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Original Research

Alkaline Protease Immobilized on Graphene Oxide: Highly Efficient Catalysts for the Proteolysis of Waste-Activated Sludge

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Abstract

This study aims to investigate the enzymatic hydrolysis of waste-activated sludge (WAS) using free and immobilized alkaline protease. Alkaline protease was immobilized on graphene oxide (GO) sheets using glutaraldehyde as cross-linking reagent. The storage stabilities, kinetic parameters, physical properties, and the free amino acid (FAA) profiles of the WAS protein hydrolysates from free and immobilized enzyme were analyzed. The immobilized enzyme showed significantly improved storage stability, whereas kinetic analysis showed that the apparent K_m for the immobilized alkaline enzyme was about 1.8-fold higher than that of free protease. Alkaline protease immobilized on GO showed significant activity towards WAS protein hydrolysates, attractive for practical applications. The FAAs formed by free protease and enzyme immobilized on GO were generally similar.

Keywords: waste-activated sludge, alkaline protease, immobilized, free amino acids, graphene oxide

Introduction

Waste-activated sludge (WAS) is a complex mixture composed of single carbohydrates, amino acids, alcohols, and volatile fatty acids mixed with polymers and heteropolymers including proteins, polysaccharides, and lipids [1]. Protein is the highest content among the three organic matters, accounting approximately for 60% [2]. The conversion of this waste biomass to higher-value products has been recognized as an attractive alternative waste management solution [3, 4]. Generally, the WAS hydrolysate has a high content of essential amino acids, indicating a high nutritional value used for food, feed, or as a nitrogen source for cotton and other cash crops [5]. After acidic, basic or enzymatic hydrolysis of protein-containing WAS, a mixture of free amino acids (FAAs) can be obtained. Therefore, it is possible to utilize WAS as a starting material to produce the FAAs. Although some researchers have investigated the dewaterability of sludge by adding an enzyme or enzymatic product (containing protease, lipase, and cellulase) as pretreatment [6-10], to the best of our knowledge, there is no reference to investigate the feasibility of WAS hydrolysis by immobilized enzyme.

Proteases have been immobilized on both natural (inorganic and organic) and synthetic supports. Several reports have demonstrated the feasibility and performance of proteolysis using the enzymes immobilized on various supports, such as chitin, polymer, sol-gel supports, mesoporous activated carbon, mesoporous silica, and glass beads [11-16]. All those studies have shown that the carrier used in the

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immobilization procedure can significantly affect kinetics and stability properties of protease. However, the immobilized enzyme systems have certain disadvantages, due mainly to mass transfer limitations of the substrate and/or product. If protease is immobilized onto the nanoparticles, which have high specific surface area and low diffusion resistance, it would be very effective for catalysis. Nanophase materials have many advantages due to their unique size and physical properties. It has been testified that the enzymes immobilized on the nanostructured materials have some advantages over the bulk solid substrates [17].

Graphene is the fundamental basis of 0D fullerene, 1D carbon nanotubes, 3D graphite, and 2D individual sheet structure composed of sp2-hybridized carbon with one-atom thickness [18]. Graphene, discovered in 2004, has become one of the hottest topics in many fields due to its unique properties, such as high thermal conductivity, excellent mechanical flexibility, and fast electron transportation [19-22]. It has demonstrated that graphene and graphene oxide (GO) show very high mechanical properties with good biocompatibility, and graphene-based biomaterials have recently been explored [23, 24]. Graphene oxide (GO) nanosheets perform better than other materials due to the planar morphology and large accessible surface area. Suspension of graphene nanosheets is stable in water at high concentrations (0.6-2 mg/ml) with the aid of polymeric dispersant, and can be used as the loading platform for enzymes [18, 25].

The objectives of this paper are to:

(1) immobilize alkaline protease on GO nanosheets by using glutaraldehyde as cross-linking reagent
(2) analyze the storage stabilities, kinetic parameters and the FAAs profiles of the WAS protein hydrolysates under free and immobilized enzyme hydrolysis
(3) characterize the alkaline protease-dispersed GO biohybrids (for simplicity hereinafter GOAP).

