Monatsh Chem (2010) 141:119–123
DOI 10.1007/s00706-009-0230-7

ORIGINAL PAPER

Sol–gel encapsulation of acid phosphatase in the presence of the ionic liquid [BMIM][BF_4]

Suhasini Kulkarni · Elizabeth Chin · Vu Tran · Maggie K.-M. Ho · Chieu Phan · Monika Sommerhalter

Received: 3 September 2009 / Accepted: 22 November 2009 / Published online: 15 December 2009

Abstract The effect of the ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4]) on acid phosphatase (APase) from wheat germ in solution and in the sol–gel-encapsulated form was investigated to explore new methods of enzyme preparation with improved catalytic performance. APase was encapsulated in hydrogel beads made from tetramethyl orthosilicate. Compared with free APase, 20–28% enzymatic activity was retained in over nine catalytic cycles. Sol–gel encapsulation improved the thermal stability of APase. Heat shock exposure at 60 °C for 1 h resulted in activity decreasing by a factor of three, only, for sol–gel-encapsulated APase, in contrast with a factor of nine decrease for free APase. Addition of 10% *v*/*v* [BMIM][BF_4] did result in a slight decrease of enzymatic activity for free and sol–gel-encapsulated APase, but resulted in a remarkable increase in alkaline pH tolerance of sol–gel-encapsulated APase.

Keywords Enzymes · Immobilization · Colorimetric assay · Thermal deactivation · pH Tolerance

Introduction

Enzymes have intriguing applications as biocatalysts for the production of fine chemicals. The confinement of enzymes to a solid matrix enables recovery and the multiple re-use of enzymes, rendering their biotechnological applications more practical and economical. Using sol–gels as immobilization matrices has advantages, including low cost, ease of preparation, chemical inertness, optical transparency, and versatility in the shape of the material [1]. A key problem, however, is that compared with the free enzyme in solution, enzyme sol–gel encapsulation typically results in a loss of activity. Incorporation of additives such as sugar molecules, polymers, or surfactants can help to recover some of the lost activity by increasing the stability of the enzyme and/or by improving the material transport properties of the sol–gel matrix [2–4]. In the latter case, the additive acts as a template that improves the pore size distribution of the sol–gel matrix.

Ionic liquids have desirable properties which make them good replacements for traditional organic solvents. Those properties include low vapor pressure, high thermal stability, and the ability to dissolve both inorganic and organic compounds [5]. The use of ionic liquids as additives in enzyme sol–gel immobilization processes is still rare, but the first exploratory results are highly encouraging [6]. For example, a 30-fold increase in the activity of horseradish peroxidase was observed when the water-miscible ionic liquid [BMIM][BF_4] was incorporated into tetraethyl orthosilicate (TEOS)-based xerogel monoliths [7]. Another study on lipase encapsulated in a TEOS-based xerogel powder in the presence of different ionic liquids resulted in more modest, but still significant, changes in enzymatic activity [8]. Depending on the type of ionic liquid, a 1.2 to 15.5-fold activity increase was observed relative to the sol–gel-encapsulated lipase without ionic liquid [8]. Both studies attribute the beneficial effect of the ionic liquids to pore-filling effects and enzyme protection from the alcohol released during the sol–gel procedure.

Acid phosphatase (APase) catalyzes the hydrolysis of monoesters and anhydrides of phosphoric acid to produce

S. Kulkarni · E. Chin · V. Tran · M. K.-M. Ho · C. Phan · M. Sommerhalter (✉)
Department of Chemistry and Biochemistry,
California State University, East Bay,
Hayward, CA, USA
e-mail: monika.sommerhalter@csueastbay.edu

inorganic phosphate [9]. APase acts on various substrates and can be used to improve the quality of feedstock or to recycle phosphate from sewage sludge [10]. Previously, APase has been successfully immobilized in tetramethyl orthosilicate (TMOS)-based xerogel monoliths in the presence or absence of the additive glucose [2]. Another study on the sol–gel encapsulation of acid and alkaline phosphatase showed that the presence of ionic surfactants can substantially extend the operating pH range of both phosphatase enzymes [4].

