J. Appl. Biol. Chem. **51**(4), 143-147 (2008)

Article

Enzymatic Properties of Atrazine Chlorohydrolase Entrapped in Biomimetic Silica

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Received May 14, 2008; Accepted July 14, 2008

Purified atrazine chlorohydrolase (AtzA) was entrapped in the nanoparticles of biomimetically synthesized silica at the ambient condition within 20 min. Entrapped AtzA in biomimetic silica was less affected by pH change and showed higher thermostability than free enzymes. The entrapped AtzA was also more tolerant against proteolysis, with 80% of the initial activity remaining and retained 82% of the initial activity even after four cycles of usage. These results suggest that entrapment of AtzA in biomimetic silica could be utilized under diverse environmental conditions with the active catalytic performance sustained.

Key words: *atrazine chlorohydrolase, biodegradation, biomimetic silica, entrapment.*

Tandem repeat units of the R5 peptide [SSKKSGS YSGSKGSKRRIL] present in the silaffin protein of *Cylindrotheca fusiformis* have been demonstrated to catalyze the formation of silica from TMOS under ambient conditions [Brott *et al.*, 2001; Kroger *et al.*, 1999]. An interesting characteristic of this silica matrix is that silica-precipitating peptides were found to be entrapped during its generation [Naik *et al.*, 2003; Naik *et al.*, 2004]. The observation of this phenomenon has led to further studies investigating the entrapment of the exogenously added enzymes such as butyrylcholinesterase in a silica matrix through the execution of biosilicification reactions [Luckarift *et al.*, 2004].

Atrazine is an herbicide with an average application of the active ingredient reaching 60 million pounds per year to the corn fields in the United States [USDA Agricultural Chemical Usage, 2006]. The prolific usage of this herbicide has resulted in an extensive contamination of the surface and the ground water sources [Graziano *et al.*, 2006; Jiang *et al.*, 2006; Shipitalo and Owens, 2003]. Mandelbaum *et al.* [1995] isolated a *Pseudomonas* sp. ADP capable of mineralizing atrazine, and De Souza *et al.* [1996] characterized AtzA, which catalyzes the formation of non-toxic, water-soluble hydroxyatrazine through dechlorination reaction. The AtzA has been investigated for applications in water treatment through immobilization in the sol-gel silica matrix [Kauffmann and Mandelbaum, 1996; 1998] and in cellulose [Kauffmann *et al.*, 2000]; although the immobilized AtzA retained 40% of the free enzyme activity, the effects of environmental factors such as temperature, pH, and proteolysis on the enzyme activity have not yet been investigated.

In the present study, a purified AtzA was entrapped into the biomimetic silica nanopartices utilizing the R5 peptide in a solution containing TMOS, and the effects of pH and protease treatment on enzyme activity, thermostability, and reusability of the entrapped AtzA were investigated to study the efficiency of entrapping AtzA in the biomimetic silica and feasibility of application of the technique under the environmental conditions.

Materials and Methods

Chemicals and bacterial strains. Atrazine (Novartis Animal Health, Greensboro, NC) and *E. coli* DH5α (pMD4) containing atrazine chlorohydrolase gene *atzA* [De Souza *et al.*, 1995] were generously provided by Professor Michael J. Sadowsky of the University of

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Abbreviations: Atrazine, 2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine; AtzA, atrazine chlorohydrolase; MOPS, 3-(*N*-morpholino)-propanesulfonic acid sodium salt; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TMOS, tetramethyl orthosilicate

doi:10.3839/jabc.2008.024

Minnesota, St. Paul, MN. Tetramethyl orthosilicate (99%) and TRIZMA base (tris-hydroxymethylaminomethane) were purchased from Aldrich (St. Louis, MO). Silica, protease (P 8811), phenylmethanesulfonyl fluoride (>99%), and MOPS (99.5%) were purchased from Sigma (St. Louis, MO). R5 peptide was synthesized by AnyGen (Gwangju Institute of Science and Technology, Gwangju, Korea).