X-ray diffraction (XRD), atomic force microscopy (AFM), and X-ray photoelectron spectroscopy (XPS) were used to characterize the functional group, size, and morphology of the GO and GOAP. This study will enable us to have a better understanding of the stable GOAP biohybrids.

Experimental Section

Materials

GO was prepared from 100 mesh natural flake graphite by the modified Hummers method [26, 27]. Amino acid standard mixture, triethylamine (TEA), norleucine internal standard substance, and phenylisothiocyanate (PITC, supplied in 25-µl vacuum-sealed ampules) were purchased from Bonna-Agela Technologies (Tianjing, China). A standard L-amino acid solution contained 2.5 µmol/ml each of Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, and Val, as well as 1.25 µmol/ml Cys in 0.1 N HCl. Alkaline protease was purchased from BeiJing AoBoXing Universeen Bio-Tech Co., Ltd. All other reagents were of analytical grade. All solutions were made by distilled water except for and the GO solution.

Enzyme Activity Assay and Synthesis of GOAP Biohybrids

Assay of enzyme activities and synthesis of GOAP biohybrids have been reported in [28].

Sample Preparation

WAS samples were obtained from the secondary sedimentation tank of a municipal wastewater treatment plant (Shanghai, China) with a capacity of 1.5×10^4 m^3 per day. This plant uses a typical anaerobic-anoxic-oxic process and the sludge is mechanically dewatered to 20-25% dry solids content by a belt press filter. The WAS samples were kept in a 4±0.5ºC fridge prior to use. The water concentration and pH of the sludge were 84.7% and 6.9, respectively. The Kjeldahl method for total nitrogen (while 6.25 for conversion coefficient) were chosen, and 58.7% (DS) protein was found.

Enzymatic hydrolysis of proteins from WAS were divided into two steps. Firstly, free alkaline protease (2wt%, activity of 25.1×10^4 U/g) was added into the WAS suspension prepared in distilled water in rations of 1:4 at pH 8.0, and then incubated at 55ºC for 4 h under vibration at middle speed. At the end of incubation, the solution was heated at 95ºC for 15 min and then centrifuged at 3,600×g for 15 min. The supernatant was collected as WAS protein hydrolysate. Secondly, free or immobilized alkaline protease (9.0 and 10.0 mg/ml for free and immobilized enzyme) was added into the WAS protein hydrolysate and pH was adjusted to 8.0 with 0.1 M HCl and 0.1 M NaOH, and incubated at 55ºC for free and 60ºC for immobilized enzyme. The suspensions were subsequently centrifuged at 15,000 g for 15min at 4ºC and the immobilized enzyme was collected and stored in phosphate buffer for reusing. For free enzyme, the samples were heated at 95ºC for 15 min and centrifuged at 15,000 g for 15 min at 4ºC. Then the supernatant was designated raw amino acid solution for the next derivatization process.

Two hundred microliters of filtered hydrolysate sample or amino acids standard solution was transferred to a 1.5-ml glass vial and 20 µl of norleucine internal standard solution was added. Thereafter, the solution was vortexed homogenously. Then, 100 µl of 1 M TEA acetonitrile solution and 100 µl of 0.1 M PITC acetonitrile solution were added into the prepared solution successively, and the solution was immediately vortexed for several seconds. The vial was closed and left to stand for 1 h at room temperature (about 25ºC). Then 400 µl n-hexane was added and vortexed for 1 min and left to stand for 10 min at room temperature. The reaction between PITC and the FAAs producing phenythiocarbamyl amino acids (PTC-AA) was allowed to complete in this course. The sublayer solution was filtered through a 0.45 µm membrane filter. A 20 µl aliquot of the filtrate was injected into the HPLC system.

Chromatographic Conditions and Instruments

FAA samples were analyzed using an Agilent 1100 system consisting of two G1312A pumps, a model 7725 manual injection valve (USA) equipped with a 20 µl sample loop, a temperature control module, and a model G1315B photodiode array detector at 254 nm wavelength. Data were recorded and analyzed by Millenmium 2011 software. A column used at 40ºC was a Venusil AA (4.6×250 mm, 100 A pore size and 5µm particle size, from Bonna-Agela Technologies). A standard solution master amino acid, to which norleucine was added as internal standard, was used for identification and quantification of FAAs in samples. A two-solvent gradient was used to run the samples. Mobile phase A comprised 93 mM sodium acetate and 7.0% acetonitrile, and pH was adjusted to 6.5 with acetic acid, and mobile phase B was 80% acetonitrile. The flow rate was 1 mL/min throughout, and the gradient consisted of the following profiles: 100% A at start, 97% A and 3% B at 4 min, 89% A and 11% B at 16 min, 79% A and 21% B at 17 min, 66% A and 34% B at 32 min, 100% B at 34 min., and 100% A at 38.01 min. The column was regenerated and equilibrated with eluent A for 20 min prior to injection.

