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Utilization of a precolumn with size exclusion and reversed-phase modes for size-exclusion chromatographic analysis of polysorbate-containing protein aggregates

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Keywords:

Size-exclusion chromatography

Polysorbate

Protein aggregation

MSpak GF-4A

Temperature-dependent retention

Abstract

Size-exclusion chromatography (SEC) is a useful method for quantification of protein aggregates because of its high throughput capacity and highly quantitative performance. One of the problems in this method concerns polysorbates, which are well-known additives for protein-containing products to prevent protein aggregation, but frequently interfere with the photometric detection of protein aggregates. We developed a new SEC method that can separate polysorbates from protein sample solutions in an on-line mode with a precolumn with size exclusion and reversed-phase mixed modes. The precolumn can effectively trap polysorbates in aqueous mobile phase, and the trapped polysorbates are easily eluted with acetonitrile-containing aqueous mobile phase to clean the precolumn. Small parts of protein aggregates may be also trapped on the precolumn depending on temperature and proteins. Setting appropriate column temperature can minimize such inconvenient trapping of aggregates.

1. Introduction

Nowadays, protein-based pharmaceutical products have rapidly been advanced. The situation increases demands for development of analytical technologies for protein aggregates, which are common sources of protein instability and are considered to concern their potential to elicit immune responses [1-5]. Various analytical methods that can evaluate protein aggregates have been developed such as analytical ultracentrifugation and asymmetric field flow fractionation [6-10]. However, these methods still have several weak points, for example, low throughput, need for professional skills, or difficulty in method development [10]. In contrast, size-exclusion high performance liquid chromatography (SEC-HPLC) with high throughput capacity and highly quantitative performance is frequently used and indispensable for quantification of protein aggregates.

In SEC-HPLC, proteins and their aggregates are separated based on the difference in the permeation property into pores of stationary phase (e.g., silica-based polymeric beads). Larger molecules (e.g., aggregates) or non-spherical (e.g., straight chain) molecules in a mixture are rapidly excluded and therefore eluted from the resin pores, while smaller molecules with greater access to the pores are eluted more slowly [11]. In many marketed biopharmaceuticals, nonionic surfactants such as polysorbate 80 (PS80) and polysorbate 20 (PS20) are included as stabilizers to protect the active protein against denaturation or aggregate formation [12–16]. The molecular mass of the polysorbates is usually lower than that of most of proteins and protein aggregates. However, the polysorbates present in some formulations are eluted at retention times close to those of protein aggregates and then interfere with aggregate quantitation in SEC-HPLC. This interference becomes a serious problem to perfome sensitive and quantitative

SEC-HPLC methods capable of quantitating protein aggregates in formulated products. Unfortunately, it is very difficult to selectively separate detergents from protein solutions. Some methods are reported to separate detergents from protein solutions [17-22], but these methods take time because they cannot be connected directly to an analytical column and the recovery of protein is not 100%. Gunturi et al. proposed to utilize potassium phosphate buffer containing isopropyl alcohol as a mobile phase in SEC-HPLC [23]. They found that the peaks of the polysorbates were completely disappeared when isopropyl alcohol content was increased up to 20–25% range in the mobile phase [23]. However, isopropyl alcohol at such high concentrations may cause some damage to proteins. Therefore, some separation methods without use of organic solvents are strongly desired for long time. In this study, we will propose a new SEC-HPLC method that can separate the polysorbates from protein samples in an on-line mode using a precolumn with mixed characteristics of size exclusion phase and reversed-phase. We will also show the significance of the temperature control in the on-line separation to perform sensitive and quantitative SEC-HPLC methods.

2. Experimental

2.1. Chemicals and reagents

Sodium monobasic phosphate, sodium dibasic phosphate and human serum albumin (HSA, lyophilized powder, Fatty acid free, globulin free, ≥ 99%, product # A3782) were from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride (NaCl) was from Wako Pure Chemicals (Tokyo, Japan). Dulbecco's phosphate-buffered saline (PBS) was from Nissui Seiyaku (Tokyo, Japan). Polysorbate 80 (PS80) was from NOF Corp. (Tokyo, Japan), while polysorbate 20 (PS20) was from Croda (East Yorkshire, England).

Synagis injection containing Palivizumab was purchased from Abbvie (IL, USA). The pure standard sample of recombinant human erythropoietin (r-HuEPO) was obtained from Kyowa Hakko Kirin (Tokyo, Japan).

2.2. Sample preparations

HSA and synagis injection were reconstituted by addition given amount of water and dialyzed with PBS for 24 h. The HSA was diluted with the PBS to 3 mg/mL, and the synagis injection was diluted with the PBS to 1 mg/mL. The commercially available r-HuEPO was also dialyzed with the PBS and diluted with the PBS to 1 mg/mL. Heat-induced r-HuEPO aggregate-containing samples were prepared by heating the diluted r-HuEPO sample for one day at 40 °C. PS80 and PS20 were diluted with the PBS to 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1 mg/mL. All the samples were stored at 2-8 °C until the HPLC characterization described in Section 2.3.

2.3. SEC-HPLC methods

SEC-HPLC was performed with an Agilent 1100 chromatography system connected to a Tosoh TSKgel G3000SW$_{XL}$ (300 × 7.8 mm, 5 μm particle size). The chromatographic control, data acquisition and data analysis were performed using Chemstation (Agilent Technologies). The samples were set on an auto-sampler (as an accessory of the Agilent 1200 chromatography system) at 4 °C in a refrigerator. The UV detector was operated at a wavelength of 215 nm. The mobile phase for the SEC-HPLC method was the PBS. The mobile phase was filtered with a 0.45-μm Millipore filter and was degassed with an online

degasser. The flow rate was 0.5 mL/min and the column was maintained at 25 °C unless otherwise noted. In order to prevent automatic stopping (due to condensation detection at lower temperatures), the leak sensor of the column oven was turned off. The method run time was set to 40 min.

A Shodex MSpak GF-4A (10 × 4.6 mm, 9 μm particle size) was used as a precolumn to separate the polysorbates. The column, when necessary, was connected to the front of the main column (Tosoh TSKgel G3000SW_{XL}) with a stainless steel capillary and fittings.

3. Results and discussion

3.1 SEC-HPLC characteristics of HSA aggregates and polysorbates

Figure 1 shows chromatograms of the HSA sample and the diluted polysorbate samples on the SEC-HPLC system without the precolumn at 215 nm. Spectrophotometric detection of proteins is frequently performed at 280 nm based on the absorption of tryptophan and aromatic amino acid residues. However, the photometric detection at 215 nm due to amide bonds is required for highly sensitive detection of protein targets at extremely low concentrations such as protein aggregates in protein-based pharmaceutical products. The main peak at a retention time of 17 min corresponds to HSA monomer, while the peaks at 15, 14.5, 14 min correspond to dimer, trimer and tetramer of HSA, respectively. Larger aggregates were eluted in the region of retention times from 11 to 14 min. In order to characterize the aggregation, the peak analysis of this region is very important. Unfortunately, PS80 and PS20 were eluted at retention times very close to that of as the aggregates of HSA. Although the molecular mass of PS80 and PS20 are smaller than that of HSA, the polysorbates were eluted at retention times earlier than the time

expected from the molecular mass. The reason may be that PS80 and PS20 can be formed in a micellar state in an aqueous solvent. This is a typical example of the interference from PS80 and PS20 in the SEC-HPLC quantification of aggregate contents as reported before [23].