Purification and activity of AtzA. *E. coli* DH5α was cultured for 12 h in a 5-L fermentor (New Brunswick, Edison, NJ) containing 4 L of LB medium and 25 μg mL^{-1} chloramphenicol. The cell culture was centrifuged, and the resulting cell pellet washed three times with 0.85% NaCl. The AtzA was purified using the modified method described by De Souza *et al.* [1996]. Ammonium sulfate precipitation was done at 20-40% saturation, instead of 20%, and Acta system (Pharmacia, Piscataway, NJ) with Hiprep 11/16 Q FF was used for anion exchange chromatography. Purity of the purification steps was determined by SDS-PAGE. Concentration of AtzA was determined by the Bradford protein assay (Biorad, Hercules, CA).

Entrappment of AtzA in biomimetic silica. TMOS (1 M) in 1 mM HCl and R5 peptide (5 mg mL^{-1} dissolved in phosphate buffer, pH 7.0) were mixed at a volume ratio of 1:5 and incubated for 20 min in Eppendorf tubes. During the formation of biomimetic silica by the R5 peptide, 30 μL of the purified AtzA (3.3 mg mL^{-1}) was added to the reaction mixture. The supernatant was removed by centrifugation at 16,000 g for 5 min at 4°C. Precipitated material was washed three times with deionized water and tested for the AtzA activity. After hydrolyzing the AtzA-containing biomimetic silica with 1 N NaOH for 10 min at room temperature, the presence of AtzA in the biomimetic silica matrix was detected by SDS-PAGE [Luckarift *et al.*, 2004; Naik *et al.*, 2004]. To investigate the possibility of adsorption, 100 μg AtzA were incubated with the pre-formed biomimetic silica for 20 min, followed by the separation of the supernatant and biomimetic silica pellets via centrifugation at 16,000 g for 5 min. Both the supernatant and the biomimetic silica pellets were tested for the AtzA activity.

Scanning electron microscopy. Biomimetic silica precipitate was washed with distilled water, mounted onto a carbon tape secured to the aluminum stub, and dried at room temperature. Images were obtained at 15 kV using the Hitachi S-4700 scanning electron microscope (Tokyo, Japan).

Enzyme assay. To determine the AtzA activity, 0.5 mL of the reaction mixture containing 30 μL of AtzA (3.3 mg mL^{-1}) and atrazine (100 mg L^{-1}) in 25 mM MOPS buffer (pH 7.0) was incubated at room temperature for 30 min. Methanol (0.5 mL) was added to the reaction vials and kept at −70°C for 4 h to denature any remaining proteins. The mixture was then centrifuged at 16,000 g for 5 min and filtered using a polyvinylidenedifluoride Whatman filter (0.2 μm). The filtrate was analyzed with a Shimadzu HPLC system. One unit (U) of the enzyme activity was defined as the amount of enzyme that degrades 1 μmol of atrazine per min. A standard curve was prepared in the range of 20-150 mg L^{-1}.

Properties of entrapped AtzA in biomimetic silica. To investigate the effect of pH on AtzA activity, free and entrapped AtzAs were incubated with different buffer solutions (pH 5, 6, 7, 8, and 9) for 30 min at room temperature, and the activities were determined by the standard activity assay method described in Materials and Methods. For the thermostability study of free and entrapped AtzAs, both enzymes were incubated at 60°C for 30 min, and the residual activities were determined.

For the study of protease treatment, the free and the entrapped AtzAs were treated with 30 μg of protease for 30 min at room temperature, and the residual activities were determined. In order to investigate the reusability of the entrapped AtzA, the activities of the entrapped AtzA of both the supernatant and the biomimetic silica pellet were determined four times consecutively with 20 min standing between each cycle. Enzymatic activities of the free and the entrapped AtzA were determined as the relative activity (%) compared to that of the free AtzA under the standard activity assay conditions.

