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A simple and sensitive detection of glutamic-pyruvic transaminase activity based on fluorescence quenching of bovine serum albumin

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It is well known that Cu(II) can coordinate with L-alanine (Cu–Ala), which can be destroyed through the addition of glutamic-pyruvic transaminase (GPT) since GPT can effectively catalyze the conversion of L-alanine into keto-acetic acid. As a result, the free Cu(II) ion can combine with bovine serum albumin (BSA) and in turn quench the fluorescence of BSA. In this context, a simple and sensitive GPT activity detection *via* fluorescence quenching method has been developed. The fluorescence intensity of the system shows a linear relationship with the GPT concentration in the range of 5 and 400 U L^{-1} with a detection limit down to 3 U L^{-1} (S/N = 3). Avoiding any labels or complicated operations, this cost-effective and convenient method holds the potential for the rapid diagnosis of GPT-related diseases.

1. Introduction

Glutamic-pyruvic transaminase (GPT; synonym alanine aminotransferase, ALT), is an important amino transferase that can catalyze the transfer of the amino group from L-alanine to α-ketoglutarate.[1] It is located in most cells in the body, but found at the highest concentrations in the liver. Early studies showed that GPT is a marker of liver disease due to the release of GPT into the blood following liver cell necrosis.[2] Being through severe damage, GPT levels could rise up to 50 times higher than the normal (about 400 U L^{-1}).[3,4] Therefore, GPT activity detection is crucial to the diagnosis of diseases. Conventional methods for GPT analysis mainly rely on colorimetric, spectrophotometry and chromatography techniques.[5] Generally, such techniques require relatively expensive reagents, including enzymes and cofactors, and complicated procedures along with the low accuracy.[6–9] Consequently, in order to address these problems, other alternative methods were explored for GPT activity determination. For instance, Rietz and Guilbault developed fluorimetric methods to determine the activity of GPT both in solution and on a solid-surface.[10] Linear responses were observed in the ranges 5–106 U L^{-1}. I. Moser *et al.* established a miniaturized liquid handling system comprising thin film biosensor array for GPT determination.[11] The system shows a linear relationship with the GPT concentration in the range of 6 and 192 U L^{-1}. Hiroaki Suzuki *et al.* fabricated a microfluidic system for GPT activity measuring based on a Y-shaped flow channel equipped with an on-chip L-glutamate sensor with the GPT activity up to 250 U L^{-1}.[12] Recently, Ruey-JenYang *et al.* developed a paper-based analytical devices for the determination of GPT activity (0–125 U L^{-1}).[13] Despite these early encouraging efforts and the extensive studies on the structure and mechanism of GPT,[14,15] sensitive and rapid methods for GPT detection with wide working range are highly desirable and urgent since the activities of GPT are being routinely determined in clinical laboratories, particularly for the diagnosis of liver diseases.

Bovine serum albumin (BSA), the most abundant protein in the circulatory system, has been one of the most extensively studied proteins.[16–19] Containing tryptophan, tyrosine, and phenylalanine residues, this protein possesses intrinsic fluorescence.[20,21] It also has an affinity site with both histidyl and carboxyl groups for Cu(II) ion. It has been reported that Cu(II) ion could bind with BSA, leading to the fluorescence quenching of BSA.[22] So the Cu(II)-catalyzed BSA fluorescence quenching has become one of the most effective methods for the detection of Cu(II), which is characterized by low cost, mild conditions and high sensitivity.[23,24] A variety of compounds including dyes, drugs, and quantum dots have been exploited for the selective detection of Cu(II) based on the fluorescence quenching of BSA.[25–29]

It is worth mentioning that, in human circulatory system, Cu(II) ions are migrated mainly in the form of complexes with amino acids.[30] The Cu(II)–amino acid complexes have attracted enormous research attentions and interests due to their unique structures and properties.[31–33] Specifically, Cu(II) complex with L-alanine (Cu–Ala) is a mononuclear complex, and the central Cu(II) ion is hexacoordinated with L-alanine.[34,35] It has been reported that the complexation between L-alanine and Cu(II) is far stronger than that between BSA and Cu(II), which can prevent

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the destruction of Cu–Ala by BSA.[36–38] On the other hand, GPT can catalyze the transfer of the amino group from L-alanine to α-ketoglutarate and sabotage the complexation of Cu(II) and L-alanine, resulting in the release of free Cu(II) ion.[39–41] We hypothesized that the subsequent combination of free Cu(II) ion with BSA would lead to the fluorescence intensity quenching of the system, which should demonstrate a direct relationship with the GPT activity. Herein, we report a sensitive and simple technique for GPT activity detection employing the novel fluorometric quenching strategy.