We tried in this work to separate the polysorbate's peaks from the protein-derived ones by using a precolumn method because on-line analysis without any specific separation pretreatment of protein samples is convenient for chromatographic analysis of protein aggregates in protein-based pharmaceutical products. One of separation modes to be utilized for this purpose may be electrostatic interaction in anion or cation exchange. However, separation conditions for ion exchange chromatography have to be tuned for every protein, because the isoelectric points of proteins are different from each other. Another separation mode may be hydrophobic interaction in reversed-phase. One of weak points of this method is that the method requires organic solvents or ammonium sulfate in elution buffer. The situation may give some damages to proteins and may cause the dissociation of some non-covalent aggregates in reversed-phase chromatography and hydrophobic interaction chromatography. As a result, we have considered that some porosity silica gel with some hydrophobic characteristics may be effective to remove hydrophobic surfactants with relatively large molecular mass such as PS80 and PS20. In preliminary experiments we tried to use two kinds of anion exchange columns (GE Healthcare, Resource Q; and Tosoh, TSK-GEL DEAE 5PW), one anion/cation multi-mode column (Poly LC, PolyMix CW) and several reversed-phase columns (Tosoh, TSKgel guard column Super AW-L; Waters, μ-Bondasphere; and Agilent Technologies, Zorbax C8) as precolumns. However, these columns were not efficient for this purpose. We finally focused on Shodex MSpak GF-4A, which has been developed for column-switching method to

eliminate surfactant from surfactant-containing protein/peptide samples. Although MSpak GF-4A is mainly used for LC-MS because surfactants degrade LC-MS columns and decrease the reproducibility of LC-MS, we tried to use MSpak GF-4A as a precolumn connected to SEC column and to develop a new online SEC-HPLC method without column-switching system.

As expected, on the SEC-HPLC system without MSpak GF-4A, the peak area of PS80 and PS20 increased in proportion to the injection amount of the polysorbates (Fig. 2A, B; black triangle). In contrast, when MSpak GF-4A was connected as a precolumn to the SEC-HPLC system, the PS80 peak was not detected at least up to 8-μg injection (Fig. 2A; gray triangle). Very small peak of PS80 was detected at 20-μg injection and clearly detected at 40-μg injection with an area of 170 mAU×sec. However, the peak area was much smaller than those observed in the separation without MSpak GF-4A. In the case of PS20, only very small peak was detected even at 20-μg and 40-μg injections when the MSpak GF-4A column was used (Fig. 2B; gray triangle). These results indicate that the MSpak GF-4A precolumn is very effective to trap PS80 and PS20 in the on-line mode. The limiting value of the trapping is about 10 μg for PS80 and 20 μg for PS20. The difference of the trapping characteristics between PS20 and PS80 seem to be ascribed to multiplier effect of mixed characteristics of size exclusion phase and reversed-phase of the precolumn. Actually the difference cannot be simply explained from the hydrophobic properties of the polysorbates, and precolumns with the revered-phase characteristics alone did not work well to trap the polysorbates selectively. When 40 μg of PS80 was injected to the precolumn-SEC-HPLC system, the precolumn must be saturated with PS80. Therefore the succeeding injection of even small amounts of PS80 (for example 8 μg) could not be trapped. However, the PS80-suturated precolumn can still trap PS20. PS20 was scarcely

detected at 20-μg injection when the PS80-saturated precolumn was use. It is noteworthy that the detergent trapped in MSpak GF-4A is easily washed out with mixed aqueous mobile phase containing organic solvents such as acetonitrile (data not shown). Actually, the precolumn was cleaned with acetonitrile (30% v/v)-containing aqueous mobile phase after each analysis unless otherwise noted, when large amounts of polysorbates were injected.

3.2 Effects of temperature on the one separation of protein aggregates on SEC-HPLC with MSpak GF-4A

As described in Section 3.1, the MSpak GF-4A is very effective to trap polysorbates. However, it was found that the chromatograms of the HSA sample depend on the column temperature (Fig. 3A). Figure 4A shows the dependence of the total peak area of the HSA aggregates on the column temperature. The peak area of the HSA aggregate increased with an increase in the column temperature from 5 °C to 20 °C and reached a constant value above 20 °C at least up to 30 °C (Fig. 4A, gray square), although the HSA monomer peak area was not affected by the column temperature (data not shown). In contrast, when only the SEC column was used without the precolumn, the total peak area of the aggregate and monomer was independent of the column temperature (data not shown). These results suggest that the HSA aggregates are specifically trapped on the MSpak GF-4A precolumn at lower temperatures predominantly by hydrophobic interaction. It has been reported that the high-order structure of protein aggregates usually differs from that of the monomer [24-26], although some protein aggregates seem to retain the high-order structure similar to that of monomer [26, 27]. In the case of HSA, the fluorescence spectral characteristics of the aggregates are different from those of the monomer (data not shown). This evidence suggests the change in the

high-order structure on the aggregation. Since the aggregates are susceptible to the adsorption on the precolumn at lower temperatures, the hydrophobic interaction between the aggregates and the stationary phase of the precolumn seems to be a major contribution to trap the aggregates on the precolumn. The hydrophobic interaction decreases with an increase in the column temperature, and the HSA aggregates can pass through the precolumn at column temperatures over 20 °C.

In contrast, the peak area of the heat-induced r-HuEPO aggregates decreased with an increase in the column temperature (Fig. 3B and Fig. 4B (gray diamond)) on the SEC-HPLC system with MSpak GF-4A. The limiting value of the peak was observed at column temperature lower than 10 °C. The peak area of the r-HuEPO monomer was independent of the column temperature at least in the range from 5 to 30 °C as in the case of the HSA monomer. These phenomena may be caused by the high-order conformational change of the heat-induced r-HuEPO aggregates at increased column temperatures to induce the exposure of these hydrophobic surface.

We also show another example of the column temperature dependence of protein aggregates. As shown in Figs. 3C and 4C, the peak shape and the area of the aggregate (dimer, peak 1) and monomer in the synagis injection are independent of the column temperature in the range from 5 °C to 30 °C. The synagis injection dimer seems to have characteristics similar to those of the monomer in view of the high-order structure and the surface property. The hydrophobic interaction between the synagis injection aggregate and the stationary phase of the MSpak GF-4A column is not so strong, as in the case of the monomer of the other proteins examined here.

Anyway, it is very important to control the column temperature to avoid the adsorption of protein

aggregetes in the MSpak GF-4A precolumn. The optimum temperature of the column must be selected for individual protein targets.

3.3 HSA with and without PS80 analyzed by SEC-HPLC with and without MSpak GF-4A

In the region from 10 to 15 min, the peak shape of HSA with PS80 was significantly different from that of HSA without PS80 by analyzing SEC-HPLC without MSpak GF-4A (Fig 5-A). Not only aggregates but PS80 were eluted in the region. While, the peak shape of the HSA sample with PS80 on SEC-HPLC with MSpak GF-4A was almost the same as that of the HSA sample without PS80 on SEC-HPLC without MSpak GF-4A (Fig 5-B). PS80 was successfully trapped by MSpak GF-4A. The results clearly show that the connection of MSpak GF-4A to SEC column and the setting of proper column temperature (e.g., 25 °C for HSA) allow to perform quantification of protein aggregate contents, because MSpak GF-4A can effectively eliminate of the interference from PS80.

4. Conclusions

We have proposed an improvement of the conventional SEC-HPLC method for analysis of protein aggregates in protein-based samples containing some detergents such as PS80 and PS20 by using MSpak GF-4A as a precolumn. The method performs an on-line analysis of protein aggregates. The mixed modes based on size exclusion phase and reversed-phase seem to effectively work to trap the detergents. However, some hydrophobic interaction between protein aggregates (especially with hydrophobic nature) and the stationary phase of the precolumn might cause to trap the protein aggregates in part. This work has

also revealed that such interference can be minimized by tuning the column temperature.

