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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]

Thus, we planned to obtain a fluorescent Kemp elimination substrate, KEMp-1 (**1**) (Fig. 1), by introducing a formyl group into

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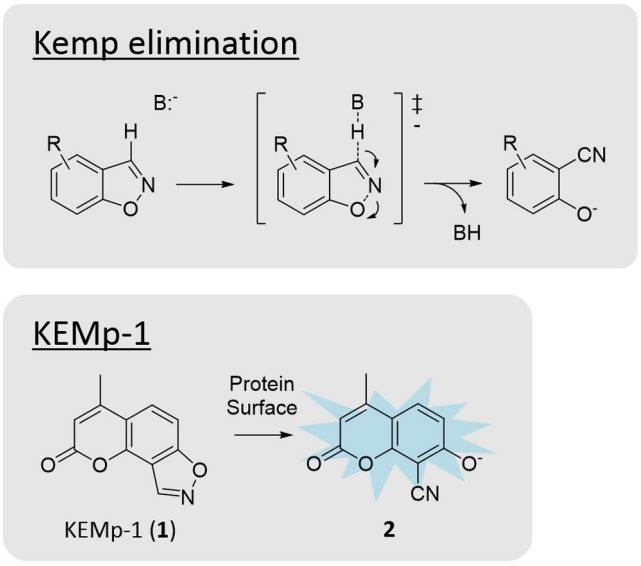

Fig. 1. Design of fluorescent Kemp elimination substrate KEMp-1.

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 (Fig. 2a). Unexpectedly, it did not exhibit fluorescence on excitation at 320 nm (Φ_{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 (Φ_{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 (Fig. 2b). LC-MS analysis confirmed clean conversion of benzisoxazole to the salicylonitrile derivative (Fig. 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 (Fig. 2c). Addition of glutathione had no effect on the reaction,

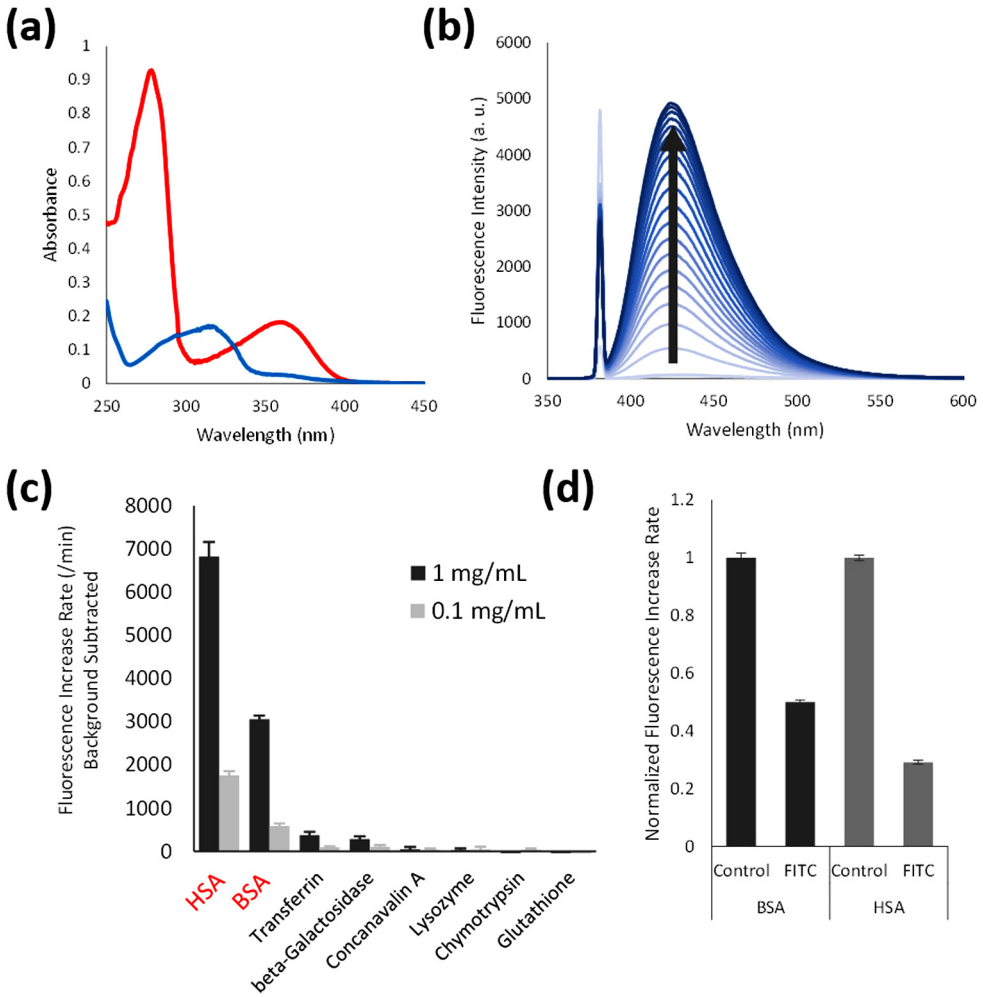

Fig. 2. (a) Absorbance spectra of KEMp-1 (10 μM) before and after addition of bovine serum albumin (BSA; 1 mg/mL) in phosphate buffer (100 mM, pH 7.4) and incubation for 18 h. (b) Fluorescence spectra of KEMp-1 (10 μM) after addition of bovine serum albumin (BSA; 1 mg/mL) in phosphate buffer (100 mM, 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.