2. Experimental

2.1. Chemicals and reagents

Cupric nitrate ($Cu(NO_3)_2 \cdot 3H_2O$), L-alanine, pyridoxal phosphate (PLP), α-ketoglutaric acid and other reagents were purchased from Aladdin China Co. Ltd (Shanghai) and used as received. Bovine serum albumin (BSA) was obtained from Sigma-Aldrich Co. LLC. Glutamic-pyruvic transaminase (GPT, EC 2.6.1.2) was purchased from Sigma-Aldrich Co. LLC. One unit will convert 1.0 μmole of α-ketoglutarate to L-glutamate per min at pH 7.6 at 37 °C in the presence of L-alanine. Cu–Ala was synthesized according to the literature.[37] All other chemicals were at least analytical grade reagents and used directly without further purification. Aqueous solutions were prepared with Milli-Q water (18.4 MΩ cm^{-1}). Unless otherwise mentioned, all experiments were carried out at room temperature.

2.2. Instruments

Fluorescence measurements were carried out on an F-7000 fluorescence spectrometer (Hitachi Ltd, Shanghai, China) using a square quartz absorption cells that can hold 4 mL of solution. Excitation and emission slits were all set for a 10.0 nm band-pass. The excitation wavelength was set at 280 nm, and the emission spectra were collected from 290 to 470 nm. The fluorescence intensity at 340 nm was used to evaluate the performances of the proposed assay strategy. UV-visible absorption spectra were recorded using a Lambda 750 UV-vis spectrophotometer. All measurements were carried out at room temperature unless stated otherwise.

2.3. Procedure for GPT activity assay

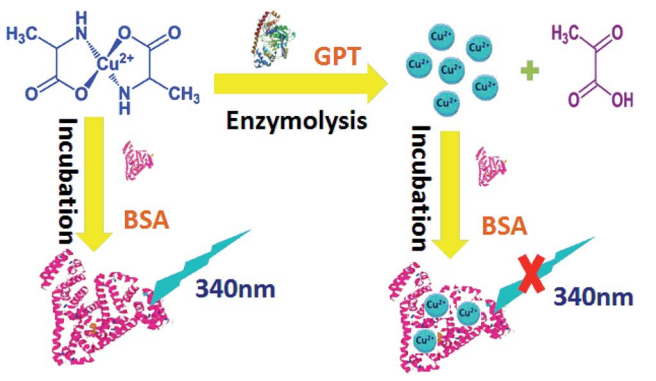
GPT activity assay was performed according to the following procedures. A 10 μL volume of GPT with a different final concentration ranging from 5 to 400 U L^{-1} was added to the aqueous dispersion consisting of Cu–Ala (20 μL, 200 mM, pH = 7.0), α-ketoglutaric acid (10 μL, 200 mM) and PLP (10 μL, 5 mM) at 30 °C followed by incubation for 20 min. BSA (2 mL, 2 mg mL^{-1}) was added to the resulting mixture, which was then incubated for 0.5 min. Subsequently, the data of fluorescence emission spectra were collected. The fluorescence quenching of BSA by Cu(II) ion was performed under the optimum conditions described in earlier reports.[22] Each point was detected for three times, and the average values were used for quantification.

3. Results and discussions

3.1. Principle of the detection system

The protocol of the proposed fluorometric quenching method is shown in Scheme 1. In the absence of GPT, since the complexation between L-alanine and Cu(II) is far stronger than that between BSA and Cu(II), it would be extremely difficult for BSA to deprive Cu(II) from Cu–Ala complex. Therefore, only a small amount of free Cu(II) ion that dissociated from Cu–Ala in solution could bind with BSA and cause the binded BSA fluorescence quenching, the system still remains strong fluorescence intensity. Through incubation with GPT, the transamination of L-alanine caused the damage to the structure of Cu–Ala, resulting in the release of more free Cu(II) ion. Subsequently, the free Cu(II) ion can combine with BSA, which results in larger degree quenching the fluorescence intensity of the system. As we know, the fluorescence quenching directly correlates with the concentration of GPT. It can be assumed that GPT activity could be rapidly and precisely determined by virtue of the fluorescence quenching of BSA.