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Figure Captions

Figure 1. Chromatograms of the HSA sample (black solid line; 3 mg/mL×10 μL), diluted PS80 sample (gray solid line; 1 mg/mL×40 μL) and the diluted PS20 sample (gray dash line; 1 mg/mL×40 μL) on the SEC-HPLC system without the precolumn at 25 °C (flow rate: 0.5 mL/min, UV detection: 215 nm).

Figure 2. Effects of the precolumn MSpak GF-4A on the peak area of (A) PS80 and (B) PS20 detected on the SEC-HPLC system; (black triangle) without or (gray triangle) with the precolumn MSpak GF-4A. The error bars indicate the standard deviation (n = 3).

Figure 3. Chromatograms of (A) the HSA sample (3 mg/mL×10 μL), (B) heat-induced r-HuEPO sample (1 mg/mL×30 μL), (C) synagis injection sample (1 mg/mL×20 μL) on the SEC-HPLC system with MSpak GF-4A at various column temperatures. Purple, blue, green, yellow, orange and red lines are each protein samples analyzed at 5, 10, 15, 20, 25 and 30°C respectively. The peaks numbered as 1, 2, 3 and 4 of the HSA aggregates correspond to the dimer, trimer, tetramer and larger oligomer(s), respectively. Peak 1 of heat-induced r-HuEPO and synagis injection corresponds to the dimer.

Figure 4. Effects of the column temperature on the total peak areas of the aggregates in (A) the HSA sample on the SEC-HPLC system with MSpak GF-4A (gray squares) and without MSpak GF-4A (black squares), (B) heat-induced r-HuEPO sample with MSpak GF-4A (gray diamonds) and without MSpak GF-4A (black diamonds) and (C) synagis injection sample with MSpak GF-4A (gray circles) and without MSpak GF-4A

(black circles). The error bars indicate the standard deviation (n = 3).

Figure 5. (A) Chromatograms of HSA by SEC-HPLC without MSpak GF-4A; black line corresponds to the HSA sample (3 mg/mL×10 μL) without PS80 and gray line is the HSA sample containing PS80 (HSA: 3 mg/mL×10 μL, PS80: 1 mg/mL×10 μL). (B) Chromatograms of HSA; black line corresponds to the HSA sample (3 mg/mL×10 μL) without PS80 on SEC-HPLC without MSpak GF-4A and gray line corresponds to the HSA sample containing PS80 (HSA: 3 mg/mL×10 μL, PS80: 1 mg/mL 10 μL) by SEC-HPLC with MSpak GF-4A.

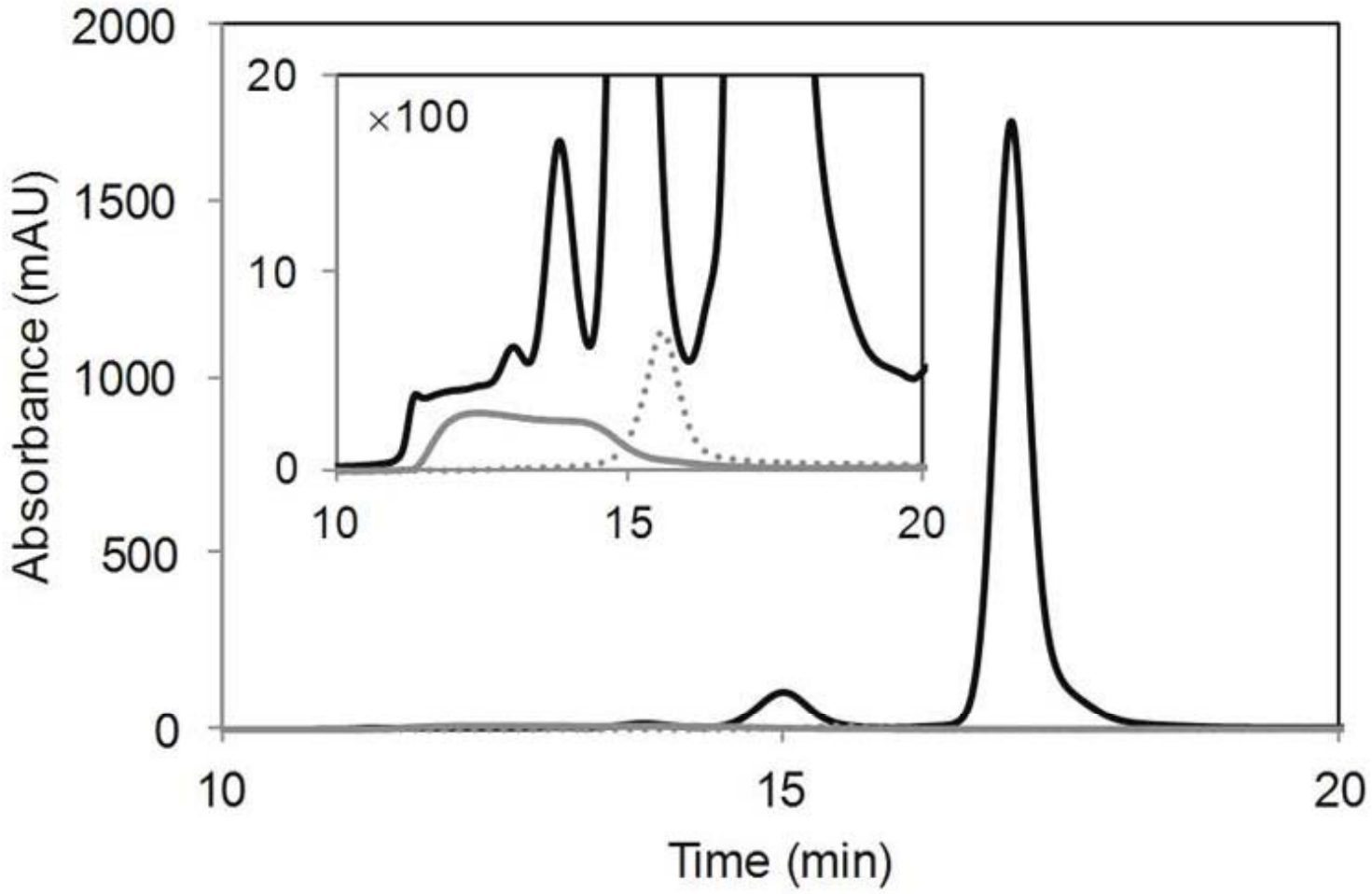
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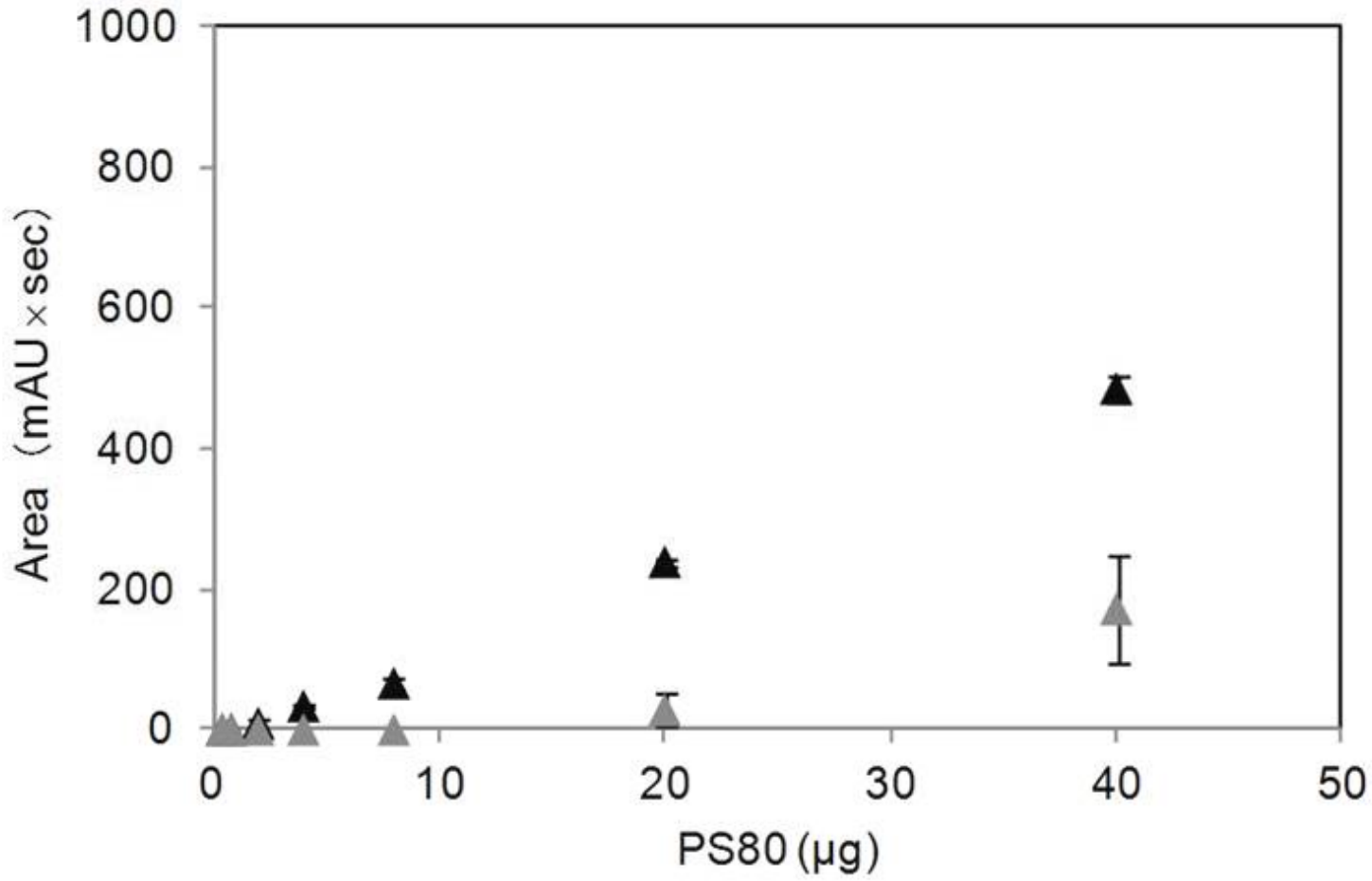
Figure 1



