Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Biotransformation of nitro-polycyclic aromatic compounds by vegetable and fruit cell extracts*

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Received Aug. 8, 2011; Revision accepted Oct. 27, 2011; Crosschecked Mar. 15, 2012

Abstract: Extracts from various vegetables and fruits were investigated for their abilities to reduce nitro-polycyclic aromatic hydrocarbons (NPAHs). The extracts from grape and onion exhibited an interesting selectivity, yielding corresponding hydroxylamines or amines as major products under mild conditions of 30 °C and pH 7.0. Grape extracts reduced the 4-nitro-1,8-naphthalic anhydride with the highest conversion rate (>99%) and the highest ratio of hydroxylamine to amine (95:5). In contrast, the onion extracts reduced 4-nitro-1,8-naphthalic anhydride with a conversion rate of 94% and a ratio of hydroxylamine to amine of 8:92. The thiol-reducing agent, β-mercaptoethanol, and metal cations, Ca^{2+} and Mg^{2+}, greatly increased the reductive efficiency. This work provides an alternative strategy for biotransformation of nitro-polycyclic compounds.

Key words: Biotransformation, Nitro-polycyclic aromatic compounds, Plant cell extracts, Hydroxylamine, Amine
doi:10.1631/jzus.B1100254 **Document code:** A **CLC number:** Q503

1 Introduction

Nitro-polycyclic aromatic hydrocarbons (NPAHs) are widely distributed environmental pollutants (Muck *et al.*, 2002; Haritash and Kaushik, 2009), and are often resistant to degradation under natural conditions. Generally, nitroreduction is the first step of remediation of nitroaromatic pollutants. Nitroreduction increases the solubility of nitro compounds greatly in aqueous environment (Spain, 1995), which facilitates microbial biodegradation. Nitroreduction products of NPAHs, including hydroxylamine or amine derivatives, are extensively used in pharmaceuticals and chemical industries (Tocher, 1997).

Bioreduction of NPAHs is a significant pathway that has shown partial success in practice (Ramos *et al.*, 2005). Despite biodegradation by a wide variety of bacteria, few microorganisms are found to be able to degrade NPAHs (Castelli *et al.*, 2008). Furthermore, the slow growth rate of the microbial community is another drawback of microbial nitroaromatic degradation, perhaps due to high toxicity and low solubility of nitro compounds (Marvin-Sikkema and de Bont, 1994; Ramos *et al.*, 2005; Yoon *et al.*, 2006; Roldán *et al.*, 2008). In addition, the uncertainties associated with changes in the indigenous microbial community remain unpredictable (Dua *et al.*, 2002; Wu *et al.*, 2008). These restrictions prompt the exploration of non-microbial biodegradation methods (Parrish *et al.*, 2004).

Plants are good choices in the removal of organics in environmental remediation (Macek *et al.*, 2000) and as biocatalysts in asymmetric reduction (Matsuda *et al.*, 2009). Several species of grass,

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* Project supported by the National Basic Research Program (973) of China (No. 2010CB126102), the National Natural Science Foundation of China (No. 31070715), the National High-Tech R&D Program (863) of China (No. 2011AA10A204), the National Key Technology R&D Program of China (No. 2011BAE06B05), and the Fundamental Research Funds for the Central Universities (No. DUT10LK33), China

legume, aquatic weeds, and transgenic plants have been shown to degrade parent polycyclic aromatic hydrocarbons (PAHs) (Abhilash *et al.*, 2009; Haritash and Kaushik, 2009), whereas they have not yet been explored in NPAHs reduction. Compared with whole-plant system, plant cell extracts offer experimental advantages in plant biodegradation capabilities, toxicity tolerance, and shorter culture periods (Doran, 2009).

Nitroreduction mainly yields corresponding hydroxylamines and aromatic amines, both of which are chemicals used in manufacturing semiconductors, pharmaceuticals, and fine chemicals. However, hydroxylamine derivatives are seldom obtained because they are immediately reduced forwards to amines. In addition, to separate a mix of hydroxylamines and amines is difficult because they have very similar polarities. Therefore, to provide a high yielding chemoselective route to produce hydroxylamine or amine is of great significance.