The XPS measurements were performed on an ESCALAB MK II (VG, U.K.) with AL Kα radiation to qualitatively and quantitatively analyze the chemical composition of the test pieces. The test samples of XPS were separated from the GO, GO-glataraldehyde, and GOAP suspension through centrifugation, and the powder was dried at 60ºC under ambient conditions. AFM images were achieved on a MultiTask AutoProbe CP/MT Scanning Probe Microscope (NanoScope, Veeco, USA) in tapping mode. Samples for AFM imaging were first sonicated in a sonic mixer (Kudos, SK3300LHC), and then deposited colloidal suspensions of GO and GOAP on freshly cleaved mica surface (about 8×8 mm diameter) and allowed to air-dry at ambient temperature. Powder XRD studies were implement on a D/Max-2550 PC (Rigaku, Japan) diffractometer with Ni-filtered Cu Kα radiation as the X-ray source in the 2θ range of 5-60º.

Results and Discussion

The Storage Stability of Immobilized Enzyme

The retention of the immobilized protease activity was inspected as described in the bioactivity assays of enzyme. The storage stabilities of free and immobilized alkaline protease were determined every 5 days by measuring the activity of the samples, stored in 0.2 M phosphate buffer at 4ºC.

Fig. 1 shows the storage stabilities of free and immobilized alkaline protease. The immobilized alkaline protease retained 89.9% of its original activity after 20 days as opposed to 5 days for that of free alkaline protease, which indicating that immobilization of alkaline on GO increased the stability of enzyme preparation. The result showed that the storage stability of immobilized enzyme is similar to other reports [29, 30].

Kinetic Studies of Free and Immobilized Enzyme

The catalytic kinetics of the free and immobilized proteases was investigated at 40ºC and pH 8. Various casein solutions at concentrations of 2.0-20.0g/L were used as substrates. Lineweaver-Burke plot was used to assess the values of Michaelis constant (K_m) and maximum effective velocity (V_{max}). The kinetic analysis showed in Fig. 2 that K_m values of free and immobilized protease were 4.85 and 8.57 mg/ml, and V_{max} were 5.7 and 6.10 µg/min, respectively. The protease immobilized on GO exhibited an apparent K_m value, which was about 1.8-fold higher than that of free protease. This increase in apparent K_m value might be due either to structural changes in the enzyme induced by the applied immobilization procedure or the lower accessibility of the substrate to the active site of the

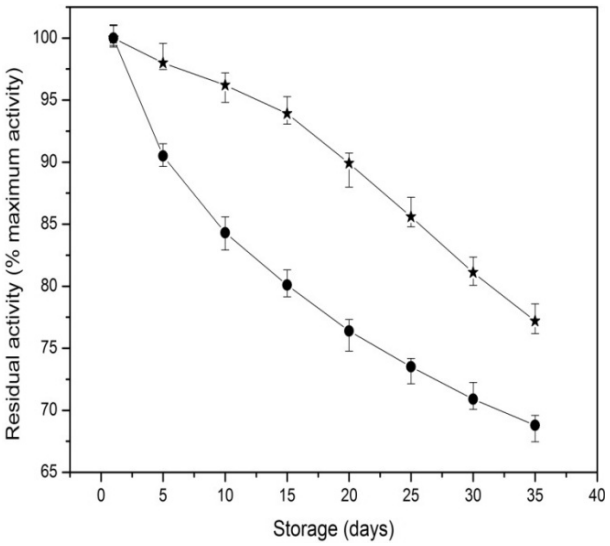

Fig. 1. Storage stability of free (roundness) and immobilized alkaline protease on GO (pentagram).