Preliminary experiments were carried out to verify our presumption. As shown in Fig. 1, the GPT solution (curve a) and the substrate solution (including α-ketoglutaric acid and PLP, curve b) and Cu–Ala solution (curve i) showed very weak fluorescence signals, suggesting that GPT and Cu–Ala and the substrate solution are non-fluorescent and hardly interfere with the assay. A very strong fluorescence signal was observed for the BSA solution (curve c). After incubation with GPT (curve d) and substrate (curve e) respectively, strong fluorescence signals were still detected. Obviously, GPT and substrate were unable to quench the intrinsic fluorescence of BSA. As for the equivalent Cu–Ala (200 mM, 20 μL, curve f) and Cu(II) (200 mM, 20 μL, curve g) incubation with BSA respectively, the fluorescence signal of the former was much stronger than the latter. An explanation for this phenomenon could be the amount of free Cu(II) ion in former system was more than the latter system, more free Cu(II) ion combine with BSA lead to more fluorescence intensity quenching of the system. It can be deduced that L-alanine can prevent the binding of Cu(II) with BSA and only free Cu(II) ion in the system can interact with BSA, in turn causing the fluorescence



Scheme 1 Structure and sensing mechanism of fluorescent sensor for GPT.

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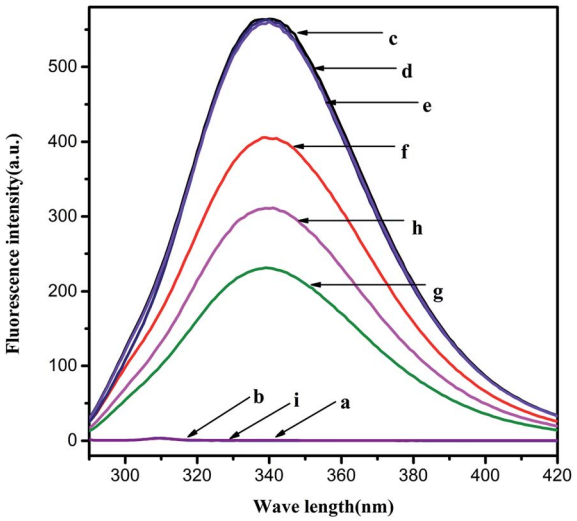

Fig. 1 Fluorescence responses of the sensing system under different conditions: (a) GPT solution (GPT: 10 μL 200 U L^{-1}; deionized water: 2.04 mL). (b) Substrate solution (α-ketoglutaric acid: 10 μL 200 mM; PLP: 10 μL 5 mM; deionized water: 2.03 mL). (c) BSA solution (BSA: 2.00 mL 2 mg mL^{-1}; deionized water: 50 μL). (d) GPT (10 μL 200 U L^{-1}; deionized water: 40 μL) incubation with BSA (2.00 mL 2 mg mL^{-1}). (e) Substrate (α-ketoglutaric acid: 10 μL 200 mM; PLP: 10 μL 5 mM; deionized water: 30 μL) incubation with BSA (2.00 mL 2 mg mL^{-1}). (f) Cu–Ala (20 μL 200 mM; deionized water: 30 μL) incubation with BSA (2.00 mL 2 mg mL^{-1}). (g) Cu(II) (20 μL 200 mM; deionized water: 30 μL) incubation with BSA (2.00 mL 2 mg mL^{-1}). (h) The mix solution containing Cu–Ala (20 μL 200 mM), α-ketoglutaric acid (10 μL 200 mM), PLP (10 μL 5 mM) and GPT (10 μL 200 U L^{-1}) incubation with BSA (2.00 mL 2 mg mL^{-1}). (i) Cu–Ala solution (Cu–Ala: 20 μL 200 mM; deionized water: 2.03 mL).

quenching. A fluorescence quenching was clearly detected after the addition of GPT into the system (curve h) and the fluorescence quenching degree was much stronger than the system without GPT (curve f). This observation means that the enzymatic reactions occurred and more free Cu(II) ions were formed. Conclusively, GPT, Cu–Ala and substrate cause negligible effect on the fluorescence of the system and the release of free Cu(II) ion from Cu–Ala catalyzed by GPT leads to BSA fluorescence quenching, which confirms the basic principle of our design.

