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Nonspecific cleavage of proteins using graphene oxide

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ABSTRACT

In this article, we report the intrinsic catalytic activity of graphene oxide (GO) for the nonspecific cleavage of proteins. We used bovine serum albumin (BSA) and a recombinant esterase (rEstKp) from the cold-adapted bacterium *Pseudomonas mandelii* as test proteins. Cleavage of BSA and rEstKp was nonspecific regarding amino acid sequence, but it exhibited dependence on temperature, time, and the amount of GO. However, cleavage of the proteins did not result in complete hydrolysis into their constituent amino acids. GO also invoked hydrolysis of *p*-nitrophenyl esters at moderate temperatures lower than those required for peptide hydrolysis regardless of chain length of the fatty acyl esters. Based on the results, the functional groups of GO, including alcohols, phenols, and carboxylates, can be considered as crucial roles in the GO-mediated hydrolysis of peptides and esters via general acid–base catalysis. Our findings provide novel insights into the role of GO as a carbocatalyst with nonspecific endopeptidase activity in biochemical reactions.

Peptides are usually hydrolyzed into individual amino acids by heating in 6 M HCl under vacuum conditions at 110 °C for 24 h [1]. Methods using chemical reagents and proteolytic enzymes cleave peptide bonds at specific sites; for example, cyanogen bromide cleaves the carboxyl side of methionine, whereas trypsin cleaves lysine and arginine [2].

Recently, graphene oxide (GO),[1] a precursor of graphene synthesis, has gained considerable attention due to its large surface area and oxygen-containing functional groups, which include alcohols, epoxides, and carboxylic acids [3,4]. GO has been shown to catalyze several oxidative reactions as a carbocatalyst [5], including oxidation of sulfides [6], C–H bond oxidation [7], and the dehydrogenation of propane to propene [8], but its low catalytic efficiency requires high GO loads. Furthermore, the catalytic activity of GO for the hydrolysis of peptide bonds is currently unknown.

In this article, we report the intrinsic catalytic activity of GO for the hydrolysis of peptides and esters. We used bovine serum albumin (BSA) and a recombinant esterase (rEstKp) from cold-adapted bacterium *Pseudomonas mandelii* as test proteins to study protein hydrolysis by GO. The 31-kDa extracellular esterase EstK (GenBank accession no. AEW10549) from *P. mandelii* consists of a single polypeptide chain with no cysteine residues and has substrate specificity for short-chain fatty acids [9,10]. We also used *p*-nitrophenyl esters with variable chain lengths (C2–C16) to study ester hydrolysis by GO. Here, we report that GO could be used as a catalyst for the nonspecific cleavage of proteins and hydrolysis of esters.

Materials and methods

Materials

GO with an average size of 74 μm was prepared by following the simplified Brodie method without any subsequent treatment [11–13]. Functional groups of GO, which include alcohols, epoxides, and carboxylic acids, were confirmed by Fourier transformed infrared spectroscopy, ^{13}C nuclear magnetic resonance, and X-ray photoelectron spectroscopy [12–14]. BSA was purchased from Sigma (St. Louis, MO, USA). Tripeptide Ala-Ala-Ala was synthesized at Peptron (Daejeon, South Korea). All other reagents were obtained from Sigma unless noted otherwise. Recombinant EstK protein without its signal peptide, rEstKp, was previously cloned and purified from *Escherichia coli* [15]. The purified enzymes were frozen in liquid nitrogen and stored at −80 °C.

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[1] *Abbreviations used:* GO, graphene oxide; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; LC–MS/MS, liquid chromatography–tandem mass spectrometry; EDTA, ethylenediaminetetraacetic acid.

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GO-mediated protein cleavage

BSA (70 μg) or rEstKp (50 μg) was incubated with 20 μg of GO in phosphate buffer (0.2 M Na_2HPO_4, pH 8.0) at 80 °C for 25 min or the indicated time. After centrifugation at 12,000*g* for 10 min, the supernatant was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with a 10% separation gel.

Top-down LC–MS/MS analysis

rEstKp (50 μg) was incubated with 20 μg of GO in phosphate buffer (pH 8.0) at 80 °C for 5 min, 25 min, and 12 h separately. After centrifugation at 12,000*g* for 5 min, the supernatant was dried using a SpeedVac and analyzed using a nano liquid chromatography–tandem mass spectrometry (nano-LC–MS/MS) instrument (linear ion trap, Thermo) by the top-down method. Amino acid composition analysis was performed at the Korea Basic Science Institute (Daejeon, South Korea) as described previously [16].

GO-mediated ester hydrolysis

Formation of *p*-nitrophenol was measured using 0.1 mM *p*-nitrophenyl esters with variable chain lengths (C2–C16) in reaction buffer (100 mM Tris–Cl, 100 mM NaCl, and 0.3% Triton X-100, pH 7.5) with 5 μg of GO. Accumulation of *p*-nitrophenol was measured using a Shimadzu UV-1800 spectrophotometer at 400 nm for 5 min.