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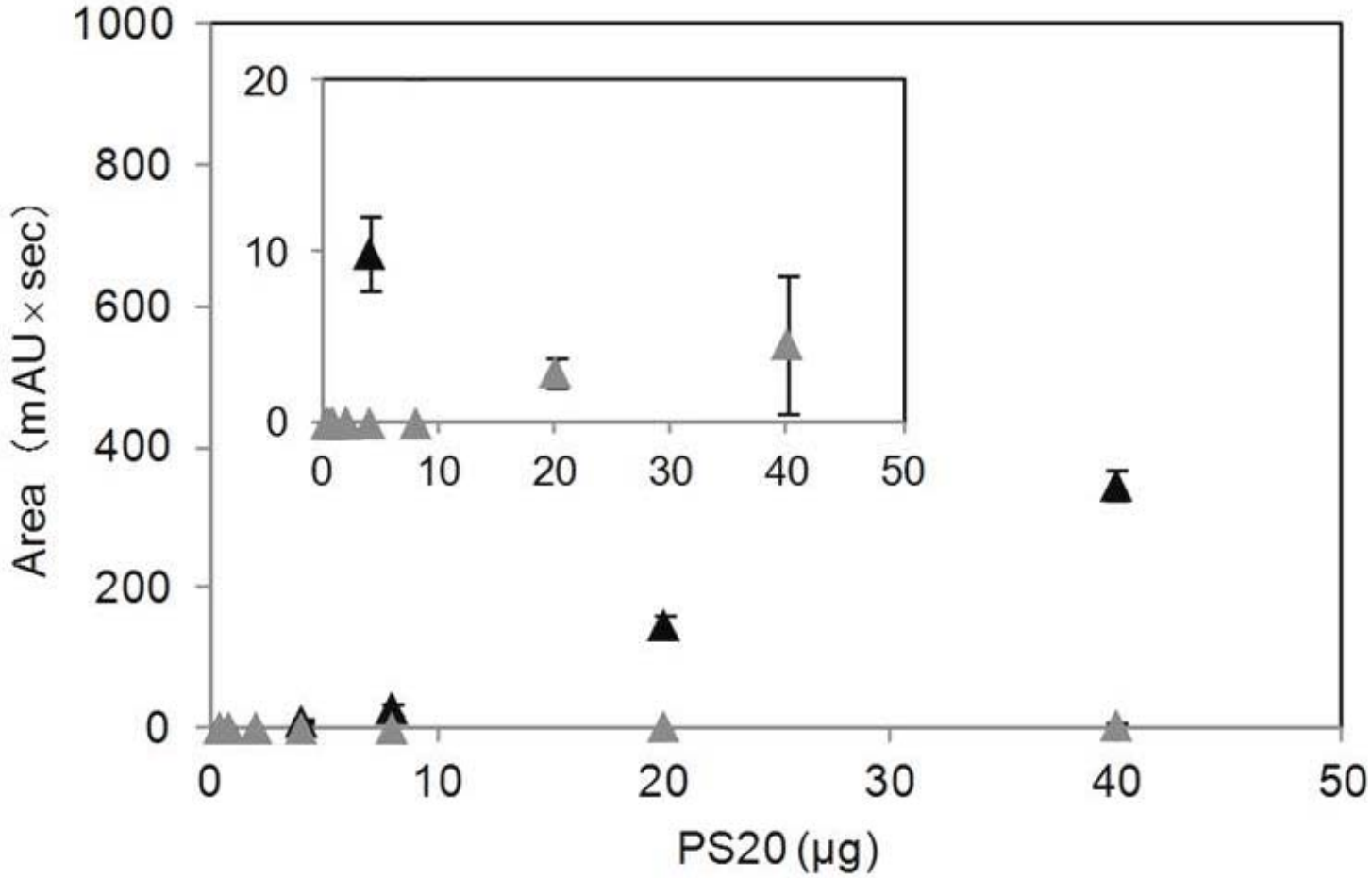
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Figure 2(B)