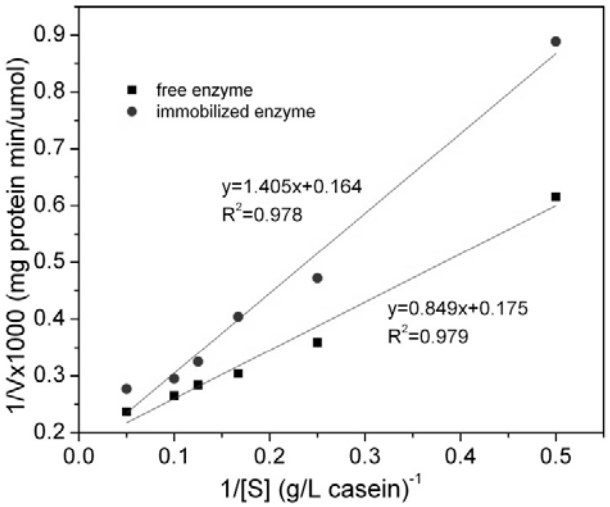

Fig. 2. Lineweaver-Burk plot for free alkaline protease (rhombus) and immobilized alkaline protease (squares) at 40ºC.

Table 1. Content of FAAs in WAS hydrolysates by free and immobilized enzyme.

Non-essential FAAs	Content (g/100g DS)		Essential FAAs	Content (g/100g DS)	
	Free enzyme	Immobilized enzyme		Free enzyme	Immobilized enzyme
Asp	0.392	0.547	Val	0.795	0.555
Glu	1.035	0.526	Met	0.278	0.063
Ser	0.657	0.493	Ile	0.463	0.413
Gly	0.665	0.333	Leu	0.707	0.693
His	0.139	0.157	Phe	0.47	0.317
Arg	0.353	0.276	Thr	0.507	0.406
Ala	1.191	0.918	Lys	0.407	0.218
Pro	0.124	0.332			
Tyr	0.368	0.273			
Cys	0.036	0.049			
Total	4.96	3.904		3.627	2.665

