Sensors and Materials, Vol. 34, No. 8 (2022) 3155–3161
MYU Tokyo

S & M 3031

Degradation of 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic Acid) in the Presence of Laccase

Takanori Tamaki,*† Tomoharu Sugiyama, and Takeo Yamaguchi

Laboratory for Chemistry and Life Science, Institute of Innovative Research, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan

(Received March 29, 2022; accepted June 1, 2022; online published June 6, 2022)

Keywords: enzymatic electrochemical devices, biocathode, laccase, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), durability

The durability of enzymatic electrochemical devices depends on the durability of enzymes and on that of mediators that shuttle electrons between enzymes and electrodes. The degradation of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in the presence of laccase was evaluated in this study. The evaluation of the ABTS-adsorbed electrode in the presence and absence of laccase showed that the redox peak area of ABTS decreased to approximately 40% of the initial value with laccase, whereas the retention of the peak area was approximately 90% without laccase. The characterization of the degradation products in a mixed solution of ABTS and laccase was then performed using Fourier transform (FT)-IR, ^{1}H-NMR, ^{13}C-NMR, and electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS). The comparison of the spectra of the products of the mixed solution of ABTS and laccase with those of the solution containing only ABTS or laccase showed the formation of nitrosamine from FT-IR spectra and newly appeared sp^2 and sp^3 carbon from ^{13}C-NMR spectra. By also considering the peaks observed in ESI-TOF-MS spectra, a possible degradation mechanism was proposed to be initiated by the adduct formation of the ABTS radical with water.

1. Introduction

Enzymatic reactions have been used in biosensors to detect substrates and in biofuel cells to convert the chemical energy of fuels into electricity under moderate operating conditions. When enzymatic reactions are coupled with electrode reactions, electron transfer between enzymes and electrodes becomes important. Except for enzymes that can undergo direct electron transfer (DET),[1,2] redox mediators are required to shuttle electrons between enzymes and electrodes. To fully use the potential of non-DET-type enzymes in electrochemical devices, the immobilization of mediators or redox polymers, in which mediators are covalently linked to polymer backbones, on the surface of electrodes becomes important. The authors have proposed the graft polymerization of redox polymers to make a thin redox polymer layer on the surface of carbon electrodes to achieve high-current-density biofuel cells. Electrodes were designed to use the high

*Corresponding author: e-mail: tamaki@cen.kagoshima-u.ac.jp
†Present address: Department of Engineering, Graduate School of Science and Engineering, Kagoshima University, 1-21-40, Korimoto, Kagoshima 890-0065, Japan

https://doi.org/10.18494/SAM3905

intrinsic activity of enzymes in combination with high-surface-area electrodes.[3–7] A numerical model that considers the reaction and diffusion processes in electrodes supported the effectiveness of a high-surface-area electrode with a thin redox polymer layer.[8] The model also shows that further increase in current density is possible by the increase in the effective concentration of the mediator, which was defined as the concentration of the mediator that effectively reacted with both the enzyme and the electrode.[9] The recent rational design of electrodes composed of nanostructured materials has enabled a high glucose-oxidizing current density above 5 mA cm^{-2}.[6,10–17]

Another important point in developing enzymatic electrochemical devices is durability. In addition to the durability of the enzymes themselves, the durability of mediators should also be considered when mediators are used in electrochemical reactions. Particularly when free mediators are not added to a fuel or oxidant solution, which means that the mediators are somehow attached to the surface of electrodes, the degradation of the mediators markedly affects the electrochemical performance of the electrodes. When 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) was immobilized through a spacer to a polymer grafted onto carbon black to construct an oxygen-reducing electrode (ABTS-grafted electrode) with laccase as the enzyme, the redox peak area of ABTS decreased by approximately 40–60% over 6 h in the presence of laccase under both N_2 and O_2. Conversely, the redox peak area is retained in the absence of laccase, which suggests that ABTS was not eluted from the electrode, and thus, ABTS undergoes some degradation reaction in the presence of laccase.[5]