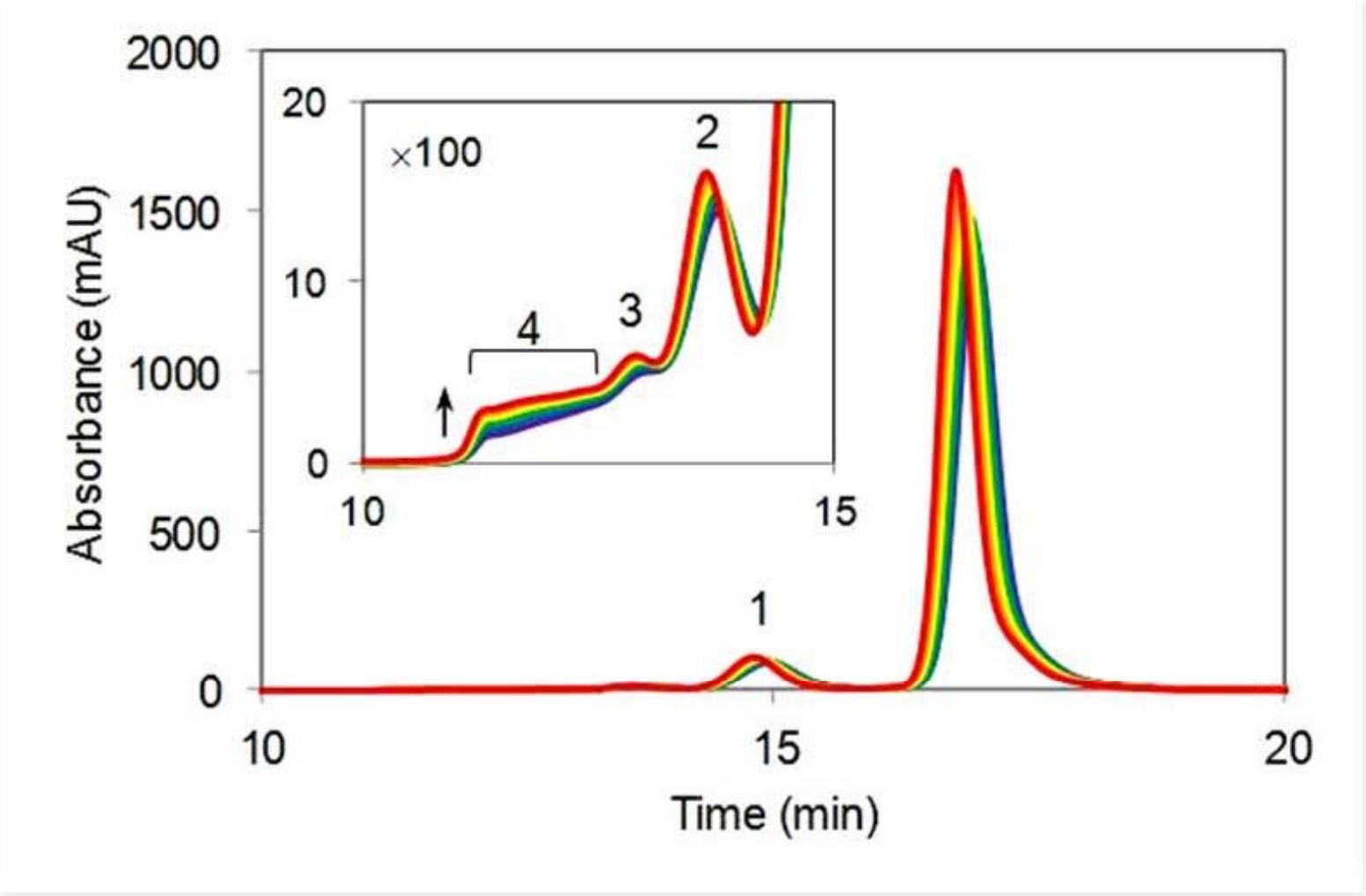
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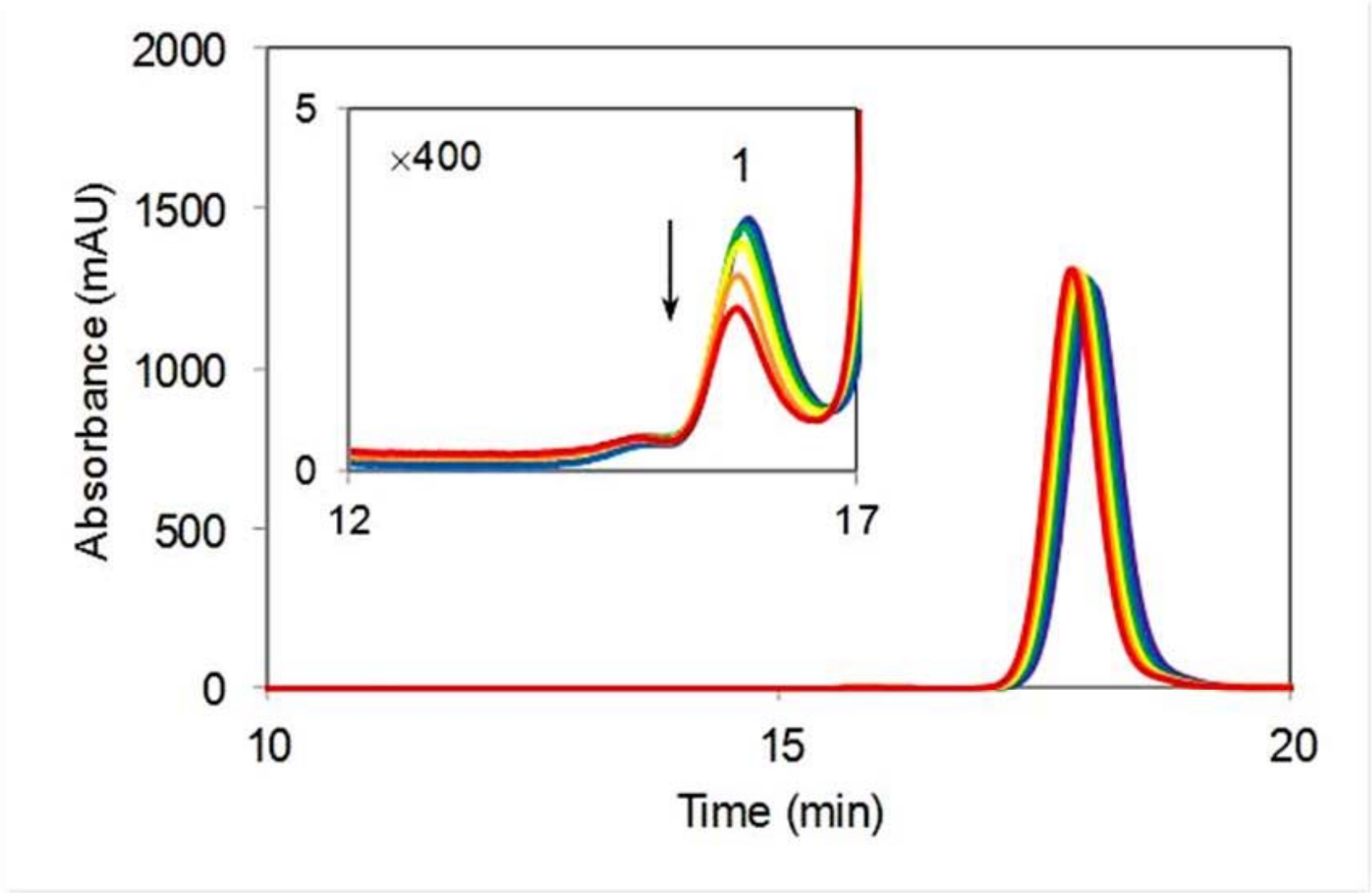
Figure 3(A)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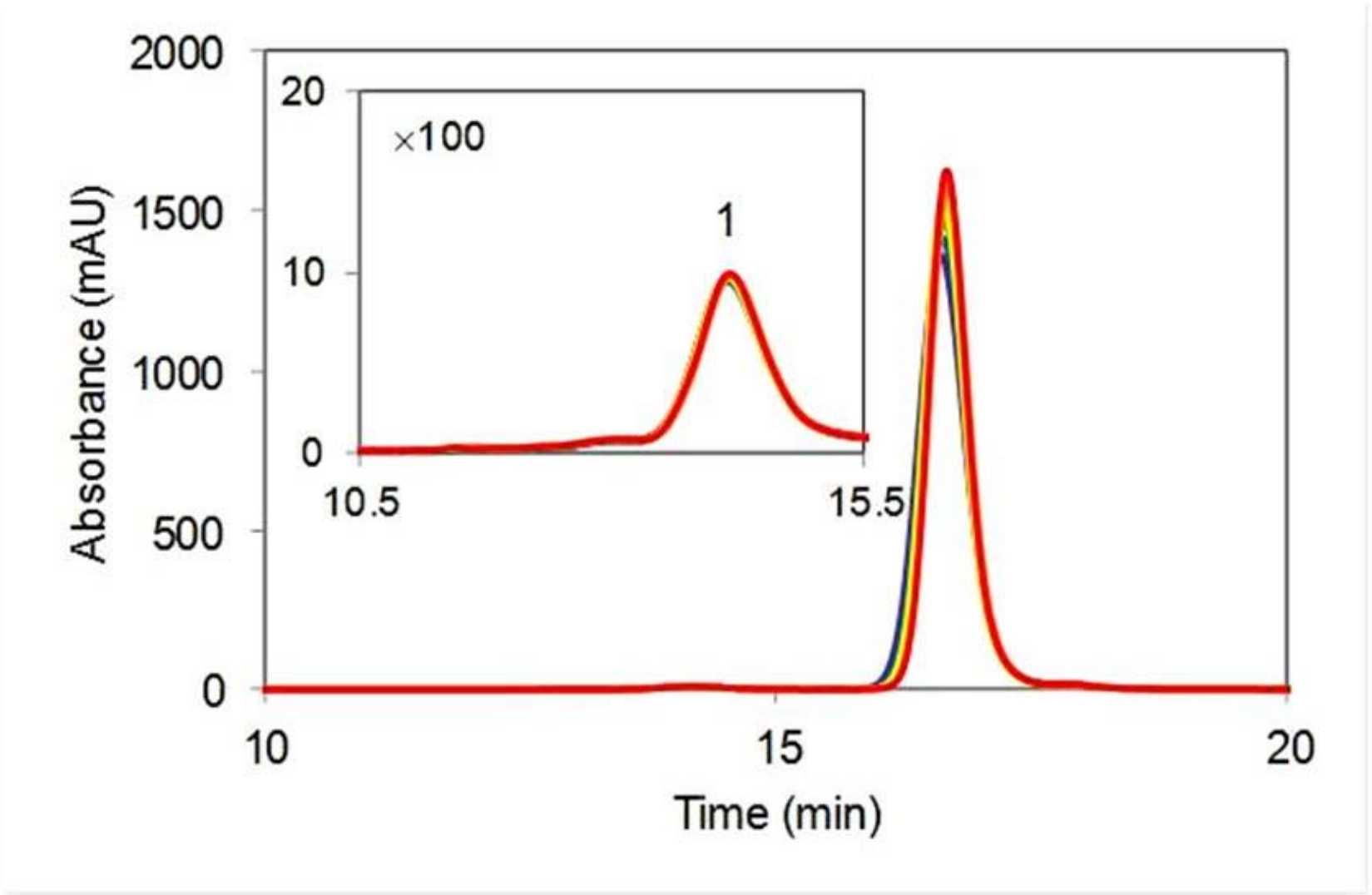
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Figure 3(C)

