A fluorogenic method for measuring enteropeptidase activity: spectral shift in the emission of GD₄K-conjugated 7-amino-4-methylcoumarin

Mal-Gi Choi², Eungyeong Lee¹, Hye-Shin Chung³, Sei-Heon Jang¹ & ChangWoo Lee¹,∗

¹Department of Biomedical Science, Daegu University, Gyeongsan 712-714, ²Division of Integrative Biosciences and Biotechnology, Pohang University of Science and Technology, Pohang 790-784, ³Department of Biotechnology, Hannam University, Daejeon 305-811, Korea

Enteropeptidase is a serine protease secreted by the pancreas and converts inactive trypsinogen to active trypsin. Enteropeptidase cleaves the C-terminal end of the substrate recognition sequence Asp-Asp-Asp-Asp-Lys (D₄K). The assay for enteropeptidase has utilized GD₄K-conjugated 2-naphthylamine (GD₄K-NA) as a fluorogenic probe over the last 30 years. However, no other D₄K-conjugated fluorogenic substrates of enteropeptidase have been reported. Furthermore, naphthalene is known as carcinogenic to humans. In this study, we used shift in the emission spectrum of GD₄K-conjugated 7-amino-4-methylcoumarin (GD₄K-AMC) as a fluorogenic method to measure enteropeptidase activity. The kinetic analysis revealed that enteropeptidase has a $K_M$ of 0.025 mM and a $k_{cat}$ of 65 sec⁻¹ for GD₄K-AMC, whereas it has a $K_M$ of 0.5 to 0.6 mM and a $k_{cat}$ of 25 sec⁻¹ for GD₄K-NA. The optimum pH of GD₄K-AMC hydrolysis was pH 8.0. Our data indicate that GD₄K-AMC is more suitable as a substrate for enteropeptidase than GD₄K-NA. [BMB reports 2011; 44(7): 458-461]

INTRODUCTION

Enteropeptidase (EC 3.4.21.9), which is also known as entero-kinase, is a serine protease responsible for the conversion of inactive trypsinogen to its active form trypsin (1, 2). Enteropeptidase consists of two subunits linked by a disulfide bond, an 82-140 kDa heavy chain that anchors enteropeptidase in the intestinal brush border membrane and a 35-62 kDa light chain that is the catalytic subunit (1). The light chain of the enteropeptidase recognizes the sequence Asp-Asp-Asp-Asp-Lys (D₄K) and cleaves the peptide bond after the Lys residue (3, 4).

The assay for enteropeptidase has utilized either trypsinogen activation or the cleavage of synthetic peptides. The trypsino-

gen activation assay measured the conversion of trypsinogen into active trypsin. Since both enteropeptidase and trypsin competed for further activation of trypsin (5), trypsinogen was not suitable as a substrate for enteropeptidase. On the other hand, the cleavage of synthetic peptides utilized the spectral properties of fluorophore which is conjugated to the C-terminal end of D₄K. The hexapeptide Gly-Asp-Asp-Asp-Asp-Lys-conjugated 2-naphthylamine (GD₄K-NA) has been used as the de facto fluorogenic substrate for enteropeptidase over the last 30 years (6, 7). This assay requires liberated 2-naphthylamine for colorimetric or fluorometric detection. In addition to 2-naphthylamine, 7-amino-4-methylcoumarin (AMC) has traditionally been used as a fluorogenic probe for many serine proteases (8). Surprisingly, D₄K-conjugated AMC has not been utilized to measure the enzymatic activity of enteropeptidase to date. Furthermore, naphthalene is toxic to human health and known as carcinogenic to humans and animals whereas AMC is known to be non-toxic.

In this study, we utilized GD₄K-AMC as a fluorogenic sub-
strate for bovine enteropeptidase light chain and used its spectral shift to measure the enzymatic activity. We demonstrated that GD₄K-AMC is more suitable as a substrate for enter-
ooseptidase than GD₄K-NA with respect to spectral read-out and enzymatic activity.

RESULTS AND DISCUSSION

The structure of 2-naphthylamine and AMC are shown in Fig. 1. The emission spectrum of 2-naphthylamine showed a max-

![Fig. 1. Structure of 2-naphthylamine (A) and 7-amino-4-methylcoumarin (B).](http://bmbreports.org)
GD4K-AMC as a substrate for enteropeptidase
Mal-Gi Choi, et al.

