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Engineering β-lactamase zymogens for use in protease activity assays

Hajin Kim, Hyun Kyung Yoon, and Tae Hyeon Yoo

In this study, the concept of autoinhibition, a mechanism of protein activity regulation, was applied to the design and engineering of a β-lactamase zymogen. Using this zymogen, a sensitive protease assay method was developed in which activation of the zymogen by proteases produces an amplified absorbance signal. The approach reported here can be adapted for engineering of zymogens as biological sensors and components of synthetic signaling pathways.

Zymogens are inactive precursors, which are converted into active enzymes via the hydrolysis of peptide bonds by proteolytic enzymes. The conversion of zymogens into active enzymes plays an important role in the regulation of various biological phenomena. In particular, proteolytic enzymes themselves often exist as zymogens that are activated by other proteases. This not only provides a means of controlling biological processes, but also overcomes the potentially detrimental effects of the presence of constitutively active proteases. In addition to the biological importance of zymogens, zymogens and more specifically engineered zymogens can be applied in the development of sensors and the construction of synthetic signaling cascades. Since zymogens are activated by the cleavage of peptide bonds, protease activity assay methods have been developed using natural and engineered zymogens. A reporter enzyme, for example, was engineered to be inactive by restricting a conformational change which is required for activity. The structural restriction is subsequently relieved by the action of a protease. Such design strategies are, however, difficult to generalize, and the engineered enzymes are used in an unpurified state, possibly because of their intrinsic instability, which may limit the development of standardized methods. Considering the roles that zymogens play in biological processes, engineered zymogens may play an important part in constructing novel signaling pathways; however, such applications have not yet been widely reported. One reason explaining the limited number of reports of such applications may be the technical difficulty associated with designing and engineering zymogens to behave as intended. In this communication, we report the engineering of a zymogen designed using the concept of autoinhibition–nature’s mechanism for the regulation of protein activity.

An autoinhibited protein is composed of an activity domain and a polypeptide pseudosubstrate blocking the active site; the two domains interact with each other via an intramolecular interaction. Because of the entropic advantage of intramolecular interactions, autoinhibited proteins exhibit low activities even when the affinity between the inhibitory- and activity-domains is low. Autoinhibited proteins are activated by the displacement of the inhibitory region via its modification, binding to other molecules, or by cleavage of the linkage between the activity- and inhibitory-domains. This autoinhibition concept has been applied in the design of protein switches. Autoinhibited enzymes, which are activated via proteolytic cleavage, are classed as zymogens, and zymogens can thus be engineered by fusion of an enzyme and its inhibitor via a linker containing a protease cleavage site.
In this study, an autoinhibited β-lactamase enzyme was engineered by fusion of a β-lactamase enzyme and its inhibitory protein (β-lactamase inhibitory protein, BLIP) via a peptide linker containing a protease cleavage site. Furthermore, since the autoinhibited proteins are activated by proteases, the engineered zymogen was used in the development of a protease assay method (Figure 1a). Abnormal protease activity is associated with various diseases, and thus such an assay method has applications in biomedical research. An advantage of the use of β-lactamase zymogens as sensors is that various chromogenic substrates of β-lactamase are available. The β-lactamase zymogen is converted into an active β-lactamase, which produces a detectable signal. In our study, absorbance detection rather than fluorescence detection was favored despite the higher sensitivity of fluorescence measurements, because, compared with fluorescence instruments, instruments for absorbance measurement are generally more readily available in research laboratories. In the assay, the signal generated by the protease is amplified via the catalytic conversion of chromogenic β-lactamase substrate into an active β-lactamase, increasing the sensitivity of the assay.

To construct an autoinhibited β-lactamase, the interaction between TEM-1, a class A β-lactamase from *Escherichia coli*, and BLIP, a potent inhibitor protein of β-lactamase (Figure 1b), was utilized. The strategy was to link two ends of two proteins via a peptide sequence including a protease cleavage site to construct an autoinhibited β-lactamase. However, as shown in Figure 1b, the two ends are far from each other, and a relatively long linker would have been ineffective for engineering autoinhibited proteins. Instead, a circularly permuted version of TEM-1 (circularly permuted β-lactamase: cpBLA) was engineered such that the new ends of the protein were close to the BLIP binding site. Based on previous reports on *Bacillus licheniformis* β-lactamase, positions M268 and A266 were selected as new N- and C-termini, respectively, and the original ends were connected via a GGGGG linker (Figure 1c). The catalytic activities of TEM-1 and cpBLA were determined and compared using CENTA as a β-lactamase substrate (Table 1). The two enzymes were found to have comparable $k_{cat}/K_M$ values (186.6 s$^{-1}$mM$^{-1}$ for TEM-1 and 178.7 s$^{-1}$mM$^{-1}$ for cpBLA), which suggests that the structure of cpBLA was intact for hydrolysis of the β-lactam ring.

![Fig. 1](image-url) (a) Scheme of the protease activity assay method based on a β-lactamase zymogen, where CENTA is used as a β-lactamase substrate. (b) Structure of the complex of TEM-1 (green) and BLIP (pink) (PDB: 1JTG); the N- and C-termini of each protein are shown in the cartoon. (c) cpBLA was engineered by connecting the N- and C-termini of TEM-1 via a linker of GGGGG (red line) and creating new N- and C-termini at M268 and A266, respectively.