In this study, we investigated reduction of nitropolycyclic aromatic compounds by cell extracts derived from various common fruits and vegetables. This work may provide an alternative strategy for bioreduction of nitroaromatic compounds.

2 Materials and methods

2.1 Plants and agents

Fresh fruits and vegetables (Table 1), including grape (*Vitis vinifera* L.), apple (*Malus pumila* Mill.), banana (*Musa balbisiana* Colla), cherry (*Prunus pseudocerasus* Lindl.), pear (*Pyrus pyrifolia* (Burm.) Nak.), strawberry (*Fragaria ananassa* Duch.), orange (*Citus reticulate* Blanco), jujube (*Zizyphus jujuba* Mill.), potato (*Solanum tuberosum* L.), plum (*Prunus* spp.), scallion (*Allium fistulosum* L. var. caespitosum), topinambur (*Hippophae Rhamnoides* Linn.), onion (*Allium cepa* L.), and garlic (*Allium saticum* L.) were purchased from a local market at typical maturity period. All chemicals were of high-performance liquid chromatography (HPLC) grade and were purchased from Sigma and Fluka (USA).

2.2 Plant extract preparation

Fresh, undamaged, firm fruits and vegetables were chosen and first cleaned with detergent, rinsed under running tap water, and then were surface disinfected by immersing in 70% ethanol for 3 min and in 5 g/L NaClO for 5 min followed by three rinses with sterile distilled water. A total of 15 g of fruit or vegetable was frozen with liquid nitrogen and then ground into a powder. The powder was dispersed in 100 ml buffer (10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 20 mmol/L NaCl, pH 7.0), and the mixture was placed on a shaker for 2 h followed by centrifugation at 5000×*g* for 30 min. The supernatant obtained was filtered extract through a 0.22-μm filter and stored at −20 °C until used.

2.3 Nitroreduction assay

All solutions and samples were kept at 4 °C (unless otherwise stated). A total of 10 ml of the extract was added to 0.1 ml of 20 mg/ml nitroaromatic compound dissolved in dimethylsulfoxyde (DMSO) and incubated at 30 °C on a shaker set at 200 r/min. The reaction was terminated by adding 20 ml of ethyl acetate. The organic solvent in the sample was removed by vacuum evaporation, and the conversion rate for nitroaromatic compound was determined by HPLC.

2.4 Conversion rate determined by reverse phase HPLC

Reverse phase HPLC (RP-HPLC) analyses of substrates and products were performed using an Agilent 1100 system (Agilent, USA) coupled to a UV detector and a Kromasil C_{18} column (4.6 mm×250 mm i.d., 5 μm, 80 Å). The column flow rate was maintained at 0.8 ml/min and the eluent was monitored at 254 nm (A_{254}). The separation conditions and the retention time for the different compounds are summarized in Tables 1 and 2.

2.5 Kinetics

For kinetic studies, 4-nitro-1,8-naphthalic anhydride was chosen as the model substrate. A total of 2 ml of the reaction mixture was taken out for RP-HPLC analysis at different time points: 0, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, and 264 h. A first-order rate equation [$\ln(c/c_0)=-k_1/t$, where c is substrate concentration, c_0 is substrate initial concentration, k_1 is velocity constant, and t is reaction time] was used to analyze the kinetic rates of nitroreduction, and the pseudo-first-order rate constant k_1 was determined.

2.6 Factors affecting nitroreduction assay

To investigate the effects of metal cations on the nitroreduction, 5 mmol/L of Mg^{2+}, Ca^{2+}, Li^{+}, Zn^{2+}, Sr^{2+}, Hg^{2+}, Cu^{2+}, Co^{2+}, Al^{3+}, Ni^{2+}, Mn^{2+}, and Fe^{3+} were independently added to the reaction mixture. To investigate the effects of inorganic anions on the nitroreduction, 5 mmol/L of IO_4^-, N^{3-}, SO_4^{2-}, SO_3^{2-}, Cl^-, and $S_2O_3^{2-}$ were independently added to the reaction mixture. To investigate the effects of other reducing chemicals, 10 mmol/L of dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), phenylmethanesulfonyl fluoride (PMSF), and β-mercaptoethanol (β-ME) were independently added to the reaction mixture. In each of the above reaction systems, the mixture including filtered plant extract in buffer (10 mmol/L HEPES, 20 mmol/L NaCl, pH 7.0) without these above mentioned compounds, or buffer blank acted as a control. Each reaction was run in triplicate. The statistical analysis of paired *t*-test was used according to the software Origin 7.0. The effect of pH on nitroreduction was also studied using either 20 mmol/L sodium phosphate (for pH 6.0, 6.5, 7.0, and 7.5) or 20 mmol/L Tris-HCl (for pH 7.0, 7.5, 8.0, and 8.5).