In a previous study, the degradation of ABTS was reported in the presence of polyphenols and laccase. The isolation and characterization of degradation products revealed that ABTS radicals formed covalent adducts with polyphenols, and the degradation mechanism of ABTS through the adducts was proposed.[18,19] In this reaction, polyphenols transferred an electron to ABTS radicals. In the ABTS-grafted electrode, an electron was transferred from an electrode to ABTS radicals, and polyphenols were not added. Thus, the degradation mechanism without polyphenols should exist in the ABTS-grafted electrode. However, in a previous study of an ABTS-grafted electrode, ABTS was immobilized to the polymer, and thus, the characterization of degradation products was difficult; the detailed mechanism in the electrochemical system without polyphenols was thereby unclear. In this study, the degradation mechanism of ABTS in the presence of laccase was evaluated before immobilization to the polymer using Fourier transform (FT)-IR, ^{1}H-NMR, ^{13}C-NMR, and electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS).

2. Materials and Methods

2.1 Materials

The carbon black used was Ketjen black (Lion Corp., Tokyo, Japan) with a particle diameter of approximately 30 nm. ABTS was purchased from Tokyo Chemical Industry Co., Ltd. Laccase from *Trametes versicolor* and poly(tetrafluoroethylene) (PTFE) suspension (60 wt% in water) were purchased from Sigma-Aldrich Co. Carbon paper (EC-TP1-060) was purchased from Toray

Industries, Inc. Conductive carbon paint was purchased from Okenshoji Co., Ltd. All other reagents were purchased from Wako Pure Chemical Industries, Ltd. and used without further purification.

2.2 Evaluation of ABTS-adsorbed electrode

The fabrication of carbon electrodes followed the procedure reported previously.[3–5] Carbon inks were prepared from unmodified carbon black, a suspension of PTFE, and isopropyl alcohol. The weight ratio of carbon black to PTFE was fixed at 3:1 in the dry state. The electrode was prepared by painting the carbon ink onto the carbon paper. After the carbon electrode had dried under ambient conditions for one night, the electrode was hot-pressed at 0.25 MPa and 130 °C. The electrode was cut to a circle of 0.66 cm diameter and attached to the rotating disk electrode (RDE) surface using conductive carbon paint. The fabricated electrodes were used as the working electrodes. A Pt line and Ag/AgCl (saturated KCl) were used as the counter and reference electrodes, respectively. Cyclic voltammetry (CV) was performed using a Hokuto Denko Dynamic Electrode HR-301 and Automatic Polarization System HZ-3000. The buffer solution used was 0.1 M acetate buffer containing 0.1 M $NaNO_3$ at pH of 4.0. All solutions had N_2 or O_2 bubbled through them for at least 15 min.

The adsorption of ABTS was performed by rotating the electrode in the buffer solution containing 1 mM ABTS at 500 rpm for approximately 1 h. The electrode was washed by rotating in the buffer solution without ABTS at 500 rpm until the change in redox peak area became negligible. Then, CV was measured once every hour in the presence and absence of 1.0 mg mL^{-1} laccase in the buffer solution at 30 °C.

2.3 Characterization of degradation products

A mixed solution of 100 mg of laccase and 100 mg of ABTS in 10 mL of reverse osmosis (RO) water was stirred for 24 h. The resultant solution was filtered using Amicon Ultra centrifugal membrane filters with a molecular weight cutoff of 30 kDa (Merck Millipore, Germany) to remove laccase from the solution. The filtered solution was then vacuum-dried at room temperature for 24 h. The resultant products were characterized by FT-IR, ^{1}H-NMR, ^{13}C-NMR, and ESI-TOF-MS.

3. Results and Discussion

The degradation of ABTS was first evaluated on the ABTS-adsorbed electrode in the presence and absence of laccase. Cyclic voltammograms (CVs) of the ABTS-adsorbed electrode in the presence and absence of laccase under N_2 are shown in Figs. 1(a) and 1(b), respectively. The retention of the redox peak area of ABTS under N_2 was calculated from the figures and is shown in Fig. 1(c). In the absence of laccase, the redox peak area is retained, which suggests that the elution of ABTS from the electrode was low, although ABTS was physically adsorbed without immobilizing to the polymer. The addition of laccase decreased the peak area. Because