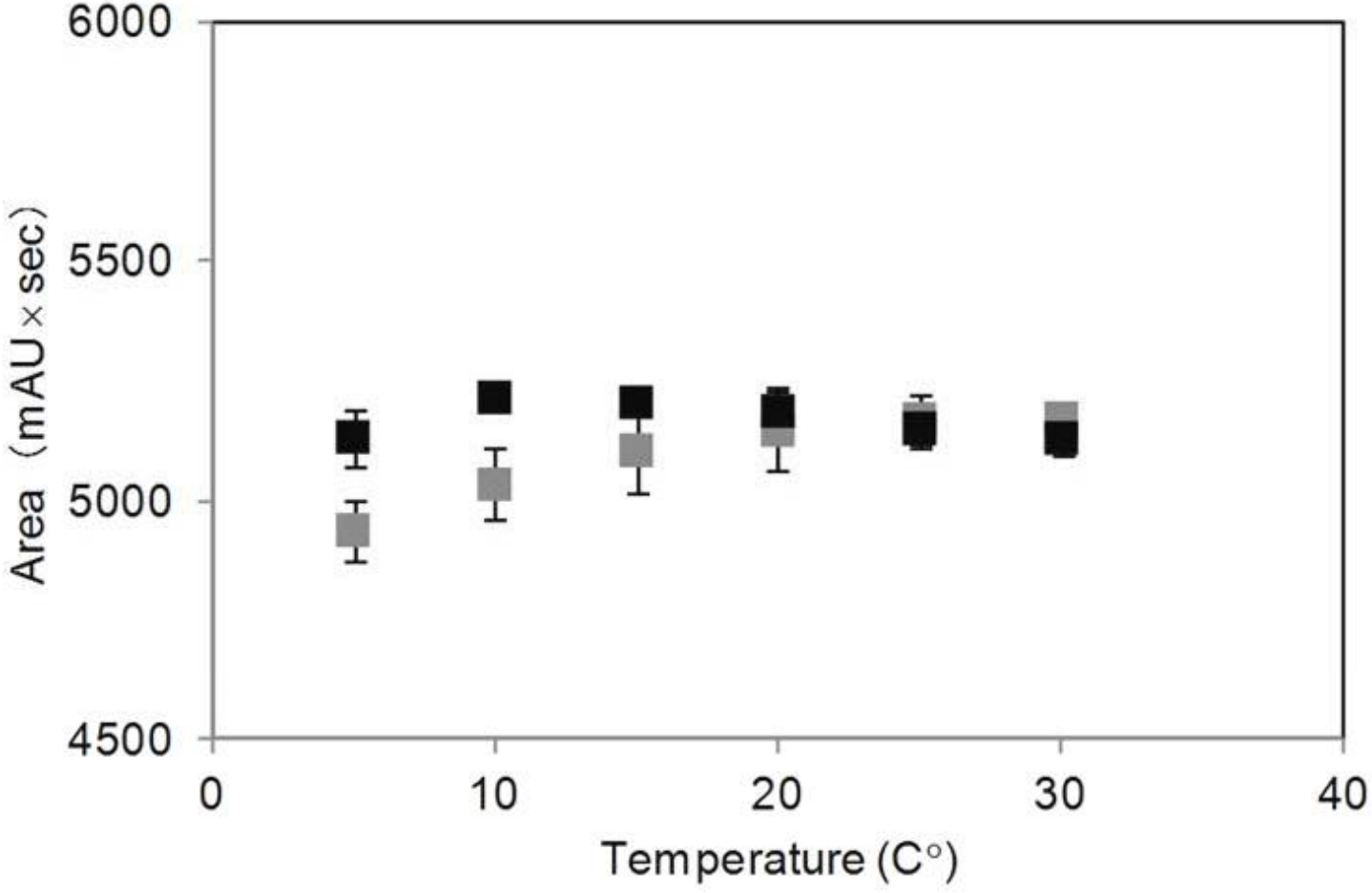
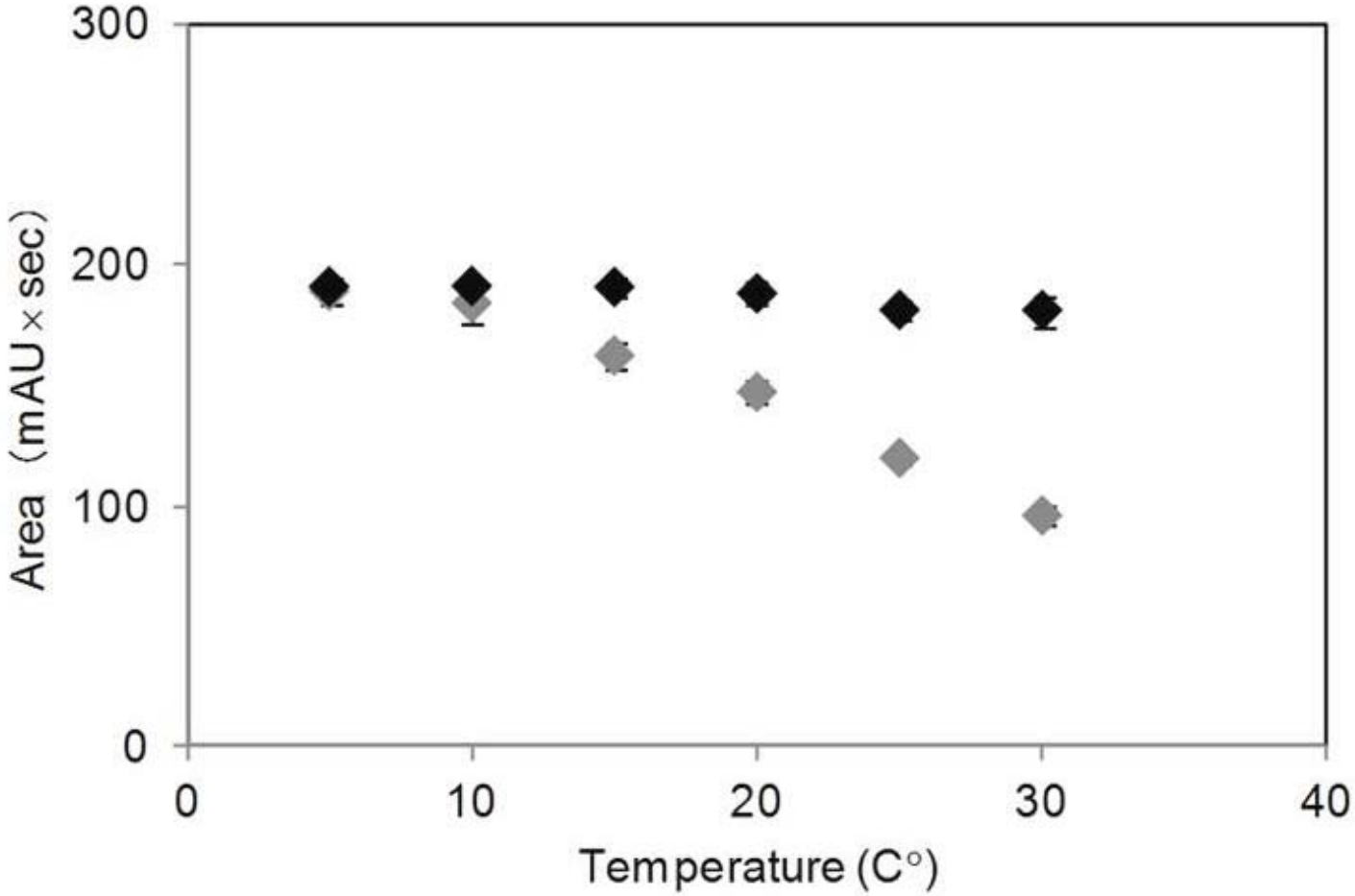