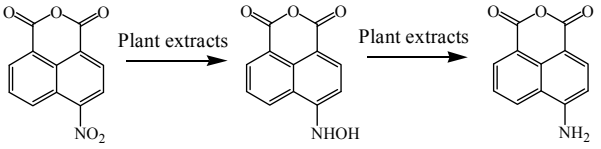
3 Results and discussion

3.1 Plants showing chemoselectivity

Nitroaromatic reduction usually proceeds through the formation of nitroso or hydroxylamine intermediates that are seldom detected due to their short lives. As shown in Table 1, except garlic (*Allium saticum* L.), which exhibited no nitroreduction activity, the extracts of all the other fruits and vegetables were able to effectively reduce 4-nitro-1,8-naphthalic anhydrides to their corresponding arylhydroxylamine, followed by arylamine products, with a conversion rate of >90%. And these plants exhibited chemoselectivity. Extracts isolated from grape (*Vitis vinifera* L.), apple (*Malus pumila* Mill.), banana (*Musa balbisiana* Colla), cherry (*Prunus pseudocerasus* Lindl.), pear (*Pyrus pyrifolia* (Burm.) Nak.), strawberry (*Fragaria ananassa* Duch.) and orange (*Citus reticulate* Blanco) yielded hydroxylamines as major products after 120 h, while other plant extracts, such as onion (*Allium cepa* L.), jujube (*Zizyphus jujuba* Mill.), potato (*Solanum tuberosum* L.), plum (*Prunus* spp.), scallion (*Allium fistulosum* L. var. caespitosum), and topinambur (*Hippophae Rhamnoides* Linn.) yielded amine products. Among them, the grape extracts reduced the nitroaromatic compounds with the

Table 1 Nitroreduction of the 4-nitro-1,8-naphthalic anhydride by plant extracts[a]

Entry	Plant extract	Conversion rate (%)[b]	–NHOH vs. NH_2
1	Grape (*Vitis vinifera* L.)	>99±0.86	95:5
2	Apple (*Malus pumila* Mill.)	90±1.09	85:15
3	Banana (*Musa balbisiana* Colla)	>99±1.21	81:19
4	Cherry (*Prunus pseudocerasus* Lindl.)	>99±0.97	80:20
5	Pear (*Pyrus pyrifolia* (Burm.) Nak.)	93±2.17	79:21
6	Strawberry (*Fragaria ananassa* Duch.)	91±3.01	65:35
7	Orange (*Citus reticulate* Blanco)	91±0.74	60:40
8	Jujube (*Zizyphus jujuba* Mill.)	92±2.65	45:55
9	Potato (*Solanum tuberosum* L.)	>99±0.66	26:74
10	Plum (*Prunus* spp.)	90±1.34	21:79
11	Scallion (*Allium fistulosum* L. var. caespitosum)	91±2.80	11:89
12	Topinambur (*Hippophae Rhamnoides* Linn.)	>99±1.08	10:90
13	Onion (*Allium cepa* L.)	94±3.27	8:92
14	Garlic (*Allium saticum* L.)	0	ND[c]

[a] Nitroreduction: 

[b] The conversion rate [mean±standard deviation (SD) of triplicate tests] was determined by RP-HPLC, based on a 120-h reaction at 30 °C and pH 7.0. [c] ND: not determined

highest conversion rate (>99%) and the highest ratio of hydroxylamine to amine (95:5). In contrast, the onion extracts reduced 4-nitro-1,8-naphthalic anhydride with a conversion rate of 94% and the ratio of hydroxylamine to amine of 8:92. The nearly complete hydroxylamine or amine products by a highly chemoselective catalysis with grape or onion cells will provide an alternative production strategy and easier purification. Because grape cells exhibit potential applications in hydroxylamine production, the following investigations were set to understand grape cells-catalyzed nitroreduction.