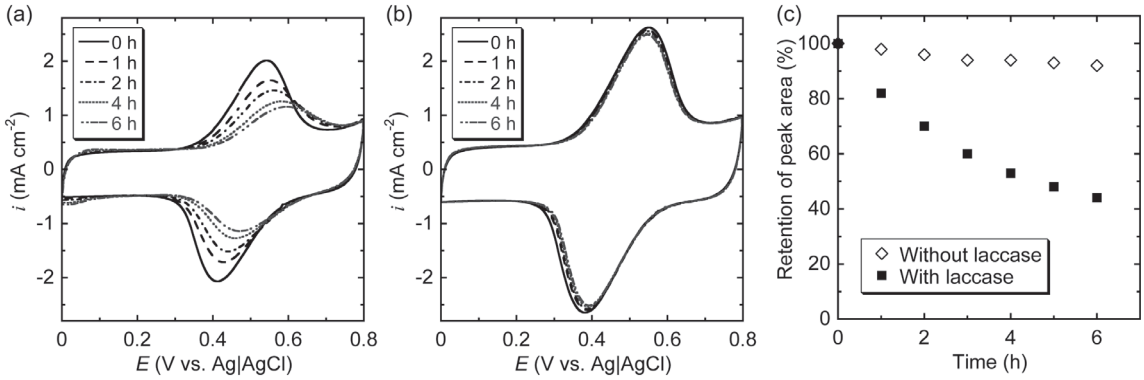

Fig. 1. CVs of ABTS-adsorbed electrode in (a) presence and (b) absence of laccase under N_2. (c) Retention of redox peak area of ABTS-adsorbed electrode in presence and absence of laccase under N_2.

the evaluation was performed without immobilizing ABTS to the polymer, the effect of the functional groups of the polymer on the degradation of ABTS was excluded. This result suggests that ABTS was degraded in the presence of laccase. It should be noted that the measurements were performed under N_2 in this study and the oxygen reduction current was not observed. The effect of the degradation of ABTS on the oxygen reduction current is important for the construction of oxygen reduction electrodes, and thus will be a topic of future studies.

Then, the degradation mechanism of ABTS in the presence of laccase was discussed. To characterize the degradation products, ABTS and laccase were mixed in a solution under N_2 or O_2 for 24 h. After filtering laccase, the products in the filtrate were analyzed by FT-IR, ^{1}H-NMR, ^{13}C-NMR, and ESI-TOF-MS. Figure 2 shows the FT-IR, ^{1}H-NMR, and ^{13}C-NMR spectra of the products. The spectra obtained for the products obtained from a solution containing only ABTS or laccase that is treated in the same manner as the mixed solution of ABTS and laccase are also included in Fig. 2. When the spectra of the products of the mixed solution of ABTS and laccase (ii and iii) were compared with those of the solution containing only ABTS (i) or laccase (iv), a peak at approximately 1400 cm^{-1} appeared in the FT-IR spectra [Fig. 2(a)] and peaks at approximately 160 and 60 ppm appeared in the ^{13}C-NMR spectra [Fig. 2(c)]. The ^{1}H-NMR spectra [Fig. 2(b)] did not change with the addition of laccase to the ABTS solution. The peak that appeared in the FT-IR spectra is ascribed to the N=O stretching vibration of nitrosamine (–N–N=O), and the peaks that appeared in the ^{13}C-NMR spectra at approximately 160 and 60 ppm correspond to sp^2 and sp^3 carbon, respectively.

Figure 3 shows the ESI-TOF-MS spectra of the product from the mixed solution of ABTS and laccase under O_2. Other than the starting material (ABTS) at m/z = 515, peaks at m/z = 289, 274, 259, and 244 were observed. The peak at 259 was also reported in a previous study when characterizing the degradation products of ABTS in the presence of polyphenols and laccase.[18,19]

On the basis of these results, the degradation mechanism of ABTS is proposed, as shown in Fig. 4. The FT-IR spectra suggested the presence of nitrosamine [product (3)]. The peak at approximately 160 ppm in ^{13}C-NMR can be ascribed to any of c(*a*) of product (2), c(*b*) of product (4), c(*c*) of product (5), and c(*e*) of product (8), whereas the peak at approximately 60 ppm can be