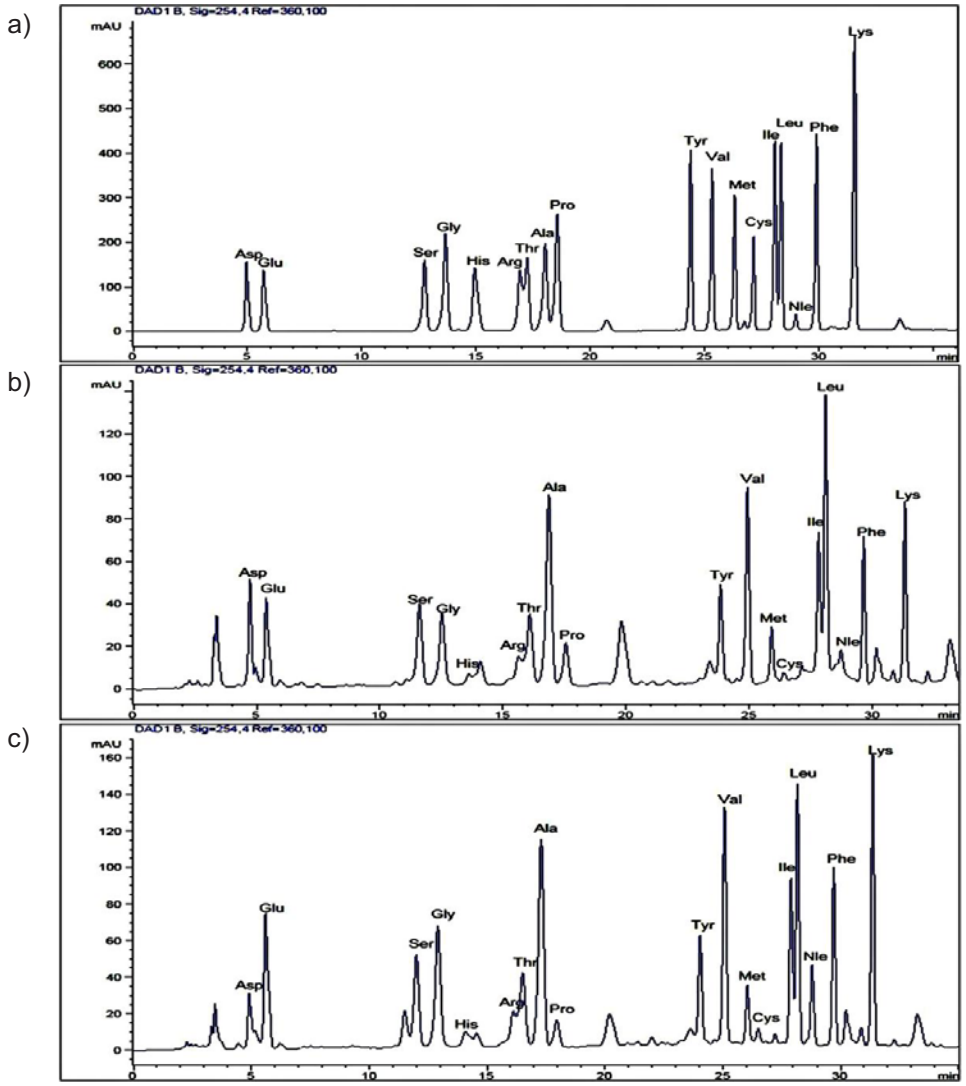

Fig. 3. Typical HPLC profiles of a mixture of standard amino acids (a), FAAs in WAS hydrolysate samples by immobilized alkaline protease (b), and free alkaline protease (c).

immobilized enzyme [31]. This may be due to GO nanosheets decorated with various oxygen functional groups such as hydroxyl, carboxyl, and epoxy groups that provide more locations for the interaction enzyme with substrate [32, 33].

FAA Profiles and Composition of the WAS Enzymatic Hydrolysis

The RP-HPLC profiles of the WAS hydrolysates with free and immobilized enzyme at pH 8 are shown in Fig. 3. The typical elution profile of an amino acid standard containing 18 amino acids shows a good separation of each component (Fig. 3a). As shown in Figs. 3 b and c, there were 17 kinds of natural amino acids emerging in the WAS hydrolysates, which consisted of seven kinds of essential amino acids and 10 kinds of non-essential amino acids. The FAAs formed with the free and the immobilized enzymes are essentially the same (Figs. 3 and 4). However there are small differences in the chromatograms such as Asp and Pro. The data in Table 1 showed that essential FAAs by free and immobilized protease were 3.627 and 2.665 g/100g DS, and non-essential FAAs were 4.960 and 3.904 g/100g DS, respectively. The total contents of FAA yield was 8.59 and 6.57 g/100g DS for free enzyme and immobilized enzyme. Similar results have been obtained previously for WAS hydrolysate produced by hot hydrochloric acid [5].

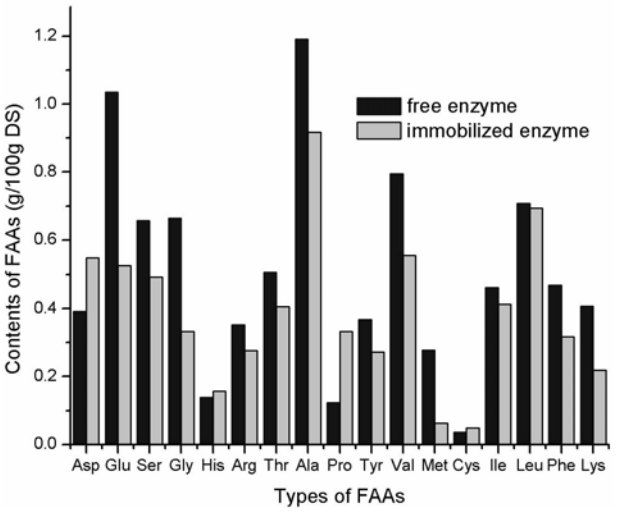

Fig. 4. Content of FAAs in WAS hydrolysates by free (dark grey) and immobilized enzymes (bright grey).

Physical Properties of GO and GOAP Hybrid Biomaterials

It is well known that in GO nanosheets the contained oxygen atoms and hydroxyl groups grafted on both sides and formed covalent bonds with the carbon atoms.