Results and Discussions

Entrapment of purified AtzA into biomimetic silica. AtzA was purified from the culture supernatant through ammonium sulfate precipitation (20 to 40%) and anion exchange chromatography. The purified enzyme showed a 55 kDa band on SDS-PAGE (data not shown), and the molecular weight was very similar to the results of De Souza *et al.* [1996]. The specific activity of the purified AtzA was 0.086 U mg^{-1}, similar to the previously published result of 0.05 U hydroxyatrazine mg^{-1} [Kauffmann and Mandelbaum, 1998]. The synthesis of biomimetic silica and the entrapment of AtzA (100 μg) were carried out at room temperature and pH 7 for 20 min. Under the reaction condition, 1 mg of biomimetic silica was synthesized, and the supernatant did not show AtzA activity, suggesting that all AtzAs were entrapped. In agreement with the findings of the previous reports [De Souza *et al.*, 1996; Kauffmann and Mandelbaum, 1998], the AtzA band was detected only from the hydrolyzed fraction of the biomimetic silica on SDS-PAGE (Fig. 1), confirming that AtzA was entrapped into the biomimetic

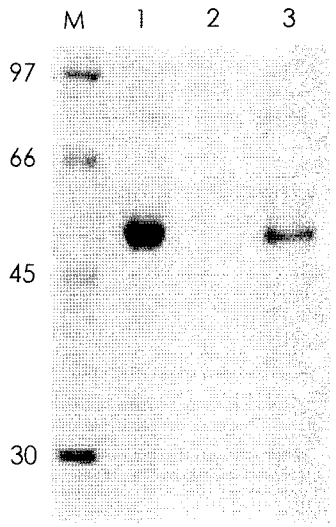

Fig. 1. Denaturing PAGE with AtzA obtained after hydrolyzing the entrapped AtzA in the biomimetic silica by sodium hydroxide. Lanes: M, marker; 1, free AtzA; 2, biomimetic silica; 3, entrapped AtzA.

silica. Additionally, when the synthesized biomimetic silica was incubated with the free AtzA, most of the activity was detected in the supernatant (97%), whereas the biomimetic silica fraction showed no activity. These findings indicated that the immobilization of AtzA into the biomimetic silica is not a simple adsorption-driven process as proposed previously [Luckarift *et al.*, 2004]. The specific activity of the entrapped AtzA was 0.034 U mg^{-1}, corresponding to 40% of the free enzyme activity. Although the activity was not much higher than the specific activity of the immobilized AtzA in the conventional sol-gel (0.02 U hydroxyatrazine mg^{-1}) [Kauffmann and Mandelbaum, 1998], the limiting factors appeared to be those other than the methanol release and hydrophobic silane effect, which decreased the enzyme performance when the sol-gel method was used [Kauffmann and Mandelbaum, 1996; 1998]. When the entrapment was carried out with different amounts of AtzA (100 to 300 μg) under the same reaction conditions, the maximum amount of AtzA entrapped was 200 μg, and the specific activity of the higher amount of entrapped AtzA was very similar to the free AtzA activity (data not shown). Butyrylcholinesterase showed a very similar loading efficiency with 220 mg g^{-1} biomimetic silica (20% w/w) [Luckarift *et al.*, 2004], much higher than those entrapped through the conventional sol-gel methodology [0.1-5% (w/w)]. The SEM images of the precipitated pellets of the biomimetic silica showed a well-organized matrix structure of nanoparticles with particle sizes that ranged from 100 to 300 nm in diameter (Fig. 2). Knecht and Wright [2003] reported that immobilization of the enzyme in the biomimetic silica