3.2. Optimization of the experimental conditions

It has been observed that GPT can catalyze the Cu–Ala to release free Cu(II) and causing BSA fluorescence quenching. If the concentration of Cu–Ala is not high enough, Cu–Ala will be quickly catalyzed by a small amount of GPT in the solution. The resulting narrow linear range won't comply with the analytical conditions of the whole enzymatic reaction. But if too much Cu–Ala is added, the excess free Cu(II) ions will cause BSA protein denaturation[42] and then interfere with the corresponding fluorescence tests. Thus, the influence of the concentration of Cu–Ala on the fluorescence quenching degree were thoroughly studied. As shown in Fig. 2A, the fluorescence intensity decreased sharply with the increasing of Cu–Ala concentration. And after 200 mM it reached a plateau, this may attributed to lack of Cu–Ala and GPT was excessive and all of the Cu–Ala was transformed into free Cu(II) ions. But if more Cu–Ala was added, the excess free Cu(II) ions will cause BSA protein denaturation. Therefore, 200 mM Cu–Ala was used in the succeeding studies.

The influence of the reaction time between Cu–Ala and GPT was also examined. As shown in Fig. 2B, the fluorescence intensity at 430 nm weakened sharply with the increase of reaction time between Cu–Ala and GPT, which tended to be increased after 20 min. The maximum fluorescence quenching was observed after 20 min, which was chosen as the incubation time. The effect of temperature on the system was also studied since the temperature can affect the activity of GPT and the stability of Cu–Ala.[43] As illustrated in Fig. 2C, the fluorescence intensity of the system (without GPT) increased from 20 °C to 50 °C, which indicated that the stability of Cu–Ala increased (a). At 30 °C, the fluorescence intensity of enzyme reaction system (with GPT) reached the minimum (b), meaning that the activity of GPT was the strongest at this point. At each temperature the fluorescence intensity of the system without GPT (a) was higher than that of the system with GPT (b). The results indicated that GPT initiate the enzymatic reaction and the transamination of L-alanine caused the damage to the structure of Cu–Ala, resulting in the release of more free Cu(II) ion. Subsequently results in a larger degree decrease in fluorescence intensity of the system. Furthermore, the biggest difference of fluorescence intensity between (a) and (b) was obtained at 37 °C. Although the activity of GPT and the stability of Cu–Ala was not the largest at this point, the fluorescence quenching degree of the system caused by GPT was largest. So the suitable temperature was found to be 37 °C. Additionally, the pH values are also important to the enzymatic processes and the stability of Cu–Ala. The results (Fig. 2D) showed that the difference of fluorescence intensity between (a) and (b) was the biggest at pH 7.0. Therefore, pH value was set at 7.0 for the following experiments.

3.3. Performance of the sensing system

The activity of GPT was tested by adding different activity units of GPT into the test solution. Fig. 3A reveals the fluorescence spectra of the sensing system at different GPT concentrations. The fluorescence intensity decreased sharply with the increasing of GPT concentration in the range of 5–400 U L^{-1}, and decreased very slowly in the range of 400–500 U L^{-1}. Fig. 3B depicts the relationship between the fluorescence intensity and GPT concentrations. In addition, a linear correlation (the linear equation is $I = 383.644 - 0.431C_{GPT}$, C_{GPT} is the concentration of GPT and I is the fluorescence intensity, $R^2 = 0.995$) existed between the fluorescence intensity and C_{GPT} in the range of 5–400 U L^{-1} under the optimal conditions. The limit of detection (LOD) was calculated according to the definition of $3\sigma_b/s$, where σ_b is the standard deviation of the blank samples and s presents the slope.[44,45] In this study, the fluorescence signal of the blank sample was measured for 10 times, and the standard deviation (σ_b) was calculated. The LOD was finally determined to be 3 U L^{-1}. It is worth noting that this obtained detection limit is clearly lower than that in the early report based on the colorimetric ultramicromethod (10 U L^{-1}).[46] Moreover, the range of 5–400 U L^{-1} complies with the analytical conditions of the whole enzymatic reaction (Fig. 4).

