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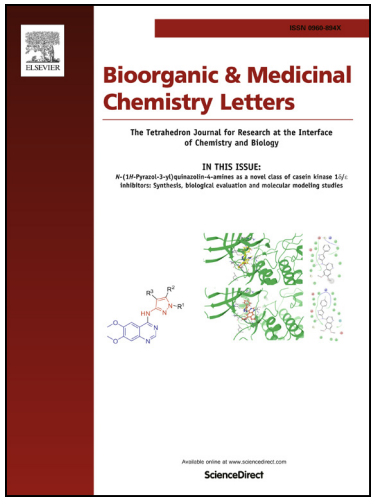

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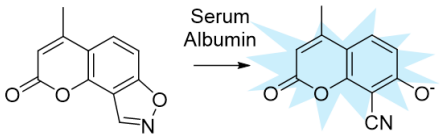

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Fluorescence detection of serum albumin with a turnover-based sensor utilizing Kemp elimination reaction

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ABSTRACT

The Kemp elimination reaction is a well-known chemical reaction that is facilitated on a protein surface microenvironment, and in particular is highly accelerated in a unique binding pocket of serum albumin. We have designed and synthesized a fluorescently activatable coumarin derivative with a benzisoxazole scaffold to enable monitoring of the Kemp elimination reaction in terms of fluorescence change for the first time. We show that this fluorescent sensor can sensitively and selectively quantitate serum albumin in blood samples. It also works in a dry-chemistry format.

Protein surfaces provide unique microenvironments due to the arrangement of reactive side chains, e.g., at enzyme active sites. Various chemical reactions are accelerated on protein surface sites, and among them, the Kemp elimination reaction has attracted many researchers as a model of enzymatic catalysis[1]. The reaction involves formation of salicylonitriles from benzisoxazoles via deprotonation of a carbon by a general base in a cooperative E2 reaction[2]. The reaction is accelerated under basic conditions and in hydrophobic environments (such as on protein surfaces), and much work has been done to develop catalytic antibodies or other protein surface structures suitable to serve as Kemp eliminases for studies of the basic mechanism of enzymatic catalysis by transition state stabilization[1, 3]. It has also been reported that certain naturally occurring proteins, such as serum albumin, catalyze the reaction very efficiently due to their unique binding properties for small molecules[4].

Here, we describe the design, synthesis and characterization of the first fluorescent Kemp reaction substrate, with the aim of developing a highly sensitive tool to study this interesting enzyme-like reaction in biological samples. Currently, Kemp elimination reactions are mainly studied by using colorimetric substrates such as 5-nitrobenzisoxazole[4]. However, compared to colorimetric assays, fluorescence assays offer greater sensitivity and wider applicability, being especially advantageous for use with bio-samples[5]. We aimed to develop a fluorescent substrate to monitor Kemp elimination by combining a fluorescent 7-hydroxycoumarin (umbelliferone) moiety with a benzisoxazole scaffold. We confirmed that the synthesized substrate works as a sensitive platform for detection of the Kemp elimination reaction. In particular, we found that this sensor can quantify serum albumin concentration in blood samples with high specificity and sensitivity. Changes of serum albumin concentration are associated with various pathophysiological states, as well as affecting free drug concentration; thus, we believe this sensor has potential for clinical application, especially since it also works in a dry-chemistry format.

In designing the Kemp elimination substrate, we required a strategy for employing the reaction to trigger fluorescence activation. Since the Kemp elimination reaction generates phenolate as the end product, we considered that this could be utilized to switch the fluorescence of umbelliferone, because the ether or ester form of umbelliferone is known to exhibit absorbance/fluorescence at shorter wavelengths than the phenolate form, and this has been employed as a basis for fluorescence switching in various sensors for ester or ether cleavage reactions[5b, 6].

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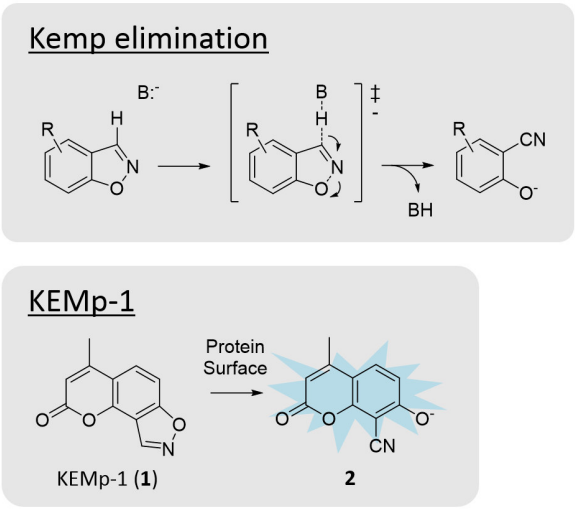

Figure 1. Design of fluorescent Kemp elimination substrate KEMp-1.

Thus, we planned to obtain a fluorescent Kemp elimination substrate, KEMp-1 (**1**) (**Figure 1**), by introducing a formyl group into 7-hydroxy-4-methylcoumarin with the Duff reaction[7], and then forming a benzisoxazole ring by oxime formation and oxidative condensation (**Scheme S1**). The expected elimination product (**2**) was also prepared by treatment of benzisoxazole in mild basic conditions.