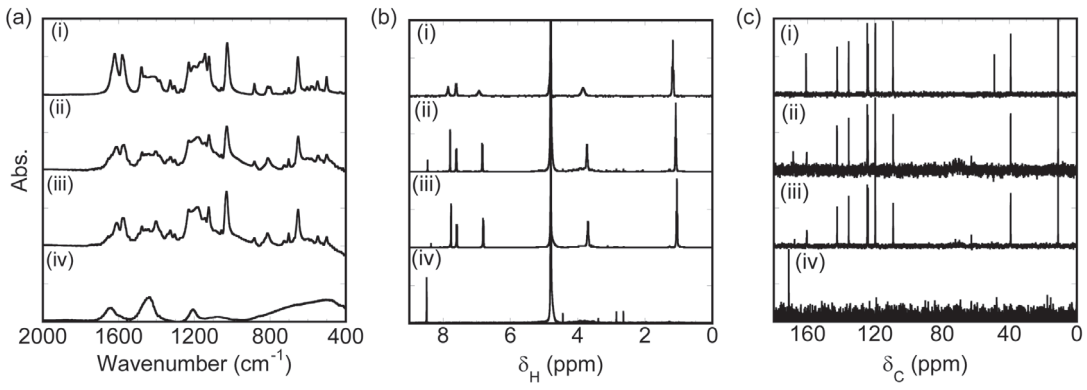

Fig. 2. (a) FT-IR, (b) 1H-NMR, and (c) 13C-NMR spectra of products of solution containing (i) only ABTS, (ii) ABTS and laccase under N_2, (iii) ABTS and laccase under O_2, and (iv) only laccase.

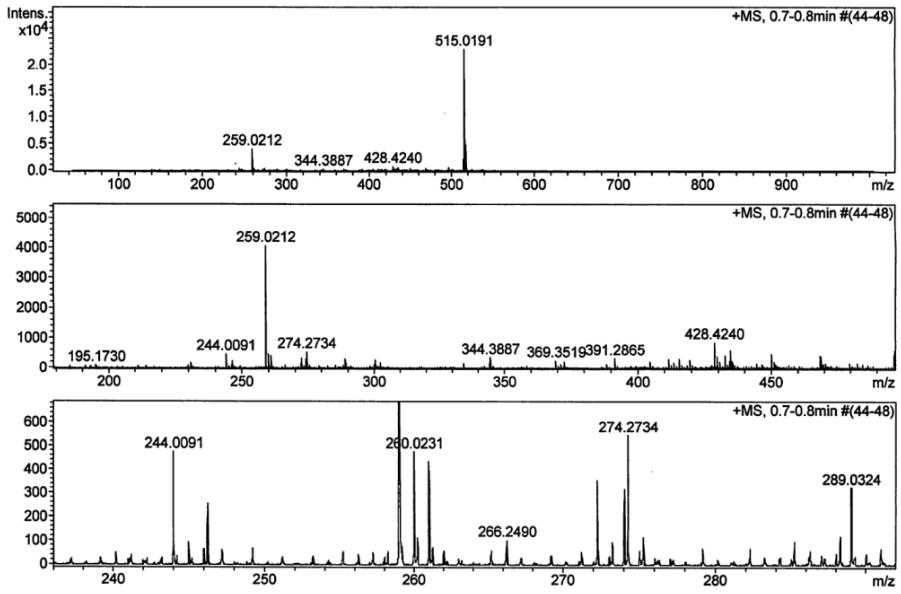

Fig. 3. ESI-TOF-MS spectra of products of mixed solution of ABTS and laccase under O_2.

ascribed to c(*d*) of product (6). If product (7), which is formed by the decomposition route to form product (8), is sufficiently stable, ^{1}H-NMR spectra should show a peak at approximately 5.4 ppm of H(*a*). However, the peak corresponding to H(*a*) did not appear in Fig. 2(b). In a previous study, products (2) and (7) were reported to be unstable and to easily undergo hydrolysis and oxidation to form product (8).[19] This rapid conversion to product (8) is considered to lead to the absence of the peak corresponding to H(*a*). The instability of products (2) and (7) reported in a previous study suggests that the peaks obtained in ESI-TOF-MAS can be ascribed to product (3) for m/z = 289, product (4) for m/z = 274, product (5) and/or product (8) for m/z = 259, and product (6) for m/z = 244.