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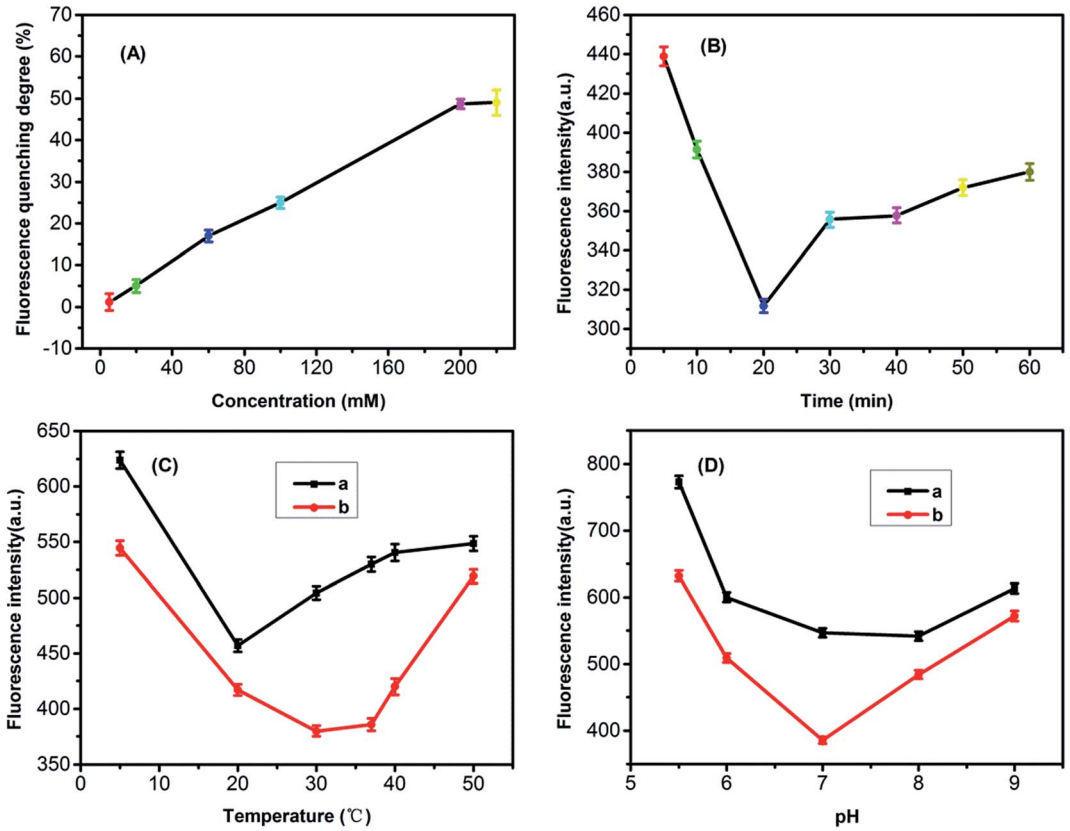

Fig. 2 (A) The concentration of Cu–Ala and the dependence of fluorescence quenching degree of the system. The concentration of GPT was 400 U L^{-1} (10 μL). (B) Effect of the reaction time between Cu–Ala and GPT on the fluorescence intensity of the system. (C) Effect temperature on the fluorescence intensity of the system: (a), without GPT; (b), with GPT. (D) Effect of media pH on the fluorescence intensity of the system: (a), without GPT; (b), with GPT.