Fig. 5 presents the XRD patterns of GO powder, GO colloidal suspension, GO-glutaraldehyde, and GOAP samples. GO has a characteristic (002) peak at 2θ=10.6º, which corresponds the interlayer distance of 8.33 Å. But the peak at 23.2º (with the interplanar distance) is about 3.8 Å, which can be ascribed to the natural graphite that incomplete oxidation of graphite with the oxygen-containing functional groups on the graphite sheets. For the biohybrid materials, this diffraction peak could not be observed because of being bound on alkaline protease. Samples prepared with phosphate buffer show diffraction peaks corresponding to (100), (-101), (011), (-111), (101), (021), (-102), (-211), and (200), similar to that of pure GO.

The tapping mode AFM images and cross-section height profiles of GO, as well as of GOAP, are shown in Figs. 6 and 7. AFM is always exploited to estimate the thickness of GO sheets. The founded thicknesses of the single-sheet GO are usually 0.9-1.3 nm from AFM analysis, although the theory thickness is only 0.34 nm [34]. Due to pristine GO suspension without surfactant addition, the agglomeration occurs, image of GO shows irregular pieces of GO sheets, but they have an obscure borderline and therefore are marking indistinct. The GO nanosheets had a 1.17±0.14 nm mean thickness. The GO sheets observed by AFM were a layered morphology constructed of 1-2 layers of a single GO that represent excellent exfoliated graphene oxide sheets according to Stankovich et al. [35]. Sample GOAP had a mean thickness of 15.8 nm, and the covalent attachment of protease on GO sheets make the shape smoother.

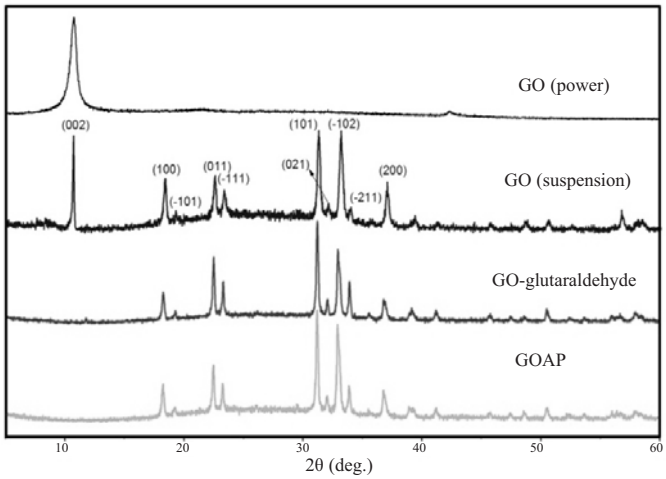

Fig. 5. XRD patterns for GO powder, GO colloidal suspension, GO-glutaraldehyde, and GOAP.

The XPS results shown in Fig. 8 confirm the elemental compositions of GO, glutaraldehyde modified GO, and GOAP samples. The peaks at about 133.1, 284.6, 399.7, 496, and 532.2 eV can be assigned to the binding energy of P 2p, C 1s, N 1s, Na 1s, and O 1s, respectively [36, 37]. Because the sample preparation involved dispersing the samples in phosphate buffer, some undesirable peak and hydroxyl group might have been introduced. This is for qualitative analysis. A quantitative analysis also was carried out to estimate the O/C atomic ratio. The O/C atomic ratio reaches as high as 42.5%, revealing a fairly high oxidation extent on the GO sheets [38]. The XPS spectrum of GO indicates that GO contains ca. 29.0 at.% of oxygen, and no obvious nitrogen signal can be detected.

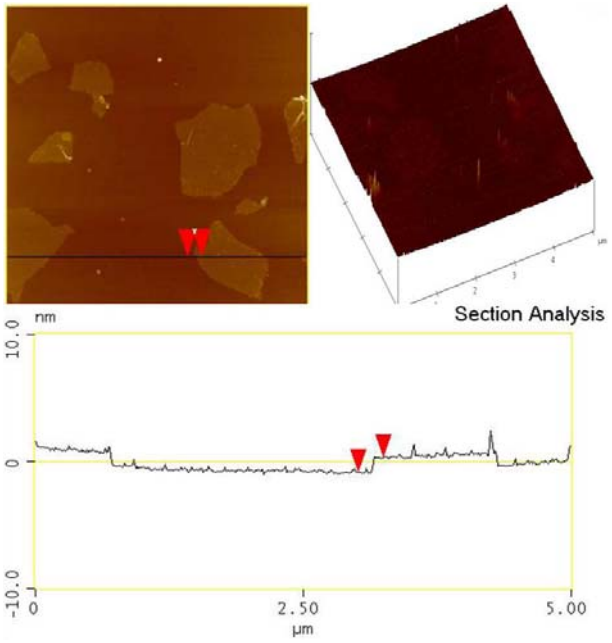

Fig. 6. Tapping-mode AFM image and cross-section height profile of GO.