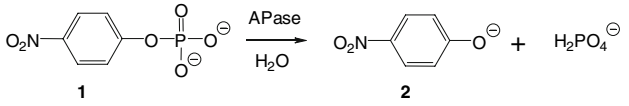
In this study, we explored the encapsulation of APase in TMOS-based hydrogel beads. Xerogels are fully dried, whereas hydrogels still retain fluid inside the pores of the sol–gel material. Casting the APase sol–gel material in the form of small beads can be advantageous for future up-scaling in bioreactors. We were also intrigued by the effect of the ionic liquid [BMIM][BF_4] on free and sol–gel-encapsulated APase. Because the [BMIM] cation bears resemblance to a short chain-length surfactant, we were particularly interested in studying the pH profile of the APase/[BMIM][BF_4]/sol–gel hybrid material.

Results and discussion

To measure the enzymatic activity of free and sol–gel-encapsulated APase, we employed kinetic and fixed-time assays based on the conversion of *p*-nitrophenylphosphate (**1**) to *p*-nitrophenolate (**2**) (Scheme 1).

The APase encapsulated in the hydrogel beads had enzymatic activity, albeit at a much lower level than free APase in solution. The activity of the APase hydrogel beads was <30% compared to that of free APase in solution. Four APase hydrogel beads (with 0.45 mg APase in total) hydrolyzed 0.027 ± 0.003 µmol of **1** per minute, corresponding to enzymatic activity of 0.060 ± 0.007 U/mg. The same amount of free APase in solution hydrolyzed 0.092 ± 0.003 µmol of **1** per minute, corresponding to an enzymatic activity of 0.204 ± 0.006 U/mg. In our kinetic assays we did not detect any lag-time with regard to the onset of the absorbance increase that detects the formation of **2**.

The apparent decline of APase activity upon sol–gel encapsulation can be caused by enzyme leakage, enzyme degradation, and/or the limited material transport properties of the sol–gel mesh. The buffer on top of the APase hydrogel beads was exchanged daily and measured for APase activity to determine whether any enzyme leaked from the sol–gel material. Typically, only 0.5–1.0% of APase leaked from the sol–gel matrix. To address the structural integrity of sol–gel-encapsulated APase we recorded circular dichroism (CD) spectra of APase free in solution and in the sol–gel-encapsulated form (Fig. 1). Both samples had very similar spectral features typical for alpha-helical proteins. Interpretation of the higher CD signal intensity of the sol–gel-encapsulated APase sample is problematical, because the sol–gel sheet in the CD cuvette shrinks during gelation as the hydrogel settles.



Scheme 1

A major advantage of sol–gel encapsulation of APase is the ability to re-use the enzyme in multiple catalytic cycles. The APase hydrogel beads were simply removed from the reaction mixture and washed three times with buffer before a new portion of **1** was added to start the next reaction cycle. This procedure was repeated in nine cycles over a period of three days without any significant loss in enzymatic activity. The activity of our sol–gel-encapsulated APase remained well above a 20% activity threshold relative to free APase in solution. The 20% activity threshold value corresponds to specific activity of 0.04 U/mg APase for sol–gel-encapsulated APase at pH 7.2 and room temperature. In a previous study that employed APase xerogel monoliths crushed into fine particles, only 7–22% enzymatic activity compared with that of free APase was achieved [2]. Wei et al. [2] discovered, however, that adding 42–60% *w/v* D-glucose to their sol–gel procedure increased the enzymatic activity of their samples by almost a factor of three.

Our additive choice ([BMIM][BF_4]) was stimulated by the report of a spectacular 30-fold activity increase for horseradish peroxidase encapsulated in a TEOS-based xerogel monolith in the presence of 12.5% *v/v* [BMIM][BF_4] [7]. Before introducing [BMIM][BF_4] as an additive into our sol–gel procedure, we tested the effect of [BMIM][BF_4] on free APase in solution. Figure 2 shows that the enzymatic

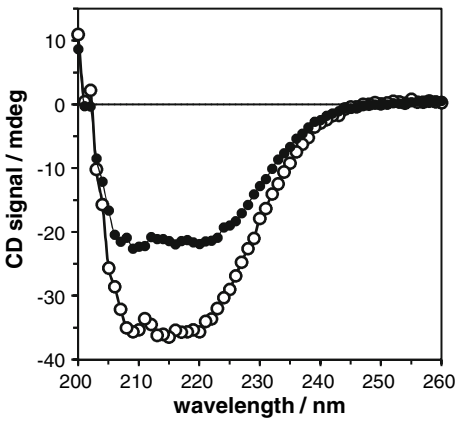


Fig. 1 CD spectra of sol–gel-encapsulated APase cast into a thin hydrogel sheet (*open circles*) and of free APase (*black circles*) in 2 mM Tris buffer, pH 7.2 with a final concentration of 0.56 mg/cm^3