3.4. Interference study

As we know, a large number of enzymes exist in the human circulatory system, which might pose the potential interference to the assay of GPT. Consequently, several representative enzymes including glutamine transaminage (TG), glucosaccharase (Glu), alcohol dehydrogenase (ADH), xanthine oxidase (XOD), and amylase were employed as the interferents to investigate the selectivity of the proposed biosensor. The concentrations of the used interferents were settled as TG 0.21 U,[47] Glu 0.5 U,[48] ADH 0.15 U,[49] XOD 0.20 U,[50] and amylase

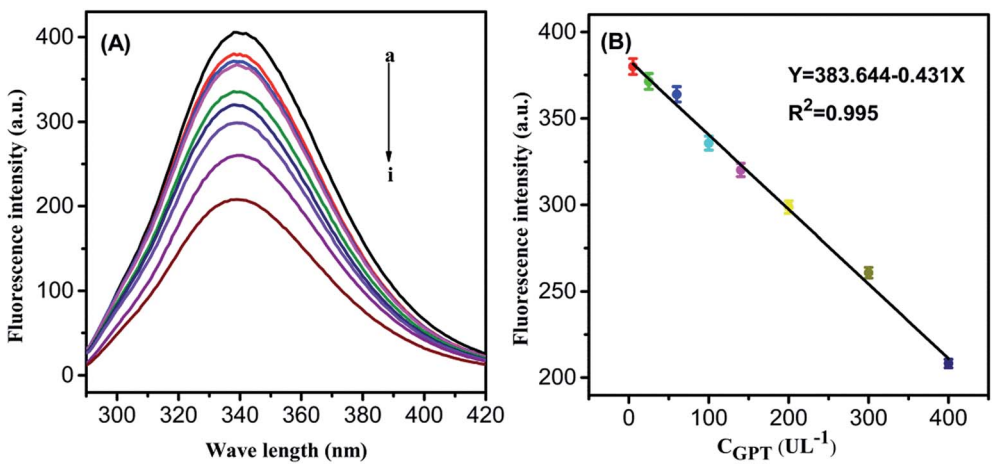

Fig. 3 (A) Fluorescence intensity of the system at different GPT concentrations. From (a) to (i): 0, 5, 25, 60, 100, 140, 200, 300 and 400 U L^{-1}. (B) Simulation curve between the fluorescence intensity and the GPT concentrations.

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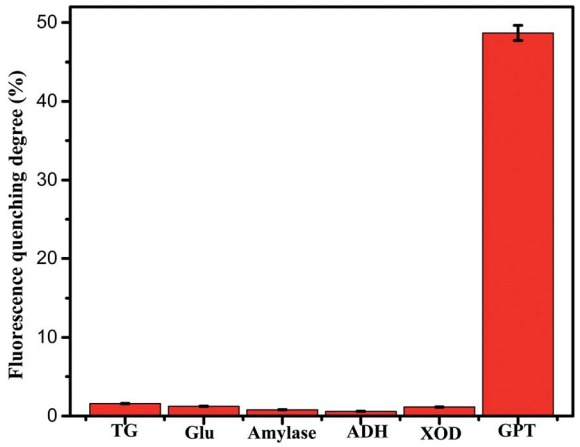

Fig. 4 Signals of the proposed biosensor at different interferents and targets. The concentration of GPT is 400 U L^{-1}, [TG] 0.2 U, [Glu] 0.5 U, [ADH] 0.15 U, [XOD] 0.20 U, [amylase] 1 mg mL^{-1}.

1 mg mL^{-1} (0.5 U)[51] respectively. It turned out that, in the presence of these selected interferents, the fluorescence quenching was hardly observed while a high fluorescence quenching was consistently achieved under the standard conditions. Presumably, these species were unable to affect the complexation between Cu(II) and L-alanine and prompt the fluorescence quenching reaction of BSA. At this stage, it can be concluded that this developed system shows an excellent selectivity for GPT detection and possesses the superior capability of resisting interference from other types of enzymes.

4. Conclusions

In summary, a fast and sensitive method for GPT activity assay based on the fluorescence quenching of BSA was developed. It has been found that GPT effectively destroyed Cu–Ala complex to release free Cu(II) ion, which subsequently combined with BSA leading to the fluorescence quenching. Various parameters of this proposed method has been extensively optimized. The superior performance of this technique including low LOD (3 U L^{-1}), wide working range (5–400 U L^{-1}) and high selectivity would pave the way for ultimately realizing the rapid and simplified diagnosis of GPT or GPT-related diseases.

Conflict of interest

The authors have declared no conflict of interest.

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