Fig. 2. Spectral properties of GD4K-NA and GD4K-AMC. Emission spectrum of 2-naphthylamine (A) and AMC (B). Emission spectrum of enteropeptidase (C). Disappearance of the emission spectrum of GD4K-NA and restoration to the spectrum of 2-naphthylamine after hydrolysis of the peptide bond between Lys and 2-naphthylamine (D). Blue-shift in the emission spectrum of GD4K-AMC and restoration to the spectrum of AMC after hydrolysis of the peptide bond between Lys and AMC (E).

imum emission at 410 nm upon excitation at 340 nm (Fig. 2A), whereas AMC showed the maximum emission at 440 nm upon excitation at 340 nm (Fig. 2B). The formation of a peptide bond between Lys and 2-naphthylamine resulted in quenching of the emission spectrum (Fig. 2D dashed line). Upon cleavage of the peptide bond after Lys by enteropeptidase, the emission spectrum was restored to the original spectrum of 2-naphthylamine (Fig. 2D solid line). As a control, enteropeptidase itself showed no emission spectrum (Fig. 2C). Contrary to GD4K-NA, the formation of a peptide bond between Lys and AMC resulted in a blue-shift in the emission spectrum, shifting the maximum emission wavelength from 440 nm to 395 nm (Fig. 2E dashed line). Cleavage of the peptide bond after Lys restored the emission spectrum to one similar to the original spectrum of AMC (Fig. 2E solid line). The fluorescence at ~400 nm indicating the presence of GD4K-AMC in samples (Fig. 2E) suggests that both GD4K-AMC and liberated AMC existed in dynamic equilibrium. Our observations suggest that shifts in the emission spectra of AMC could be a fluorogenic method for measuring enteropeptidase activity.

Next, we determined the kinetic parameters of enteropeptidase for GD4K-AMC. As a control experiment, GD4K-NA was used as a substrate for bovine enteropeptidase light chain and the results agreed with those of previous studies (9-11), with the $K_m$ of GD4K-NA being 0.5 to 0.6 mM. The initial reaction rates of GD4K-AMC hydrolysis were measured and plotted on the Lineweaver-Burk plot (Fig. 3A). The Michaelis-Menten constant, $K_m$, determined in this study was 24.7 μM for GD4K-AMC (Table 1), which was ~20-fold lower than the $K_m$ of GD4K-NA. The bovine enteropeptidase light chain was reported to have a $K_m$ of 25.8 μM for trypsinogen at pH 8.4, whereas the native form of heavy and light chain dimer has a $K_m$ of 1.2 μM (9). Our data indicate that trypsinogen and GD4K-AMC have a similar affinity to the bovine enteropeptidase light chain. The $k_{cat}$ of GD4K-AMC was 64 sec⁻¹, which was ~2.5-fold higher than that of GD4K-NA (9). Overall, GD4K-AMC serves as a substrate with a lower $K_m$ and a higher $k_{cat}$ for enteropeptidase than GD4K-NA.

We further characterized the optimal pH and calcium concentration of enteropeptidase for GD4K-AMC. As shown in Fig. 3B, the optimal pH of enteropeptidase for GD4K-AMC was pH 8.0, which was also similar to the optimal pH 8.0 for GD4K-NA as reported by Hesford et al. (6). We also examined the effect of calcium ions on the activity of enteropeptidase. Grant and Hermon-Talor showed that the $K_m$ of enteropeptidase for GD4K-NA decreased from 0.525 mM to 0.28 mM when calcium concentration was changed from 0.1 mM to 10 mM (12). However, as shown in Fig. 3C, when GD4K-AMC was used as a substrate, the enteropeptidase activity was higher at lower calcium concentration, 0 to 1 mM, and the enzymatic activity was decreased 50% at 10 mM calcium concentration. We speculate that this difference might be due to distinct substrate specificity of enteropeptidase for GD4K-
GD4K-AMC as a substrate for enteropeptidase
Mal-Gi Choi, et al.

460 BMB reports

http://bmbreports.org

Fig. 3. Characterization of GD4K-AMC as a substrate for enteropeptidase. (A) Lineweaver-Burk plot. The GD4K-AMC concentration was 0-30 μM. The initial reaction rates of enteropeptidase were measured at 440 nm for hydrolyzed AMC. (B) pH optimum. The hydrolysis of 5 μM GD4K-AMC in 50 mM Tris·Cl buffer (pH 6.0-9.5) was measured for 5 min at 35°C. (C) Effect of calcium. The effect of varying concentrations of CaCl2, 0 to 10 mM, on the hydrolysis of 5 μM GD4K-AMC in 50 mM Tris·Cl, pH 8.0 was measured for 5 min at 35°C.