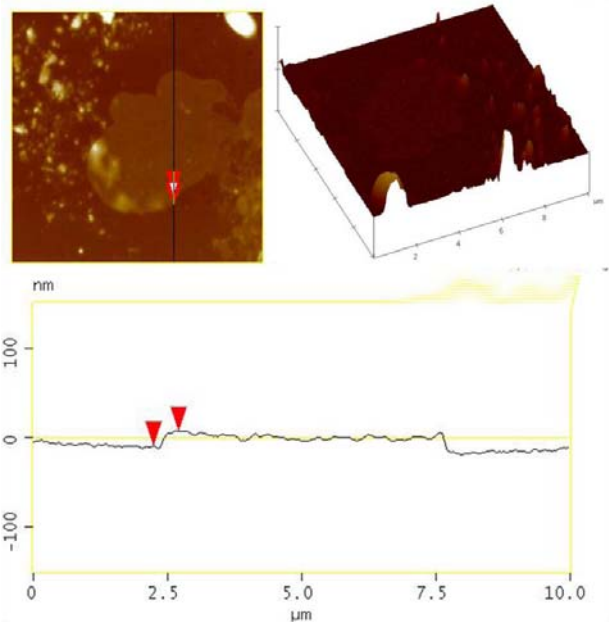

Fig. 7. Tapping-mode AFM image and cross-section height profile of GOAP.

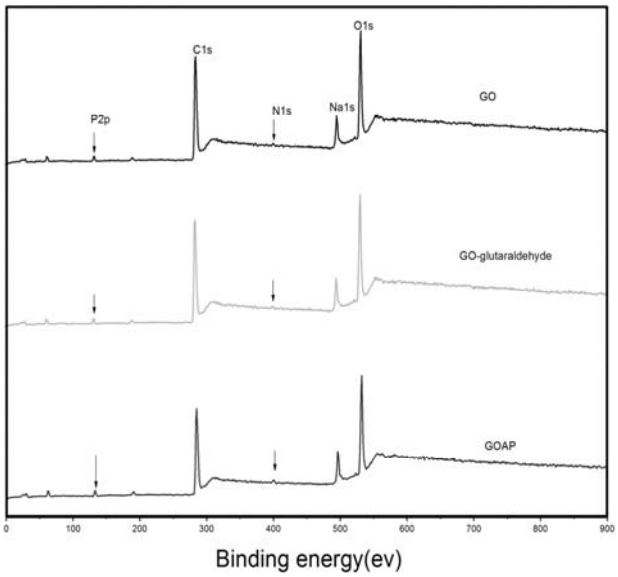

Fig. 8. Wide region XPS spectra of GO, GO-glutaraldehyde and GOAP.

Conclusions

GOAP hybrid biomaterials with excellent biocatalytic activity and operational stability properties have been successfully prepared from GO through an effective and scalable cross-linking method. Alkaline protease immobilized on GO showed significant activity toward WAS proteins. The FAAs formed with free enzyme and enzyme immobilized on GO were the same, which indicates that GO can be used as carriers for alkaline protease to produce hydrolysates with FAAs similar to those obtained with the free enzyme. The GO sheets were occupied by oxygen functionalities such as hydroxy, carbonyl, and alkoxy groups, and highly oxidized based planes and edges were generated. The results of XRD, AFM, and XPS have stated that alkaline protease was successfully immobilized. The results illustrate that the GO-immobilized enzyme systems should be promising in wastewater treatment, and in other enzyme catalytic protocols. In addition, our simple, straightforward, and soft strategy can be further extended to create a wide range of various functional hybrid biomaterials.

Acknowledgements

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