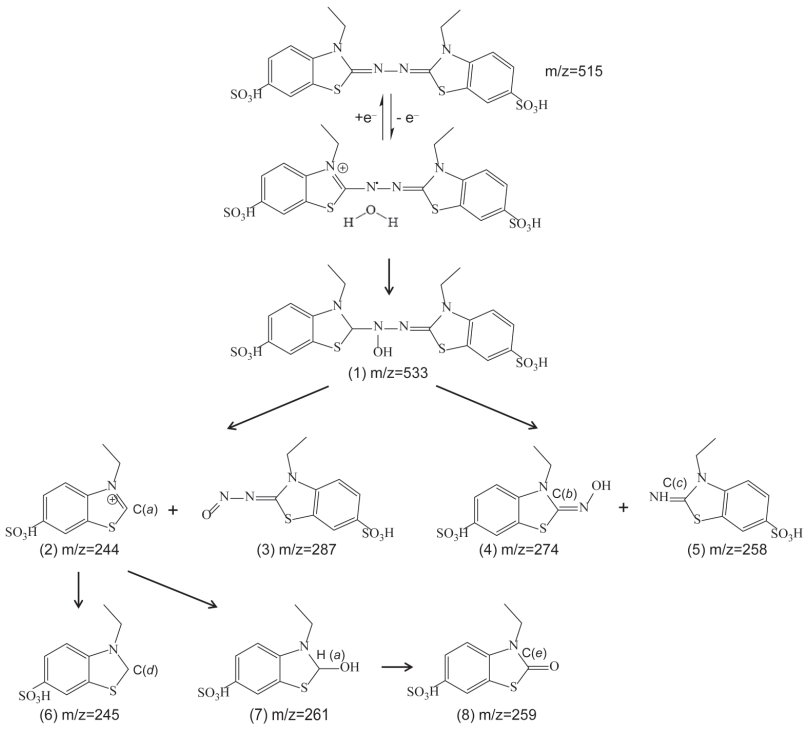

Fig. 4. Proposed degradation mechanism of ABTS in electrochemical system with laccase.

The formation of products (5) and (8) from ABTS was identified in the presence of polyphenols after purification by reversed-phase chromatography and by characterization using UV–vis, mass spectrometry, and ^{1}H-NMR.[18] In the degradation mechanism with polyphenols, the degradation reaction is initiated from the formation of polyphenol-derived adducts with ABTS radicals. The initially formed adducts are unstable and degraded to form product (5) or (8) and other covalent adducts with polyphenols.[19] Although polyphenols were not used in this study, the same degradation products (5) and (8) were observed, which suggests that the formation of the adduct with water (the second step in Fig. 4) in the electrochemical system initiates the degradation of ABTS. Considering that the presence of laccase degrades ABTS, as shown in Fig. 1 and in a previous study,[5] the ABTS radical generated by the reaction between ABTS and laccase (the first step in Fig. 4) forms an adduct with water. Because the laccase used in the study that showed the degradation of ABTS in the presence of polyphenols was from *Aspergillus oryzea* provided by Novozymes (51003) and was different from ours, the degradation mechanism of ABTS proposed in this study may not be limited to laccase from *Trametes versicolor*. However, further studies are necessary to discuss whether the degradation mechanism can be applied to other laccases.

These results indicate that the degradation of ABTS occurred in the electrochemical system with laccase. Thus, to develop stable enzymatic electrochemical devices using non-DET-type enzymes, it is important to select or molecularly design a mediator that is sufficiently stable under operating conditions based on the understanding of the degradation mechanism of the mediator.

4. Conclusions

In this study, we clarified the degradation mechanism of ABTS in the presence of laccase. The evaluation of the ABTS-adsorbed electrode in the presence and absence of laccase showed that the addition of laccase decreased the redox peak area of ABTS. The degradation products in a mixed solution of ABTS and laccase were then characterized using FT-IR, ^{1}H-NMR, ^{13}C-NMR, and ESI-TOF-MS. The comparison of the spectra of the products of the mixed solution of ABTS and laccase with those of the solution containing only ABTS or laccase showed the formation of nitrosamine from FT-IR spectra and newly appeared sp^2 and sp^3 carbon from ^{13}C-NMR spectra. By also considering the peaks observed in ESI-TOF-MS spectra, a possible degradation mechanism was proposed to be initiated by the adduct formation of the ABTS radical with water.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Numbers JP18K04822 and JP21K04767, and was also supported in part by the “Five-star Alliance”.