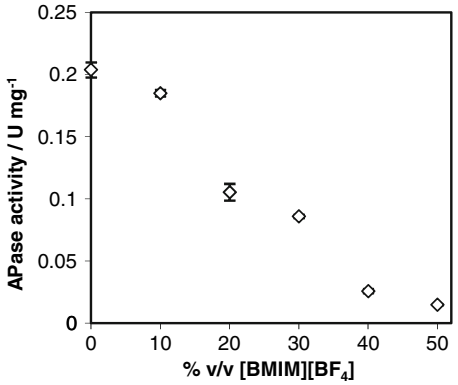


Fig. 2 Activity of free APase in 20 mM Tris buffer, pH 7.2 at room temperature with increasing amounts of [BMIM][BF_4]

activity of free APase declines with increasing amounts of [BMIM][BF_4]. At 10% *v/v* [BMIM][BF_4] this effect is still modest and we therefore decided to prepare our hydrogel beads with 10% *v/v* [BMIM][BF_4]. It should be noted that [BMIM][BF_4] also has an unfavorable effect on free horseradish peroxidase, albeit at higher concentrations of the ionic liquid. Machado et al. [11] reported that addition of 25% *v/v* [BMIM][BF_4] to horseradish peroxidase solution reduced the enzymatic activity to 25% of the original value. Nevertheless, [BMIM][BF_4] was a successful additive for the sol–gel encapsulation of horseradish peroxidase [7].

APase hydrogel beads with and without 10% *v/v* [BMIM][BF_4] were prepared in parallel to study the effect of the ionic liquid on the catalytic performance of the encapsulated enzyme (Fig. 3). Addition of 10% *v/v* [BMIM][BF_4] did not improve the catalytic performance of sol–gel-encapsulated APase. In fact, the enzymatic activity was slightly lower than the activity of sol–gel-encapsulated APase without the additive. After three catalytic cycles, the

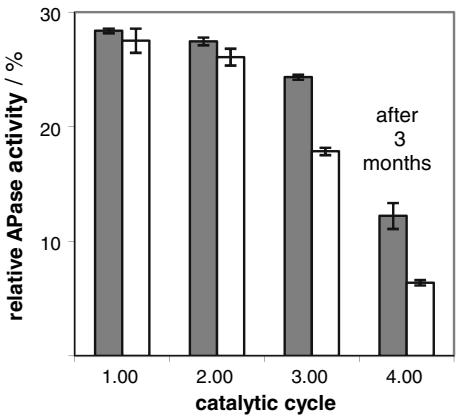


Fig. 3 Relative activity of sol–gel-encapsulated APase without any additive (*grey bars*) and with 10% *v/v* [BMIM][BF_4] (*white bars*) in 20 mM Tris buffer, pH 7.2, at room temperature. For normalization, the activity of free APase in 20 mM Tris, pH 7.2, at room temperature was set to 100%. Under these conditions, 1 mg free APase hydrolyzes 0.20 μmol **1** per minute

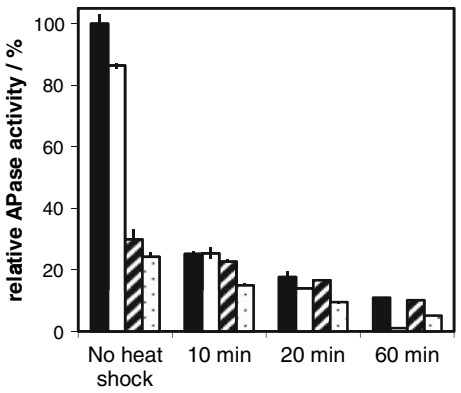


Fig. 4 Thermal deactivation at 60 °C of free APase (*black bars*), free APase with 10% *v/v* [BMIM][BF_4] (*white bars*), sol–gel-encapsulated APase (*black bars with white diagonal stripes*), and sol–gel-encapsulated APase with 10% *v/v* [BMIM][BF_4] (*white bars with black dots*)

activity of the APase hydrogel beads with 10% *v/v* [BMIM][BF_4] fell below a 20% activity threshold relative to free APase in solution. Nonetheless, this study showed that both hydrogel APase materials can be used after being stored for 3 months at 4 °C.