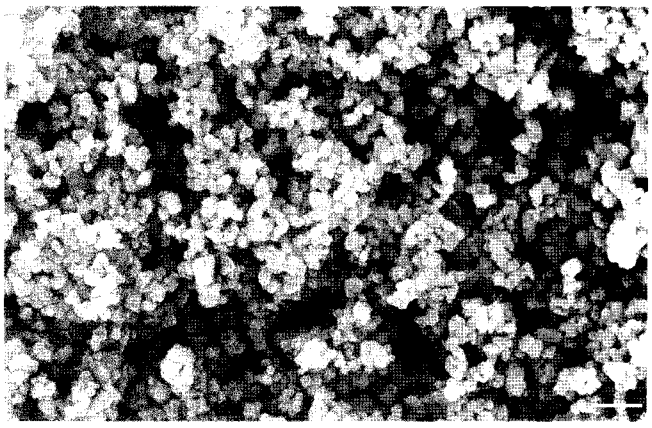

Fig. 2. SEM images of biomimetic silica nanoparticles entrapping AtzA, scale bar 1 μm.

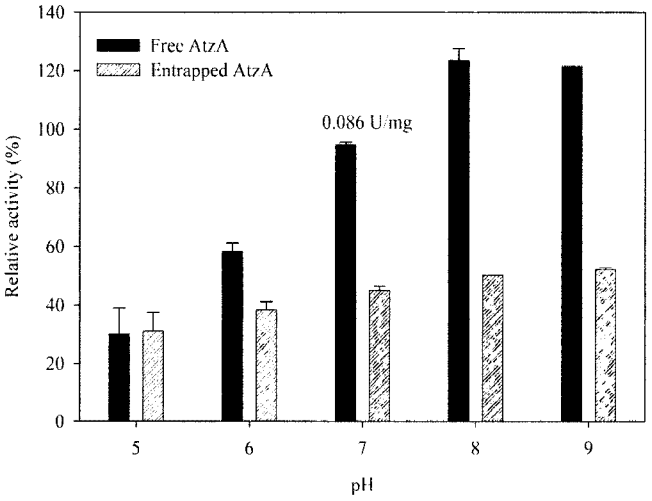

Fig. 3. Effects of different pH on the relative activities of free and entrapped AtzAs.

slightly affected the matrix structure, which enabled the small silica nanoparticles to form aggregates; this finding indicated that the matrix of the aggregates turned into a barrier to lower the activity of the enzyme entrapped through the biomimetic silica method.

Effects of pH, temperature, reusability, and protease on enzymatic activity of entrapped AtzA in biomimetic silica. Based on the chemical reactions under the environmental conditions, the enzymatic activities of the entrapped AtzA were investigated at pH ranges from 5 to 9. The free AtzA and the entrapped AtzA had unusually high optimal pH at 8.0 and at 9.0, respectively (Fig. 3). Activity of the free AtzA at pH 5 was approximately 32% of that at pH 7, indicating the sensitivity of the free AtzA to acidic conditions. In contrast, the entrapped AtzA kept 66% of activity at pH 5.0, suggesting that the entrapped AtzA is less affected by pH change. Figure 4 shows the thermostabilities of the free and the entrapped AtzAs after heat treatment at 60°C

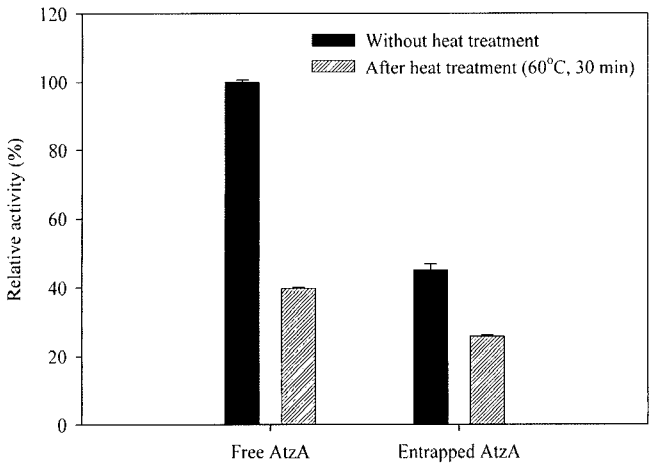

Fig. 4. Effects of heat treatment at 60°C for 30 min on the relative activities of free and entrapped AtzAs.

