7

The stability of the peptides against endopeptidase was investigated. Since MIPE-1686 was developed from peptide 1 by substitutions, mainly with hydrophobic residues, chymotrypsin C (CTRC) was chosen as a representative endopeptidase because it preferentially cleaves peptide bonds at the carboxyl side of hydrophobic residues such as leucine (Leu), Tyr, phenylalanine (Phe), methionine (Met), Trp, glutamine (Gln) or asparagine (Asn). Peptides were incubated with CTRC (2 µg/mL) for 90 min (Table 2). MIPE-1686 displayed a strong resistance (90.1%) to CTRC with no degraded peptide peak appearing in the HPLC analysis (Fig. 2), but peptide 1 was easily degraded, only 18.1% remaining intact together with the appearance of two degraded fragments (1ca, 1cb) (Fig. S3A). MS showed that the main degradation site was the carboxyl side of Leu11. This degradation pattern was also seen in peptide 3 (Fig. S3B), which had however, a remarkably improved survival rate of 71.4%. Enhanced stability was also observed in peptide 2 (38.1%) when compared with peptide 1. However, the HPLC analysis of hydrolyzate 2cb demonstrated that the main cleavage site in this case was the carboxyl side of Leu14 (Fig. S4A). The same cleavage pattern was also observed in peptide 4 and its E31R derivative (Figs. S4B and S5A). Derivatives having both substitutions in peptides 2 and 3 showed enhanced stabilities of 92.3 and 81.1%, respectively. However, the HPLC analysis of hydrolyzate 2cb demonstrated that the main cleavage site in this case was the carboxyl side of Leu14 (Fig. S4A). The same cleavage pattern was also observed in peptide 4 and its E31R derivative (Figs. S4B and S5A). Derivatives having both substitutions in peptides 2 and 3 showed enhanced stabilities of 92.3 and 81.1%, respectively. Calculation of the latter value includes the NSB loss since the peak area of E31R was decreased to 79–81% after 90 min incubation without CTRC. In peptide 5 where Leu14, recognized by CTRC is replaced by Chg14 (Chg: cyclohexylglycine), no degradation of the peak was detected (Fig. S5B). The observed slight reduction of remaining rate (74.4%) is probably caused by NSB loss similar to that observed in E31R. Finally, MIPE-1686 bearing the same Chg14 displayed remaining rates of 90 and 85% at 90 and 270min, respectively, higher than peptide 5 (Fig. S8), indicating that MIPE-1686 has strong CTRC-resistance, and probably a slight improvement of NSB loss compared to E31R or peptide 5.

Next, the stability of peptides against trypsin 3 (0.1 µg/mL), which preferentially cleaves peptide bonds at the carboxyl side

| Peptide | Sequence\(^a\) | % Peptide remaining after 90 min\(^b\) |
|---------|----------------|--------------------------------|
| 1       | SRIEA IKIQI L*SKLR L-NH\(_2\) | 18.1 ± 2.5 |
| 2       | SRIEW IKIQI WSKL*R L-NH\(_2\) | 38.1 ± 0.4 |
| 3       | WYIEA IKIQI L*SKLR L-NH\(_2\) | 71.4 ± 0.6 |
| 4       | WYIEW IKIQI WSKL*R L-NH\(_2\) | 92.3 ± 1.8 |
| E31R    | WYIRW IKIQI WSKL*R L-NH\(_2\) | 81.1 ± 0.4 |
| 5       | WYIRW IKIQI WSKXR L-NH\(_2\) | 74.4 ± 1.8 |
| MIPE-1686 | WYIRW IKXQI WSKXR L-NH\(_2\) | 90.1 ± 5.0 |

\(a\) Space is inserted every five residues from N-terminus. Substituted residues are underlined. \(b\) Data are presented as mean values ± S.D. (n = 3). \(c\) X = Chg, w = d-Trp. \(d\) This value includes non-specific binding (NSB) loss. \(e\) This value probably includes non-specific binding (NSB) loss without detection of any fragment peaks.
of basic residues such as arginine (Arg) or lysine (Lys), was examined (Table 3). Peptide 1 was easily degraded in 90 min incubation. From MS analysis of the main degraded fragment 1ta (Fig. S6A), the cleavage occurred preferentially at the carboxyl side of Arg15. This cleavage style was the same as that in peptide 2 which gave the fragment 2tb, although an additional hydrolysate 2ta, formed by the degradation at the carboxyl side of Lys13 was detected (Fig. S6B). The stability of peptide 2 was 67.8%, and clearly increased when compared with that of peptide 1 (17.7%). A similar improvement was observed in peptide 3 (73.7%), but no cleavage at Arg15 was observed (Fig. S7A). Furthermore, no degradation of E31R bearing an Arg4-modification was occurred (Fig. S7B). Finally, similar to E31R, MIPE-1686 was highly stable (92.8%) against trypsin 3 (Fig. 3) with no hydrolysate detected after 400 min (Fig. S8B), suggesting that MIPE-1686 is extremely resistant to trypsin 3.

**Conclusion**

Here, we showed that the linear 16-mer myostatin inhibitory peptide MIPE-1686 with an unprotected N-terminus has strong enzymatic stability to APN, CTRC and trypsin 3. These results suggest that MIPE-1686 has the potential to be effectively used in in vivo experiments, and also propose the new methodology to modify the degradation pattern and stability based on regulating the secondary structure of linear peptides. Therefore, this study would contribute to expand the range of choices for peptidic platform in current mid-sized drug development. Namely, it would be a valuable paradigm for the design of bioactive linear peptides that can pass the preclinical drug discovery process.

**Experimental**

**Materials** Reagents and solvents were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), Watanabe Chemical Ind. (Hiroshima, Japan), and Tokyo Chemical Ind. (Tokyo, Japan). Human recombinant APN, CTRC and trypsin 3 were purchased from R&D Systems (Minneapolis, MN, U.S.A.), and used as received. All peptides were prepared as described previously.

**Enzymatic Stability Analyses**

**Analysis of Stability toward APN**

Peptides (50 µM) were incubated in APN solution (1 µg/mL) in 50 mM Tris buffer (pH 7.4) at 37°C for a measured time. An aliquot of the peptide solution (20 µL) was mixed with 0.1% trifluoroacetic acid (TFA)-containing H₂O-CH₃CN (4:1 solution) (80 µL). Then 20 µL of this solution, which was strictly defined with injection loop volume, was analyzed by HPLC using a C₁₈-bound reverse-phase column (4.6 × 150 mm; COSMOSIL 5C18-AR-II) with a binary solvent system: a linear gradient of CH₃CN (25–40%, 30 min) in 0.1% aqueous TFA (flow rate = 1.0 mL/min), with detection by UV spectroscopy (220 nm). The identity of degraded fragments was determined by LCMS-2020 (Shimadzu, Kyoto, Japan).

**Analysis of Stability toward CTRC**

Peptides (50 µM) were incubated in CTRC solution (2 µg/mL) in 25 mM Tris buffer (pH 8.0) containing 0.5 mM CaCl₂ at 37°C for a recorded time. The following analyses were the same as those in “Analysis of Stability toward APN.”

**Analysis of Stability toward Trypsin 3**

The peptide (50 µM) was incubated in trypsin 3 solution (0.1 µg/mL) in 50 mM Tris buffer (pH 7.5) containing 0.15 M NaCl, 10 mM CaCl₂ and 0.05% Brij-35 (w/v) at 37°C for a recorded time. The following analyses were the same as those described in “Analysis of Stability toward APN.”

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**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

Supplementary Material containing Figs. S1–S8, is available online.
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