Next, we tested the heat-shock tolerance of free and sol–gel-encapsulated APase in the presence or absence of 10% *v/v* [BMIM][BF_4] (Fig. 4). This test showed that sol–gel-encapsulated APase without ionic liquid additive was most resistant to thermal deactivation. After 1 h of exposure to 60 °C, this sample retained 34% of its original activity. Its specific activity decreased from 0.059 ± 0.006 to 0.020 ± 0.001 U/mg. Under the same conditions, sol–gel-encapsulated APase with 10% *v/v* [BMIM][BF_4] retained 21% of its original activity. This corresponds to a decrease in specific activity from 0.048 ± 0.002 to 0.010 ± 0.001 U/mg. Both free APase samples retained much less of their original activity. The activities of free APase and free APase with 10% *v/v* [BMIM][BF_4] decreased to 11 and 1% of their original values, respectively. After 1 h of exposure to 60 °C, the free APase sample had a specific activity of 0.022 ± 0.001 U/mg and the free APase sample with 10% *v/v* [BMIM][BF_4] had a specific activity of 0.002 ± 0.0006 U/mg. All samples with 10% *v/v* [BMIM][BF_4] (in free or in sol–gel-encapsulated form) were consistently less active than their corresponding samples without additive. Encapsulation of APase in the confined cages of the sol–gel matrix, however, had a protective effect against the thermal denaturation of the enzyme.

Figure 5 compares the pH profiles for free APase, free APase with 10% *v/v* [BMIM][BF_4], sol–gel-encapsulated APase, and sol–gel-encapsulated APase with 10% *v/v* [BMIM][BF_4]. All measurements are based on fixed-time assays as described in the Experimental section. The pH optimum for all four APase samples remained close to pH 6.

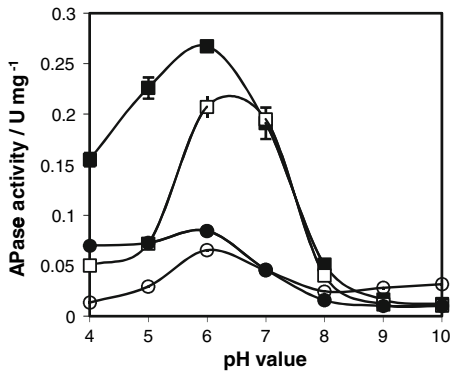


Fig. 5 pH profile for free APase (*solid squares*), free APase with 10% *v/v* [BMIM][BF_4] (*empty squares*), sol–gel-encapsulated APase (*solid circles*), and sol–gel-encapsulated APase with 10% *v/v* [BMIM][BF_4] (*empty circles*)

Addition of 10% *v/v* [BMIM][BF_4] to free or sol–gel-encapsulated APase reduced the catalytic performance at more acidic pH values. Notably, the combination of sol–gel encapsulation and ionic liquid addition enhanced the catalytic performance of APase in the basic pH region. At pH 10, sol–gel-encapsulated APase with 10% *v/v* [BMIM][BF_4] had enzymatic activity of 0.031 ± 0.001 U/mg, whereas all other samples had values at or below 0.012 U/mg. Extension of the operating pH range was also observed in a study on the sol–gel encapsulation of acid and alkaline phosphatase in the presence of ionic surfactants [4]. Sol–gel encapsulation of APase in the presence of the cationic surfactant cetyltrimethylammonium bromide rendered APase active at pH 13, whereas the anionic surfactant sodium bis(2-ethylhexyl)sulfosuccinate caused alkaline phosphatase to be active at pH 1 [4]. These changes in the pH tolerance of the two phosphatase enzymes were attributed to ionic interactions between the enzymes, the surfactants, and the solid sol–gel matrix. At alkaline pH we also observed a synergistic effect of spatial confinement and addition of an ionic compound. Addition of [BMIM][BF_4] caused a remarkable increase in alkaline pH tolerance of sol–gel-encapsulated APase.