Figure 4(A)



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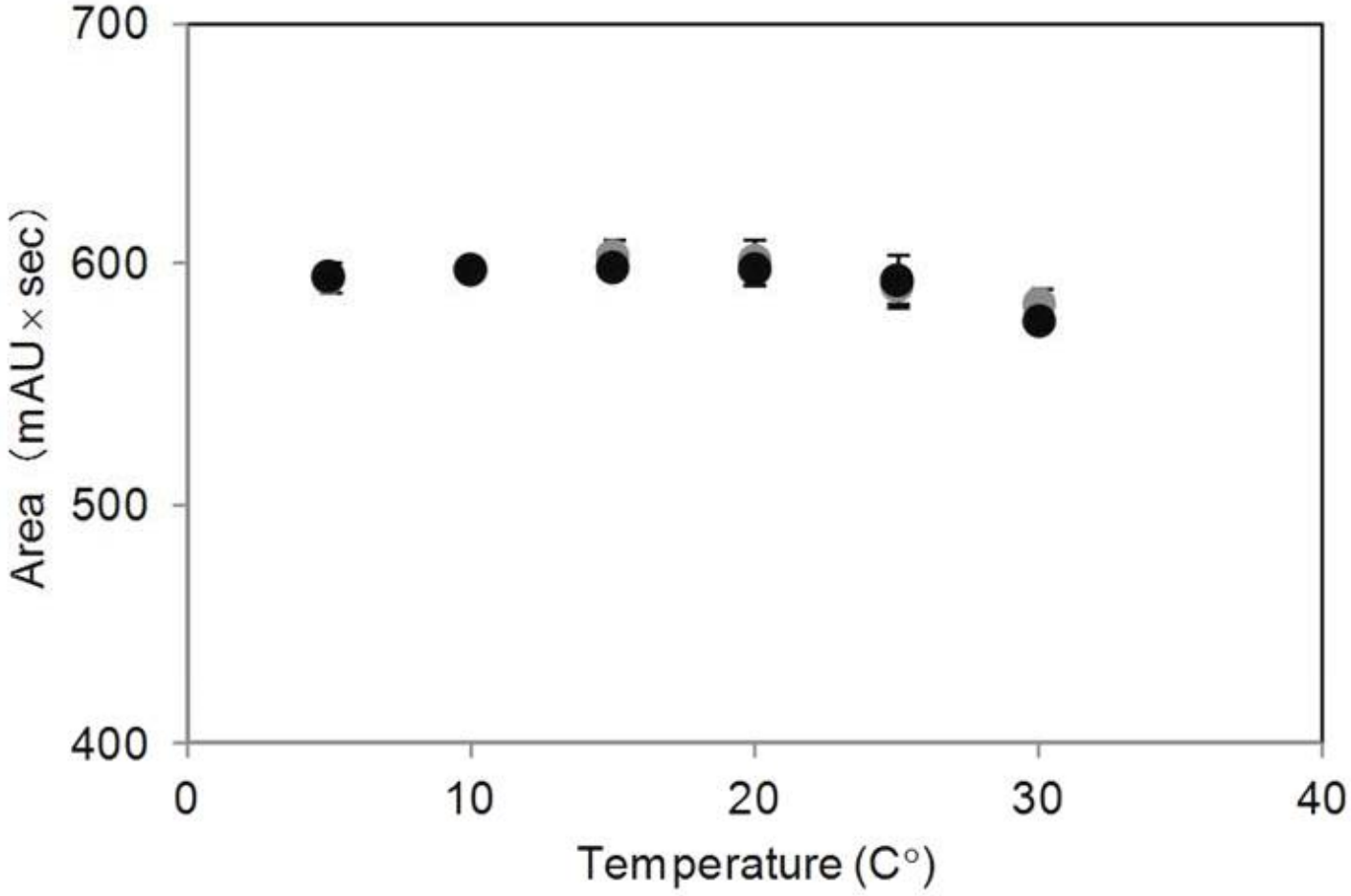