Results

Peptide hydrolysis

To investigate the effect of GO on peptide hydrolysis, BSA (70 μg) was incubated with 20 μg of GO in phosphate buffer (pH 8.0) at various temperatures (4, 25, 40, 60, and 80 °C) for 25 min. The BSA content of the supernatant after centrifugation dramatically decreased by 11, 41, 78, and 87% at 25, 40, 60, and 80 °C, respectively, compared with the sample at 4 °C (Fig. 1A, upper panel). Because GO has been shown to form protein complexes via noncovalent interactions [17], we examined whether or not reduction of BSA content in the supernatant could be attributed to noncovalent interactions between GO and BSA. Pellets from the experiment shown in Fig. 1A (upper panel) were analyzed by SDS–PAGE. The results show that the amount of BSA was similar in all samples (data not shown), indicating that the reduction of full-length BSA was due to cleavage of BSA and not adsorption of BSA to GO.

We also characterized cleavage of BSA by GO at 80 °C with different time intervals. The rate of BSA (70 μg) cleavage increased in a time-dependent manner (Fig. 1B), with approximately 90% of BSA disappearing in 25 min using 20 μg of GO at 80 °C. The rate of BSA cleavage was also proportional to the amount of GO (Fig. 1C). These data are consistent with previous findings requiring high GO loads in GO-mediated oxidative reactions [6–8]. X-ray photoelectron spectroscopy analysis has previously shown that GO contains no metal ions during preparation [13]. To confirm that the observed catalytic effects were due to GO and not to metal ions, BSA was incubated with GO in the presence of 1 mM ethylenediaminetetraacetic acid (EDTA) at 80 °C. However, EDTA had no effect on the cleavage of BSA (data not shown). Taken together, our data demonstrate that BSA was cleaved by GO, and the reaction rate increased as temperature, time of incubation, and GO amount increased, as is the case for a catalyst.

Because BSA used in this experiment (BSA fraction V) was prepared by acetone precipitation and contained other proteins, another protein purified in our laboratory, rEstKp from *P. mandelii* [15], was used to investigate the hydrolyzed fragments. As shown in Fig. 2A, rEstKp was cleaved in a time-dependent manner (5 min, 25 min, and 12 h), which was similar to the cleavage of BSA. Next, we carried out top-down LC–MS/MS analysis using rEstKp hydrolysates. Peaks of the cleaved rEstKp peptides increased at 12 h (Fig. 2B), whereas no distinct peaks were observed in the 5- and 25-min samples (data not shown). Peptide sequences were obtained from all three samples also by LC–MS/MS analysis (Fig. 2C). More peptides were obtained on longer incubation time

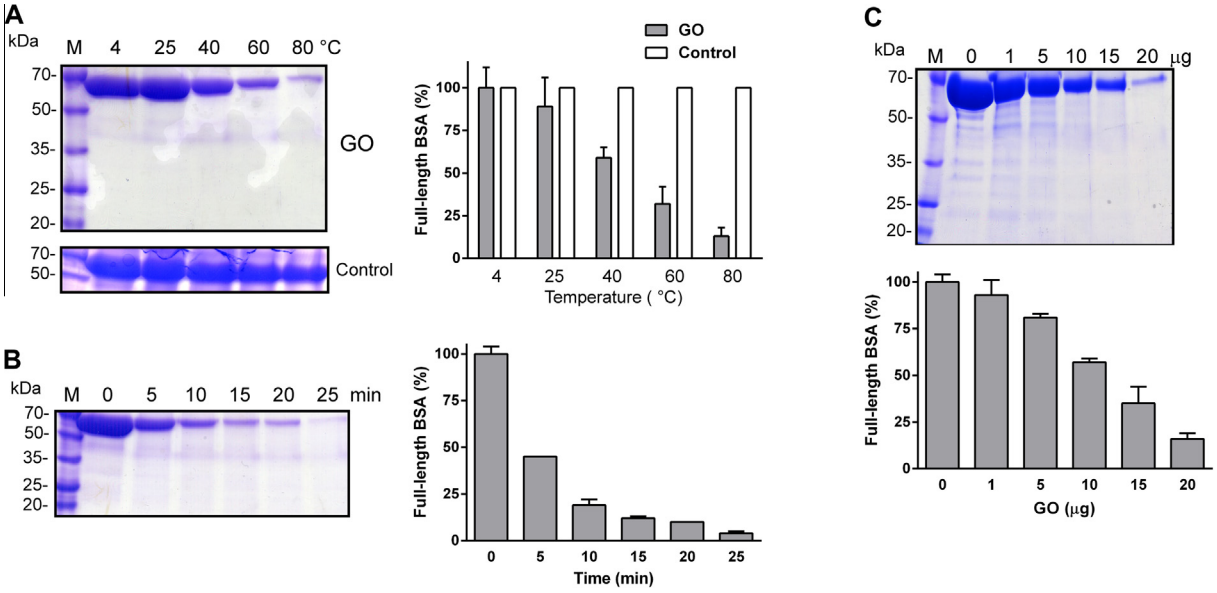

Fig.1. GO-mediated cleavage of BSA. (A) Effect of temperature. BSA (70 μg) was incubated with 20 μg of GO in phosphate buffer (0.2 M Na_2HPO_4, pH 8.0) at various temperatures (0–80 °C) for 25 min. (B) Effect of incubation time. BSA (70 μg) was incubated with 20 μg of GO in phosphate buffer at 80 °C for the indicated time. (C) Effect of GO amount. BSA (70 μg) was incubated with increasing amounts of GO (0–20 μg) in phosphate buffer at 80 °C for 25 min. The supernatant after centrifugation at 12,000*g* was separated by 10% SDS–PAGE. M, molecular weight marker. Experiments were repeated three times. Representative blot is shown. Data correspond to means ± standard deviations for three experiments.