3.2 Kinetic properties of 4-nitro-1,8-naphthalic anhydride reduction

The reduction of 4-nitro-1,8-naphthalic anhydride by grape extracts exhibited a pseudo-first-order reaction with a rate constant of 0.010 h^{-1} and a half-life of 60 h. In the process of nitroreduction, the concentration of hydroxylamine reached the highest level when the reaction was carried out for 120 h (Fig. 1). After 264 h, all products were transformed into amine. Thus, the production of hydroxylamine or amine was time-dependent and these two products could be obtained easily through controlling the reaction time.

3.3 Factors affecting nitroreduction assay

Inorganic anions, such as chloride and sulfate, have also shown their effects on redox reactions (Lewis and Ray, 1984). In this study, strong oxidized inorganic anion IO_4^- and electron transporting inhibitor N^{3-} inhibited the nitroreduction significantly, while others, including SO_4^{2-}, SO_3^{2-}, Cl^-, and $S_2O_3^{2-}$, did not strongly affect this reduction (Fig. 2).

The effects of other chemicals include thiol-reducing agents (DTT and β-ME), serine protease inhibitor (PMSF), and metal chelator (EDTA) on the reduction of NPAHs were investigated. As shown in Fig. 2, the nitroreduction was dramatically increased in the presence of β-ME, but inhibited by EDTA.

Active metals, such as magnesium, aluminum, and zinc, are usually good reducing agents (Laine and Cheng, 2007). We investigated the effects of metal cations on reduction of NPAHs by grape extracts. As shown in Fig. 2, the nitroreduction process was significantly increased by Ca^{2+} and Mg^{2+}, but inhibited by Hg^{2+}, Co^{2+}, Al^{3+}, Cu^{2+}, and Ni^{2+}.

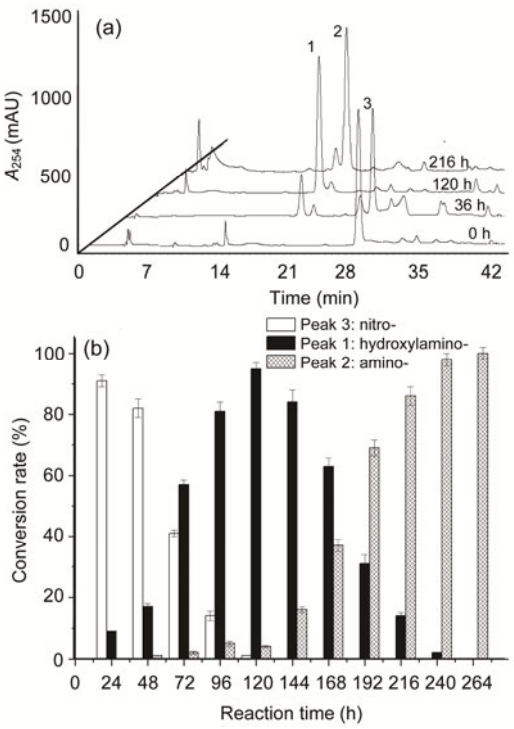


Fig. 1 Analysis of the active components of grape extracts by RP-HPLC

(a) HPLC results for grape extracts reducing the nitro group. Peak 1: 4-hydroxylamino-1,8-naphthalic anhydride; Peak 2: 4-amino-1,8-naphthalic anhydride; Peak 3: 4-nitro-1,8-naphthalic anhydride. (b) Time-course study results for grape extracts reducing the nitro group. The results represent mean±SD of triplicate tests. The conversion rate was determined by RP-HPLC

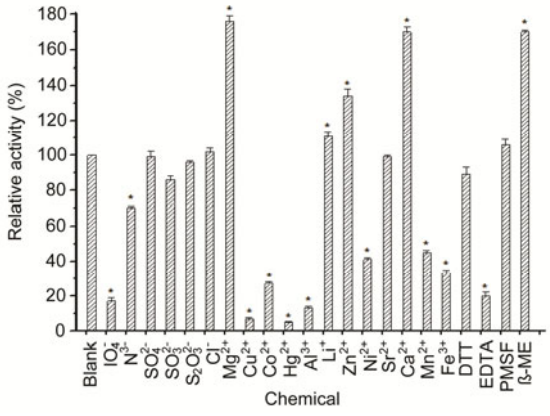


Fig. 2 Effects of chemicals including inorganic anions (5 mmol/L), metal cations (5 mmol/L), and common reagents (10 mmol/L) on the nitroreduction activity by grape extracts