To summarize, our sol–gel encapsulation process turned APase into a biocatalyst that can be easily recovered from the reaction mixture and re-used multiple times. However, in comparison with free APase in solution, the activity of the encapsulated enzyme diminished. Although addition of the water-soluble ionic liquid [BMIM][BF_4] did not help to recover the lost activity under the typical neutral to acidic operating conditions of APase, it extended the operating pH range of APase to alkaline conditions.

Experimental

The enzyme APase from wheat germ was purchased from Sigma as a brown powder. The specific activity of this starting material was 0.4 units/mg solid. According to the vendor, one unit of enzyme hydrolyzes one μmol *p*-nitrophenylphosphate per minute at pH 4.8 and 37 °C. The ionic liquid [BMIM][BF_4] was procured from Fluka. Water was purified and de-ionized with a Millipore Milli-Q filtration system (dH_2O). All other reagents were obtained from commercial suppliers (Fisher Scientific, Sigma).

The preparation of the APase hydrogel beads was similar to a procedure described by O' Neill et al. [3]. To measure the enzymatic activity of free and sol–gel-encapsulated APase, kinetic and fixed-time assays that monitor the hydrolysis of the substrate *p*-nitrophenylphosphate were employed.

APase sol–gel encapsulation

To prepare the APase hydrogel beads TMOS, dH_2O, and 0.01 M hydrochloric acid were mixed in the ratio 50:10:1 by volume and sonicated for 20 min in an ice–water bath. The resulting homogeneous sol was divided into 28 mm^3 portions. To each portion 74 mm^3 20 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.2, 22 mm^3 dH_2O, and 100 mm^3 5 mg APase/cm^3 dissolved in 20 mM Tris, pH 7.2 were added. After vortex mixing, the solution was pipetted as 50 mm^3 drops on to parafilm sheets. The drops condensed into hydrogel beads in about 2 h. These beads were transferred into test tubes containing 20 mM Tris, pH 7.2. The APase hydrogel beads were stored at 4 °C, and the buffer on top of the beads was exchanged daily for three consecutive days. APase hydrogel beads containing 10% *v/v* [BMIM][BF_4] were prepared as described above, except that 22 mm^3 ionic liquid was added in place of dH_2O. All samples were prepared in triplicate.

Enzyme activity assays

Kinetic assays were performed in 20 mM Tris buffer, pH 7.2 using **1** as substrate. The pH value of 7.2 represents a compromise between the catalytic activity of APase (with an acidic pH optimum) and strong color development. Only the deprotonated form of the product, **2**, with a pK_A of 7.2, is yellow. Its extinction coefficient was determined to be 12.0 ± 0.2 mM^{-1} cm^{-1} at pH 7.2 and 410 nm. Because kinetic assays monitor the progress of the enzyme-catalyzed reaction continuously, it is possible to detect lag-times that occasionally occur in reactions catalyzed by encapsulated enzymes. To conduct assays on free APase, a stock solution of 5 mg/cm^3 APase was diluted in 20 mM Tris buffer pH 7.2 to a final volume of 2.4 cm^3. The reaction was initiated by adding 0.6 cm^3 50 mM stock solution of **1** to the APase sample and absorbance readings were taken every 15–30 s at 410 nm with a Milton Roy

Spectronic 20D digital UV–visible spectrophotometer. Our blank, a control sample without APase, showed no measurable increase in absorbance at 410 nm. To conduct assays on sol–gel-encapsulated APase, four APase sol–gel beads containing 0.45 mg APase in total were placed as wet hydrogel beads in a test tube with 2.4 cm^3 20 mM Tris buffer, pH 7.2. The reaction was initiated by adding 0.6 cm^3 50 mM stock solution of **1**. The contents of the test tube were gently mixed by inversion between the absorbance readings that were recorded every 30 s at 410 nm. As control, we prepared sol–gel beads without APase. A blank prepared with these empty beads showed no measurable increase in absorbance at 410 nm. All samples were prepared in triplicate. The standard deviation did not exceed 20% of the mean value. APase activity is reported in U/mg. One U/mg corresponds to the hydrolysis of 1 μmol *p*-nitrophenylphosphate/min by 1 mg APase.

All samples for the thermal deactivation study were subjected to heat shock by placing them in a 60 °C water bath for varying periods of time. The heat-shocked samples were dipped into a 25 °C water bath for 5 min and kinetic assays were conducted immediately thereafter at room temperature.