**Table 1. Kinetic characterization of β-lactamases and β-lactamase zymogens.**

<table>
<thead>
<tr>
<th>Construct</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}/K_M$ (s$^{-1}$mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-1</td>
<td>23.4 (±3.0)</td>
<td>125 (±16)</td>
<td>186.6 (±0.8)</td>
</tr>
<tr>
<td>cpBLA</td>
<td>73.5 (±13.1)</td>
<td>418 (±95)</td>
<td>178.7 (±28.8)</td>
</tr>
<tr>
<td>I</td>
<td>0.35 (±0.08)</td>
<td>656 (±157)</td>
<td>0.532 (±0.070)</td>
</tr>
<tr>
<td>II</td>
<td>0.69 (±0.12)</td>
<td>602 (±138)</td>
<td>1.17 (±0.13)</td>
</tr>
<tr>
<td>II(K74A)</td>
<td>2.43 (±1.01)</td>
<td>723 (±190)</td>
<td>3.66 (±1.89)</td>
</tr>
<tr>
<td>II(F142A)</td>
<td>1.49 (±0.10)</td>
<td>534 (±104)</td>
<td>2.84 (±0.41)</td>
</tr>
<tr>
<td>II(W150A)</td>
<td>3.20 (±1.09)</td>
<td>821 (±75)</td>
<td>3.94 (±1.45)</td>
</tr>
<tr>
<td>II(F142A)-1</td>
<td>1.38 (±0.12)</td>
<td>488 (±60)</td>
<td>2.86 (±0.52)</td>
</tr>
<tr>
<td>II(F142A)-2</td>
<td>1.15 (±0.12)</td>
<td>485 (±116)</td>
<td>2.50 (±0.81)</td>
</tr>
</tbody>
</table>

Two β-lactamases zymogens were constructed by fusing the C-terminus of BLIP to the N-terminus of cpBLA and the C-terminus of cpBLA to the N-terminus of BLIP via a linker containing an MMP2 cleavage site (Figure 2a). MMP2 plays an important role in cancer metastasis, and assay methods for the quantification of this enzyme have been developed. The engineered autoinhibited β-lactamases were expected to exhibit lower catalytic activities than cpBLA, and the $k_{cat}/K_M$ values for CENTA were measured for constructs I and II. The two constructs were shown to act as zymogens; however, to obtain larger signal increases following activation by MMP2, which is critical for the development of a highly sensitive protease assay, the engineered zymogens were further optimized. Since the expression levels of construct I were low, construct II was selected for further optimization. The strength of intramolecular interactions...
is known to be affected by two factors: linker features including length, composition, and flexibility, and the binding affinity between the inhibitory domain and the activity domain. A high binding affinity between the inhibitory and activity domains is advantageous in that the activity of the autoinhibited protein is suppressed; however, because the inhibitory domain is still present in the solution following cleavage, a high binding affinity may also decrease the activity of the activity domain with protease treatment. Too strong a binding of the inhibitory domain to the activity domain may thus not yield optimal results. Several mutations of BLIP have been reported to decrease the binding affinity between BLIP and TEM-1. Three such mutations (F74A, F142A, and W150A) were thus introduced into construct II (Figure 2a) and, as expected, the three mutated constructs exhibited higher $k_{cat}/K_M$ values than those of construct II (Table 1). Of these three constructs, construct II(F142A) exhibited the largest signal increase following MMP2 treatment (Figure 2). A variant of construct II with a longer linker (GGGGSGSPGVLGRGGGSGGS) was found to have a higher $k_{cat}/K_M$ value than that of construct II (2.36 vs. 1.17 s$^{-1}$mM$^{-1}$), which suggested that optimization may be achieved by reducing the linker length to be shorter than that of the original construct. Constructs with linkers shorter than the one used in construct II(F142A) were thus generated (Figure 2a), and in fact, larger signal increases were observed following MMP2 treatment in constructs with shorter linkers, with the largest signal increase being observed with construct II(F142A)-2 containing a medium length linker.

Using the optimized construct II(F142A)-2, the relationship between MMP2 concentration and initial hydrolysis velocity of CENTA was investigated. As shown in Figure 3, a linear relationship was observed for low concentrations of MMP2 (≤ 1 nM), while a hyperbolic curve was obtained when the entire MMP2 concentration range was included. The signal saturation observed at high MMP2 concentrations may result from the technical limitations in measuring $V_0$ at high $\beta$-lactamase concentrations. For concentrations of MMP2 as low as 25 pM, the signal was found to be distinguishable from background signal.

In this study, autoinhibited $\beta$-lactamase enzymes, which can be activated by proteases and used as highly sensitive protease assay sensors, were engineered. Thezymogens were constructed by fusing a circularly permuted $\beta$-lactamase and its inhibitor BLIP via a linker containing a protease cleavage site. Many pairs of enzymes and their protein or peptide inhibitors have been reported, and peptide or protein inhibitors can be found via high-throughput screening technologies such as phage and cell-surface display platforms. We thus anticipate that the strategy employed in this study can be widely applied to engineering zymogens that can be developed into sensors or components of synthetic signaling pathways.

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Notes and references