KEMp-1 (**1**) showed an absorbance maximum at 320 nm, like other umbelliferone derivatives with protection of the 7-OH group (**Figure 2a**). Unexpectedly, it did not exhibit fluorescence on excitation at 320 nm ($\Phi_{FL} < 0.01$). In contrast, the hydrolysis product, 8-cyano-7-hydroxy-4-methylcoumarin (**2**), had an absorbance maximum at around 380 nm, and it exhibited strong fluorescence ($\Phi_{FL} = 0.59$). Therefore, the Kemp elimination reaction can be monitored in terms of fluorescence activation under 380 nm excitation. Indeed, after the addition of bovine serum albumin (BSA), a protein known to have high catalytic activity toward benzisoxazoles[4a], a dramatic absorbance change was observed at pH 7.4, and the reaction resulted in more than 80-fold fluorescence activation (**Figure 2b**). LC-MS analysis confirmed clean conversion of benzisoxazole to the salicylonitrile derivative (**Figure S1**).

Next, we examined the reactivity of the KEMp-1 with various proteins. Interestingly, the fluorescence increase was highly selective for serum albumin, especially for human serum albumin (**Figure 2c**). Addition of glutathione had no effect on the reaction, suggesting that KEMp-1 binds specifically to the surface of albumin for the catalytic elimination. The k_{cat}/K_m value was not high compared to those of general enzymatic reactions (**Table 1**). However, the reaction of conventional benzisoxazoles[4b] with serum albumin is blocked by the modification of key lysine residues [8], and the modification of lysine residues with fluorescein isothiocyanate (FITC) was confirmed to dramatically reduce the reactivity of KEMp-1 (**Figure 2d**). Therefore, the reaction of KEMp-1 may proceed via a similar mechanism to that of conventional benzisoxazoles. Aiming to take advantage of the high sensitivity of fluorometric assay with turnover-based signal amplification, we next examined whether this system could be applied to monitor Kemp elimination reaction catalyzed by serum albumin in blood samples.

Serum albumin is a major blood protein[9], serving as a carrier of hydrophobic compounds such as steroids, hemins, and fatty acids. Many therapeutic drugs also bind to albumin, which consequently influences their efficacy by reducing the free (effective) drug concentration[10]. Since albumin is produced in the liver, its blood concentration is often altered in liver disease[11], and therefore monitoring of changes in serum albumin concentration is important for clinical management of patients. At present, colorimetric assays that utilize the binding of organic dyes (e.g. bromocresol green) to albumin are widely used to measure blood albumin levels, but these assays have limited sensitivity and selectivity[12]. We expected that a fluorescent sensor would offer superior performance.

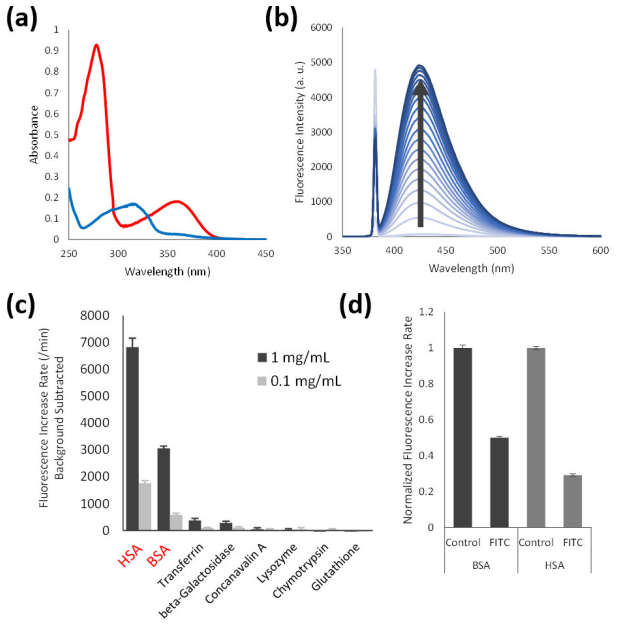

Figure 2. (a) Absorbance spectra of KEMp-1 (10 μM) before and after addition of bovine serum albumin (BSA; 1 mg/mL) in phosphate buffer (100 mM, pH 7.4) and incubation for 18 hr. (b) Fluorescence spectra of KEMp-1 (10 μM) after addition of bovine serum albumin (BSA; 1 mg/mL) in phosphate buffer (100 mM, pH 7.4). (c) Fluorescence increase rates of KEMp-1 (10 μM) after addition of various proteins (1 mg/mL or 0.1 mg/mL). n = 4. Error bars represent S.D.. (d) Normalized fluorescence increase rates of KEMp-1 (10 μM) after addition of human serum albumin (HSA; 1 mg/mL) modified with or without fluorescein isothiocyanate (FITC) in phosphate buffer (100 mM, pH 7.4). n = 4. Error bars represent S.D..

Table 1. Kinetic parameters of KEMp-1 reactivity with human serum albumin (HSA) and bovine serum albumin (BSA).