The relative activity was compared with the control without these compounds. The results represent mean±SD of triplicate tests. $^*P<0.05$, significant differences according to paired *t*-tests

As shown in Fig. 3, the optimum pH for the nitroreduction catalyzed by grape extracts was 7.0. The conversion rate was kept above 50% between pH 6.0–8.5. Compared with specific enzymes, such as nitroreductase from *E. coli* (Dai *et al.*, 2009), *Rhodococcus* sp. (Shen *et al.*, 2009), and other sources (Hallas and Alexander, 1983; Schackmann and Muller, 1991; Medina *et al.*, 2004; Teramoto *et al.*, 2004; van Aken, 2009), with pH ranging between 6.5–7.5, the plant extracts exhibited advantages over a wide range of reaction pH.

3.4 Substrate spectra

Several nitropolycyclic aromatic compounds, including 3-nitro-1,8-naphthalic anhydride, 4-nitro-1,8-naphthalic anhydride, 3-nitrophthalimide, and 4-nitrophthalimide, were investigated for substrate spectra of grape extracts. They could be reduced to corresponding hydroxylamine or amine by grape extracts with very high conversion rate (>86%) and chemoselectivity (Table 2).

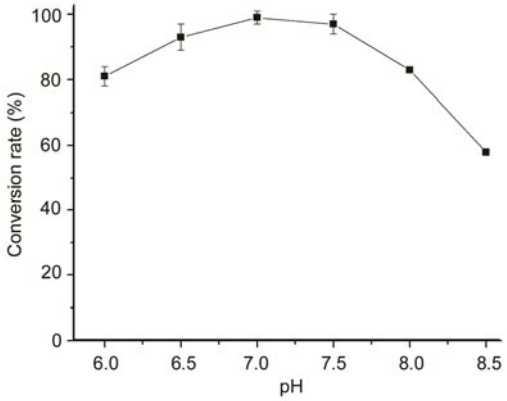
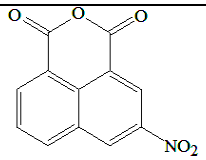
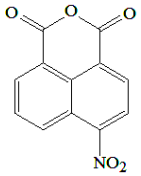
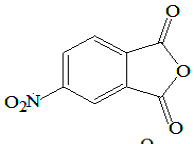
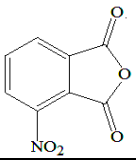


Fig. 3 Effect of pH on the nitroreduction by grape extracts
The results represent mean±SD of triplicate tests. The conversion rate was determined by RP-HPLC

Table 2 Substrate specificity of nitroreduction catalyzed by grape extracts

Entry	Substrate	HPLC condition	Retention time of –NHOH/–NH_2/–NO_2 (min)	Conversion rate (%)	–NHOH:–NH_2
1		30%–100% CH_3OH, 60 min	19.5/21.2/26.8	>99±1.88A	94:6D
2		30%–100% CH_3OH, 60 min	20.5/22.0/27.2	>99±3.07A	95:5D
3		30%–51% CH_3OH, 18 min	6.5/7.7/14.3	93±2.19B	88:12E
4		30%–60% CH_3OH, 15 min	6.4/7.2/7.4	86±3.98C	83:17F

The conversion rate (mean±SD of triplicate tests) was determined by RP-HPLC. Different letters in the same column indicate significant differences ($P<0.05$) according to paired *t*-tests

4 Conclusions

Plant-based biotransformation exhibited advantages over a wide range of mild conditions. Under 30 °C, 200 r/min, and pH 7.0, nitro-polycyclic aromatic compounds were reduced into the corresponding hydroxylamine or amine products within 120 h in the presence of grape or onion extracts. The nearly complete hydroxylamine or amine products by a highly chemoselective catalysis with grape or onion cells will provide an easier preparation of high-value hydroxylamine or amine compounds in chemical

industries. The identification of the key active components in the future will facilitate full understanding and potential applications in biotransformation of nitroaromatic compounds.

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