Fixed-time assays were used to determine the pH profile of APase. Each test tube contained 500 mm^3 buffer at a given pH, 500 mm^3 15.2 mM **1**, and 100 mm^3 APase sample. The amount of free APase was 0.05 mg per test tube and the amount of sol–gel-encapsulated APase was 0.225 mg distributed in two hydrogel beads each with a volume of 50 mm^3. The buffers used were: 50 mM acetate, pH 4.0, 50 mM acetate, pH 5.0, 50 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.0, 50 mM Tris, pH 7.0, 50 mM Tris, pH 8.0, 50 mM 2-(*N*-cyclohexylamino)ethanesulfonic acid, pH 9.0, and 50 mM 3-(*N*-cyclohexylamino)propanesulfonic acid, pH 10.0. After 10 min, 4 cm^3 100 mM NaOH was added, and the absorbance at 410 nm was recorded. Under these conditions (pH 12) the extinction coefficient of **2** at 410 nm is 18.3 ± 0.2 mM^{-1} cm^{-1}.

Circular dichroism

To prepare an APase hydrogel sample that fits into a 1-mm circular dichroism (CD) cuvette, 3.5 cm^3 sol–gel solution were gently mixed with 5.25 cm^3 APase stock solution containing 0.93 mg/cm^3 APase in 2 mM Tris, pH 7.2. This mixture was cast into a plastic cassette (Novex/Invitrogen) 1 mm thick and the cassette was sealed with parafilm. The sol–gel solution was prepared by sonicating 7.37 cm^3 TMOS, 0.92 cm^3 dH_2O, and 0.88 cm^3 0.01 M hydrochloric acid for 20 min in an ice–water bath. The final protein concentration after dilution with the sol–gel solution was 0.56 mg/cm^3. A sample containing 0.56 mg/cm^3 free APase in 2 mM Tris buffer pH 7.2 was prepared from the same APase stock solution and at the same time as the sol–gel sample. Both samples were stored for 2 weeks at 4 °C. Shortly before the CD measurement, the cassette housing the APase sol–gel was opened. A small rectangle was cut with a razor blade and transferred into a CD cuvette. Gelation of the APase sol–gel resulted in a slightly shrunk hydrogel sheet that easily fit into a CD cuvette 1 mm thick. The same cuvette was used for the APase solution sample. CD spectra were recorded with an AVIV Model 215 circular dichroism spectrometer in 1 nm increments. The temperature was set to 25 °C and controlled with a thermostat.

Acknowledgments The principal investigator (Monika Sommerhalter) received a Faculty Research Grant and an Interdisciplinary Sieber-Tombari Research Award from California State University, East Bay. The first author (Suhasini Kulkarni) received a graduate student research grant from California State University, East Bay. The second author (Elizabeth Chin) gratefully acknowledges financial support from the CSU-LSAMP program which is supported by the National Science Foundation under Grant No. HRD-0331537. CD measurements were performed in the laboratory of Professor Daryl Eggers at San Jose State University. We thank Dr Eggers for his kind hospitality and support during the measurements.

References

1. Gill I (2001) Chem Mater 13:3404
2. Wei Y, Xu JG, Feng QW, Lin MD, Dong H, Zhang WJ, Wang C (2001) J Nanosci Nanotechnol 1:83
3. O'Neill H, Angley CV, Hemery I, Evans BR, Dai S, Woodward J (2002) Biotechnol Lett 24:783
4. Frenkel-Mullerad H, Avnir D (2005) J Am Chem Soc 127:8077
5. Kragl U, Eckstein M, Kaftzik N (2002) Curr Opin Biotech 13:565
6. Karout A, Pierre AC (2007) J Non-Cryst Solids 353:2900
7. Liu Y, Wang MJ, Li J, Li ZY, He P, Liu HT, Li JH (2005) Chem Commun 1778
8. Lee SH, Doan TTN, Ha SH, Koo YM (2007) J Mol Catal B Enzym 45:57
9. Joyce BK, Grisolia S (1960) J Biol Chem 235:2278
10. He ZQ, Griffin TS, Honeycutt CW (2004) J Environ Qual 33:367
11. Machado MF, Saraiva JM (2005) Biotechnol Lett 27:1233