	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m (M^{-1} s^{-1})
BSA	8.5×10^{-6}	7.2×10^{-6}	1.2
HSA	2.4×10^{-5}	7.0×10^{-6}	3.4

Indeed, KEMp-1 was able to detect human serum albumin (HSA) at a concentration as low as 0.02 mg/mL (**Figure 3a**); this sensitivity is comparable to that of conventional Kemp elimination substrates, and is greater than that of a commercial albumin detection kit, which failed to detect HSA at concentrations below 0.04 mg/mL (**Figure S2**). We were able to detect a fluorescence increase even in the human serum diluted to an albumin concentration of 0.04% (**Figure S3**). Our serum sample contained 75 mg/mL of proteins (determined by Bradford assay using HSA as a standard), of which approximately 60-70%

was considered to be albumin, as judged from SDS-PAGE analysis (**Figure 3b**, **S4**). We found that calibration curves prepared with diluted HSA solution and with diluted serum calculated to contain equivalent amounts of HSA coincided very closely, suggesting that most of the fluorescence increase in the latter case originated from albumin in blood (**Figure 3b**). We also examined a HeLa cell lysate, which contained no albumin. The cell lysate showed dramatically reduced Kemp elimination activity (about 14% of that of serum), supporting the idea that the fluorescence increase observed with human serum is predominantly due to albumin (**Figure S5**).

In chronic hepatic failure, albumin concentration is known to decrease from 3.8-5.3 g/dL to 2-3 g/dL. Therefore, we prepared model samples by mixing human serum (5 g/dL HSA) and HeLa cell lysate (0 g/dL HSA) having the same protein content in different ratios to obtain final HSA concentrations of 0, 1, 2, 3, 4, and 5 g/dL. These solutions were clearly differentiated by the fluorometric assay using 100-fold diluted samples, and we obtained a good linear relationship between fluorescence increase and HSA concentration (**Figure S6**).

Finally, in order to test the potential utility of this system as a dry-chemistry platform for on-site detection of serum albumin in blood samples in the clinical context, we applied a solution of KEMp-1 to a filter paper and dried it. Application of a 5 µL serum sample to the dried paper induced a dramatic fluorescence increase, which was easily detectable with the naked eye under excitation with a handy UV lamp (**Figure 3c**).

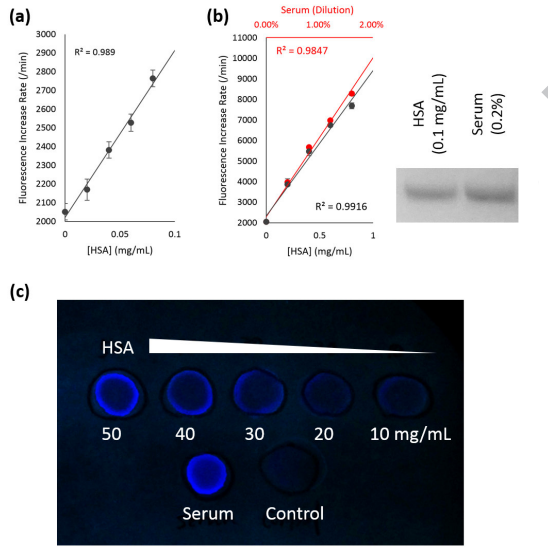

Figure 3. (a) Fluorescence increase rate of KEMp-1 (10 µM) after addition of HSA in phosphate buffer (100 mM, pH 7.4) and incubation for 60 min. n = 4. Error bars represent S.D.. (b) (Left) Fluorescence increase rate of KEMp-1 (10 µM) after addition of HSA (black) or serum (red) in phosphate buffer (100 mM, pH 7.4) and incubation for 60 min. n = 3. Error bars represent S.D.. (Right) SDS-PAGE analysis of human serum (0.2%) and HSA solution (0.1 mg/mL). (c) Photo of dried filter paper with KEMp-1 (50 pmol per spot) incubated with HSA (10-50 mg/mL), human serum, or phosphate buffer for 20 min. Illumination was done with a handy UV lamp (365 nm).

In conclusion, we have developed a Kemp elimination substrate that enables fluorescence-based visualization of the reaction in bio-samples. Like conventional benzisoxazoles, it showed high selectivity for serum albumin, and it also provided very high sensitivity. While the current molecule is not optimized, structural modifications should enable further improvement of the specificity and sensitivity, and it may also be possible to synthesize derivatives targeting other proteins. Such sensors could be useful for detection of various small molecule-protein engagements in complex biological systems, since turnover-based detection enables dramatic signal amplification; for example, turnover-based strategies are often employed to detect even very low levels of enzyme activity[5b, 13]. We wish to emphasize that KEMp-1 can specifically quantify serum albumin concentration in blood samples with high sensitivity, and is also applicable in a dry-chemistry format. Therefore, we believe this sensor has potential to be developed for clinical application. Further studies are ongoing in our laboratory.

Acknowledgments

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Supplementary material

Synthesis and characterization of KEMp-1, methods, and supplementary data.

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