Table 1. Kinetic parameters of bovine enteropeptidase light chain for GD4K-conjugated fluorogenic substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km (μM)</th>
<th>kcat (s⁻¹)</th>
<th>kcat/Km (s⁻¹μM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsinogen</td>
<td>25.8 ± 1.6</td>
<td>2.3 ± 0.2</td>
<td>89 Lu et al. (9)</td>
</tr>
<tr>
<td>GD4K-NA</td>
<td>600 ± 0.12</td>
<td>24.9 ± 2.5</td>
<td>42 Lu et al. (9)</td>
</tr>
<tr>
<td>GD4K-AMC</td>
<td>24.7 ± 6.0</td>
<td>64.8 ± 1.0</td>
<td>2,623 This study</td>
</tr>
</tbody>
</table>

(13). Fusion proteins are usually expressed in host cells with an affinity tag such as His6 followed by a protease cleavage sequence for protein purification. Since enteropeptidase leaves no extra amino acids on the N-terminus of target proteins upon cleavage after the substrate recognition sequence, the use of enteropeptidase is expected to increase with advances in the biotech industry.

In conclusion, we showed that the spectral shift in the emission of GD4K-AMC could be used in monitoring the enzymatic activity of enteropeptidase. We also determined the Michaelis-Menten constant, Km, and kcat of enteropeptidase for GD4K-AMC. Our data indicate that GD4K-AMC is more suitable as a substrate for enteropeptidase in place of possibly carcinogenic GD4K-NA.

MATERIALS AND METHODS

Materials. 2-Naphthylamine, 7-amino-4-methylcoumarin, and GD4K-NA were purchased from Sigma (USA). GD4K-AMC was synthesized at Peptron (Korea). Bovine enteropeptidase light chain was purchased from New England Biolabs (USA). All other reagents were obtained from Sigma unless otherwise noted.

Fluorescence Measurements. Experiments were conducted using 76 pM of bovine enteropeptidase light chain in 50 mM Tris·Cl buffer (pH 6.0-9.5) was measured for 5 min at 35°C. The effect of varying concentrations of CaCl2, 0 to 10 mM, on the hydrolysis of 5 μM GD4K-AMC in 50 mM Tris·Cl, pH 8.0 was measured for 5 min at 35°C.

AMC and GD4K-NA.

In recent years, many fusion proteins have been developed as bio-drugs to increase the half-life of therapeutically important proteins, which are susceptible to degradation by proteases in circulation, by tethering macromolecules such as human serum albumin or immunoglobulin heavy chain gamma (13). Fusion proteins are usually expressed in host cells with an affinity tag such as His6 followed by a protease cleavage sequence for protein purification. Since enteropeptidase leaves no extra amino acids on the N-terminus of target proteins upon cleavage after the substrate recognition sequence, the use of enteropeptidase is expected to increase with advances in the biotech industry.

In conclusion, we showed that the spectral shift in the emission of GD4K-AMC could be used in monitoring the enzymatic activity of enteropeptidase. We also determined the Michaelis-Menten constant, Km, and kcat of enteropeptidase for GD4K-AMC. Our data indicate that GD4K-AMC is more suitable as a substrate for enteropeptidase in place of possibly carcinogenic GD4K-NA.

MATERIALS AND METHODS

Materials. 2-Naphthylamine, 7-amino-4-methylcoumarin, and GD4K-NA were purchased from Sigma (USA). GD4K-AMC was synthesized at Peptron (Korea). Bovine enteropeptidase light chain was purchased from New England Biolabs (USA). All other reagents were obtained from Sigma unless otherwise noted.

Fluorescence Measurements. Experiments were conducted using 76 pM of bovine enteropeptidase light chain in 50 mM Tris·Cl, 0.1 mM CaCl2, pH 8.0 buffer at 35°C. The substrate concentration was in the range of 0-1 mM for GD4K-NA and 0-30 μM for GD4K-AMC, respectively. Fluorescence was measured using a Varian Cary Eclipse fluorescence spectrophotometer between 350-550 nm upon excitation at 340 nm in a 100 μl quartz cuvette placed in a thermostatted cuvette holder. The initial reaction rates of enteropeptidase were measured at 440 nm for liberated AMC. The Michaelis-Menten constant, Km, and kcat were determined from the Lineweaver-Burt plot. All experiments were repeated three times.

Optimal pH and calcium concentration. Fluorescence was measured to determine pH optimum upon hydrolysis of 5 μM GD4K-AMC by enteropeptidase in 50 mM Tris·Cl buffer (pH 6.0-9.5) for 5 min at 35°C. Fluorescence was also measured in varying concentrations of CaCl2, 0 to 10 mM, upon hydrolysis of 5 μM GD4K-AMC by enteropeptidase in 50 mM Tris·Cl, pH 8.0, for 5 min at 35°C. Experiments were repeated three times. A representative data was shown for pH optimum (Fig. 3B) and the results were presented as mean ± S.D. for calcium concentration (Fig. 3C).
Acknowledgements
We thank Dr. Tai Jong Kang for helpful discussions. This work was supported by the Daegu University Research Grant, 2008 (to C. L.).

REFERENCES