References

1 J. A. Cracknell, K. A. Vincent, and F. A. Armstrong: Chem. Rev. **108** (2008) 2439. https://doi.org/10.1021/cr0680639
2 R. D. Milton and S. D. Minteer: J. R. Soc. Interface **14** (2017) 20170253. https://doi.org/10.1098/rsif.2017.0253
3 T. Tamaki and T. Yamaguchi: Ind. Eng. Chem. Res. **45** (2006) 3050. https://doi.org/10.1021/ie051142a
4 T. Tamaki, T. Ito, and T. Yamaguchi: J. Phys. Chem. B **111** (2007) 10312. https://doi.org/10.1021/jp074334n
5 T. Sugiyama, T. Tamaki, and T. Yamaguchi: J. Chem. Eng. Jpn. **47** (2014) 704. https://doi.org/10.1252/jcej.13we319
6 T. Tamaki, T. Sugiyama, M. Mizoe, Y. Oshiba, and T. Yamaguchi: J. Electrochem. Soc. **161** (2014) H3095. https://doi.org/10.1149/2.0181413jes
7 T. Tamaki, T. Sugiyama, Y. Oshiba, and T. Yamaguchi: J. Phys.: Energy **3** (2021) 034002. https://doi.org/10.1088/2515-7655/abe2f6
8 T. Tamaki, T. Ito, and T. Yamaguchi: Fuel Cells **9** (2009) 37. https://doi.org/10.1002/fuce.200800028
9 T. Tamaki, R. Nishigaya, R. Yamazaki, and T. Yamaguchi: Ind. Eng. Chem. Res. **61** (2022) 4504. https://doi.org/10.1021/acs.iecr.1c04210
10 S.C. Barton, Y.H. Sun, B. Chandra, S. White, and J. Hone: Electrochem. Solid State Lett. **10** (2007) B96. https://doi.org/10.1149/1.2712049
11 H. Sakai, T. Nakagawa, Y. Tokita, T. Hatazawa, T. Ikeda, S. Tsujimura, and K. Kano: Energy Environ. Sci. **2** (2009) 133. https://doi.org/10.1039/b809841g
12 V. Flexer, N. Brun, R. Backov, and N. Mano: Energy Environ. Sci. **3** (2010) 1302. https://doi.org/10.1039/c003488f
13 S. Yoshino, T. Miyake, T. Yamada, K. Hata, and M. Nishizawa: Adv. Energy Mater. **3** (2013) 60. https://doi.org/10.1002/aenm.201200422
14 S. Tsujimura, K. Murata, and W. Akatsuka: J. Am. Chem. Soc. **136** (2014) 14432. https://doi.org/10.1021/ja5053736
15 D. P. Hickey, A. J. Halmes, D. W. Schmidtke, and D. T. Glatzhofer: Electrochim. Acta **149** (2014) 252. https://doi.org/10.1016/j.electacta.2014.10.077
16 A. Niiyama, K. Murata, Y. Shigemori, A. Zebda, and S. Tsujimura: J. Power Sour. **427** (2019) 49. https://doi.org/10.1016/j.jpowsour.2019.04.064
17 P. Y. Blanchard, P. H. M. Buzzetti, B. Davies, Y. Nedellec, E. M. Girotto, A. J. Gross, A. Le Goff, Y. Nishina, S. Cosnier, and M. Holzinger: ChemElectroChem **6** (2019) 5242. https://doi.org/10.1002/celc.201901666
18 A. M. Osman, K. K. Y. Wong, S. J. Hill, and A. Fernyhough: Biochem. Biophys. Res. Commun. **340** (2006) 597. https://doi.org/10.1016/j.bbrc.2005.12.051
19 A. M. Osman, K. K. Y. Wong, and A. Fernyhough: Biochem. Biophys. Res. Commun. **346** (2006) 321. https://doi.org/10.1016/j.bbrc.2006.05.118