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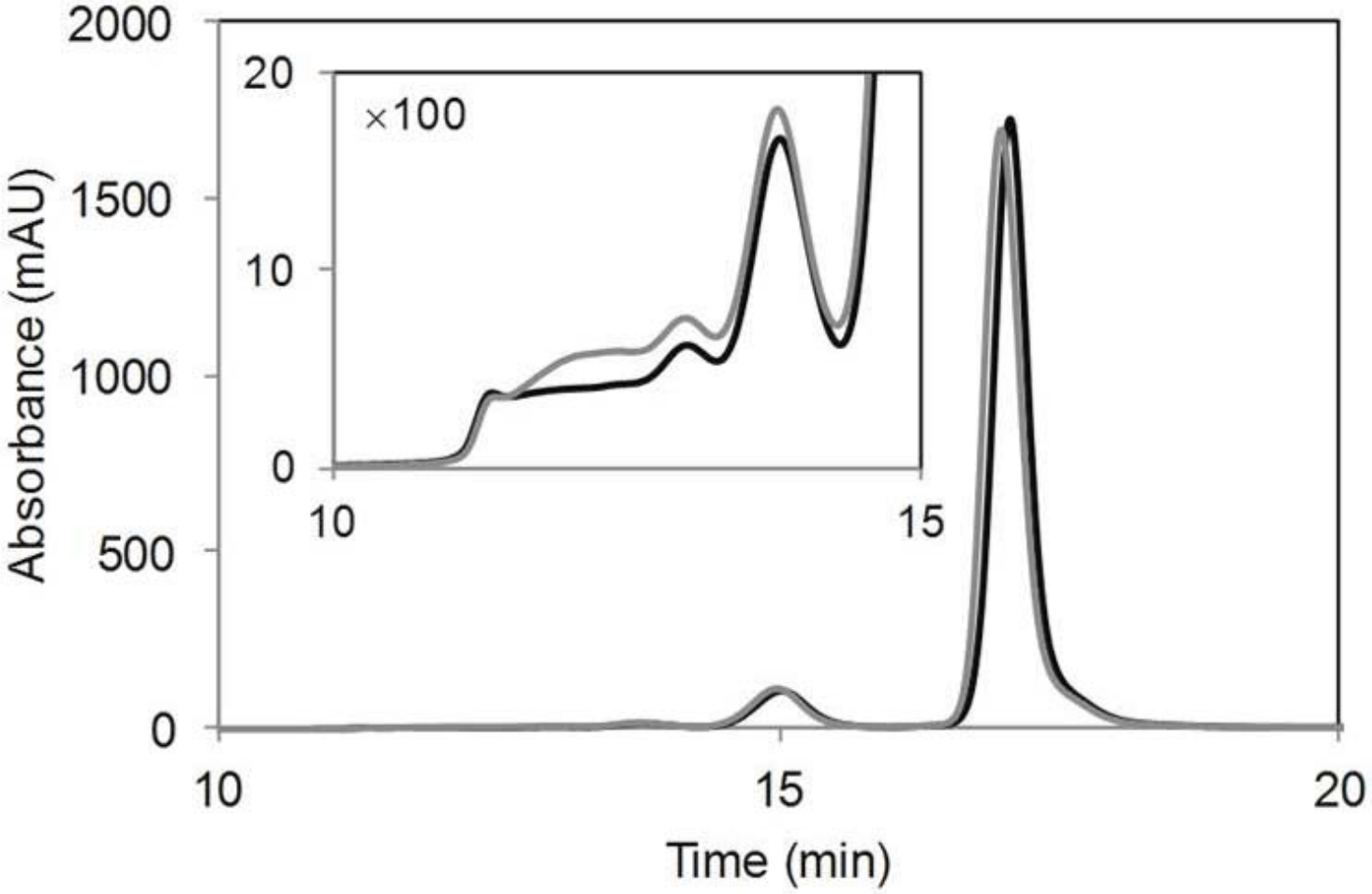
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15

Figure 5(A)

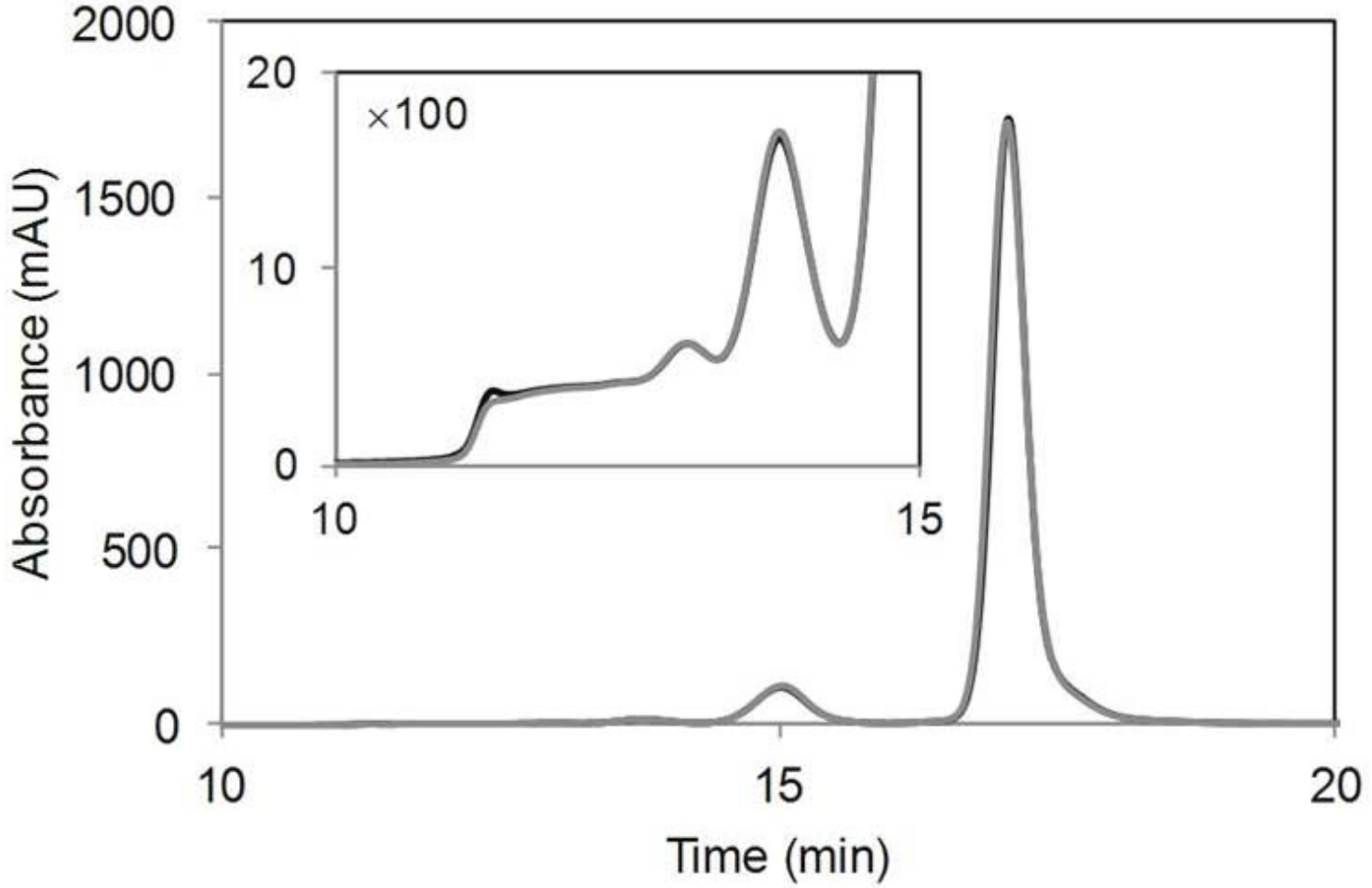
2000
1500
1000
500
0
Absorbance (mAU)
10
15
20
Time (min)
20
×100
10
0
10
15

Figure 5(B)