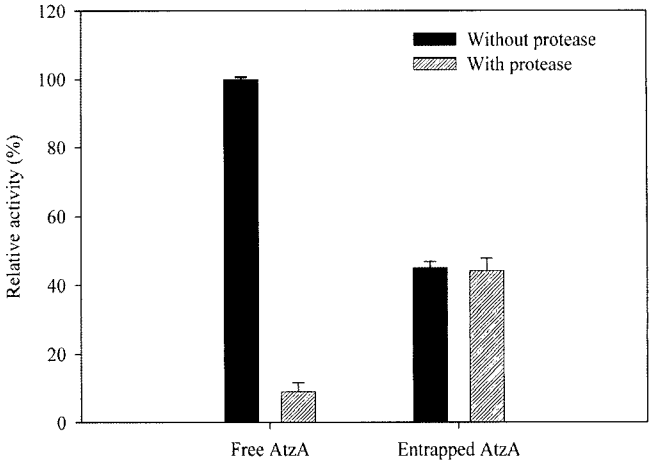

Fig. 5. Effects of proteolysis activity on the relative activities of free and entrapped AtzAs.

for 30 min; the entrapped AtzA lost 37% of the initial activity, whereas the free AtzA lost 56%. This improved thermostability of the enzymes on the biomimetic silica is possibly due to the stabilizing effect of the support matrix through the prevention of conformation changes of the bound enzymes as suggested by Shtelzer *et al.* [1992] and Luckarift *et al.* [2004].

Enzymes decompose (or are inactivated) very rapidly when added to soils [Bremner and Mulvaney, 1978]. Moreover, Ladd and Butler [1972] demonstrated that, when exposed to diverse environmental conditions, a substantial proteolytic degradation of the enzymes in soils occurred. Therefore, in the present study, the protection effect of biomimetic silica on AtzA against the protease treatment was evaluated (Fig. 5). Even after incubation of the entrapped AtzA with protease at room temperature for 30 min, 78% of the initial activity was retained, as compared to the 16% of the free AtzA. Wang and Caruso [2004] also reported a similar protective effect of the encapsulation protocol with catalase. The results of the present suggest that the biomimetic silica matrix could directly protect AtzA from proteolysis under the environmental conditions. The factors allowing the immobilized enzymes to be more favorable than the free enzymes are the potential for reusability and the long-term usage [Luckarift *et al.*, 2004]. In the present study, the entrapped AtzA repeatedly used up to four times still kept 82% of the initial activity after the cycle. The AtzA activity was not detected in the washing solutions, suggesting that loss of particles or inactivation of AtzA, rather than dislodgement of AtzA from the biomimetic silica particles into the solution, may be the main reason for the decreased activity after reuse. Luckarift *et al.* [2004] also reported that the entrapped butyrylcholinesterase in the biomimetic silica in a continuous flow-through system retained almost 100% enzymatic activity. In contrast to the biomimetic silica, previous studies have indicated that the entrapped crude extract of AtzA in a conventional sol-gel matrix resulted in a significant loss of AtzA activity after four cycles [Kauffmann and Mandelbaum, 1996]. In the present study, the free and the entrapped AtzAs stored for one month at room temperature sustained 79 and 87% of the initial activity, respectively.

In conclusion, AtzA, which catalyzes the formation of non-toxic, water-soluble hydroxyatrazine through dechlorination reaction, was easily entrapped in the biomimetic silica within 20 min and the entrapment resulted in an increased enzymatic performance at lower pH and thermosatbility. The biomimetic silica matrix could directly protect the enzyme from the proteolytic activity. Even after the fourth reuse, the entrapped AtzA retained 82% of the initial activity. Those results suggest that biomimetic silica produced by R5 peptide under ambient conditions could provide promising tools for the environmental application of AtzA.

Acknowledgments. This study was supported by National Core Research Center program (Grant #: R15-2003-012-02002-0). We thank Dr. Kenneth Widmer (International Environmental Research Center, Gwangju Institute of Science and Technology, Korea) for his assistance in editing this manuscript.

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