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Acrylamide production using encapsulated nitrile hydratase from *Pseudonocardia thermophila* in a sol–gel matrix

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ABSTRACT

The cobalt-type nitrile hydratase from *Pseudonocardia thermophila* JCM 3095 (*Pt*NHase) was successfully encapsulated in tetramethyl orthosilicate sol–gel matrices to produce a *Pt*NHase:sol–gel biomaterial. The *Pt*NHase:sol–gel biomaterial catalyzed the conversion of 600 mM acrylonitrile to acrylamide in 60 min at 35 °C with a yields of >90%. Treatment of the biomaterial with proteases confirmed that the catalytic activity is due to the encapsulated enzyme and not surface bound NHase. The biomaterial retained 50% of its activity after being used for a total of 13 consecutive reactions for the conversion of acrylonitrile to acrylamide. The thermostability and long-term storage of the *Pt*NHase:sol–gel are substantially improved compared to the soluble NHase. Additionally, the biomaterial is significantly more stable at high concentrations of methanol (50% and 70%, v/v) as a co-solvent for the hydration of acrylonitrile than native *Pt*NHase. These data indicate that *Pt*NHase:sol–gel biomaterials can be used to develop new synthetic avenues involving nitriles as starting materials given that the conversion of the nitrile moiety to the corresponding amide occurs under mild temperature and pH conditions.

1. Introduction

Nitriles are used extensively to produce a broad number of specialty chemicals containing amines, amides, amidines, carboxylic acids, esters, aldehydes, ketones, and heterocyclic compounds [1,2]. These compounds are used in a wide array of reactions as chemical feedstocks for the production of solvents, extractants, pharmaceuticals, drug intermediates, pesticides (dichlobenil, bromoxynil, ioxynil, buctril), and polymers [1]. For example, acrylonitrile and adiponitrile are used in the production of polyacrylamide and nylon-66, respectively, the latter of which is one of the most important industrial polyamides derived from petroleum feedstocks [3–6]. However, the harsh industrial conditions needed to hydrate nitriles to their corresponding amides (either acid or base hydrolysis), are often incompatible with the sensitive structures of many industrially and synthetically relevant compounds, which decreases product yields and consequently increases production costs [2,4]. There are two metabolic pathways found in microorganisms for nitrile degradation (Scheme 1). In the first pathway, the nitrile is directly hydrolyzed to their corresponding carboxylic acid and ammonia by nitrilase (EC 3.5.5.1) [3]. In the second pathway, nitriles are first hydrated to their corresponding amide by nitrile hydratase (EC 4.2.1.84), then the amide is subsequently hydrolyzed to carboxylic acid and ammonia by amidase (EC 3.5.1.4) [3].

Microbial nitrile hydratase (NHase) has great potential as a biocatalyst for organic chemical processing because of its ability to convert nitriles to amides under physiological conditions. However a major issue in the use of enzymes, in general, and NHases specifically in organic synthetic processes is the difficulty in separating the enzyme from the synthetic reaction mixture [7]. A related issue involves the use of aprotic solvents in organic synthetic reaction mixtures, which renders most enzymes including NHase inactive [7–9]. One way to overcome both of these hurdles is through the encapsulation of enzymes within silica glasses derived through sol–gel processing [10–13]. Encapsulated enzymes have resulted in the generation of novel functional materials that are optically transparent and sufficiently porous to permit small substrates access to the entrapped enzyme [11,14–19]. Recent studies have demonstrated that encapsulated proteins retain their solution structure and native function while residing in the hydrated pore of the sol–gel [11,20–22]. Moreover,

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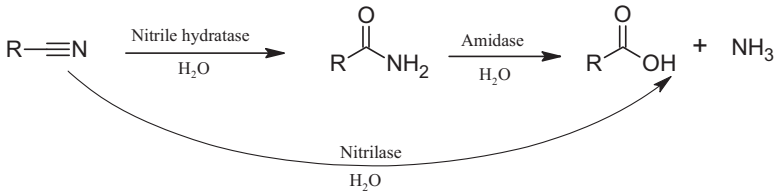

Scheme 1.

nanoscopic confinement within sol–gels stabilizes proteins against thermal and proteolytic degradation [11,17]. These physical properties permit the broad application of sol–gel:protein materials as chemical sensors, separation media, and heterogeneous catalysts [23].

For industrial applications (i.e. acrylamide production) whole cells containing NHase and some isolated, purified, NHases have been immobilized on various supports by means of adsorption, entrapment, cross-linking, and membrane immobilization [4]. While the immobilization of whole cells containing NHase have been successful for the production of various commodity chemicals, purified NHases are necessary for the specific hydration of nitriles with other hydrolysable groups that will be susceptible to side-reactions within a bacterial cell. In addition, reaction systems that cannot tolerate carboxylate side products also require purified NHase since other enzymes in the bacterial nitrile degradation pathway, such as nitrilases can convert nitriles to carboxylates [24]. Purified enzymes also eliminate the need to have nitrile substrates pass across cell membranes; this would otherwise decrease the yields of recoverable products [4,25]. Herein we report the immobilization of the Co-type NHase from *Pseudonocardia thermophila* JCM 3095 (*Pt*NHase) using the sol–gel process in tetramethyl orthosilicate (TMOS) gels. This novel biocatalytic material is capable of specifically hydrating acrylonitrile to acrylamide in high yields and under mild conditions.

2. Experimental

2.1. Materials

Tetramethyl orthosilicate (TMOS, ≥99%), acrylonitrile, acrylamide, Type I Trypsin from bovine pancreas, and Type II α-chymotrypsin from bovine pancreas, were purchased from Sigma–Aldrich. All reagents were of the highest purity available and used as received without further purification.

2.2. Plasmid construction

A pUC18-NHase plasmid encoding the α-subunit and β-subunit genes of *Pt*NHase [26,27], was used as a template for the polymerase chain reaction (PCR). A polyhistidine (His_6) affinity tag was engineered onto the C-terminus of the α-subunit gene using Phusion DNA polymerase. The following primers were used for the PCR: forward 5′-GCC ATG GGA AAC GGC GTG TAC GAC GTC GGC GG-3′ and reverse 5′-GGT ACC AAG CTT TCA ATG ATG ATG ATG ATG ATG CGC GAC CGC CTT-3′. The PCR product (βα-His_6 genes) was subcloned into the pSC-B-amp/kan vector using the Strataclone Blunt PCR cloning kit. The βα-His_6 genes were subsequently subcloned between the *Nco*I and *Hind*III restriction sites of the kanamycin resistant pET28a$^+$ to create the *Pt*-His_6/pET28a$^+$ plasmid. The nucleotide sequence for the *Pt*NHase activator gene was obtained from the GeneBank, ID HV233497.1, and used to synthesize the gene with *E. coli* codon usage and cloned into a pIDT-SMART (Integrated DNA Technologies, Inc.) ampicillin resistant vector with *Nde*I and *Hind*III restriction sites. Subsequently, the *Pt*NHase activator gene was inserted between the *Nde*I and *Hind*III restriction sites of the ampicillin resistant pET21a$^+$ vector to create the *Pt*-activator/pET21a$^+$ plasmid. All plasmid sequences were confirmed using the automated DNA sequencing at the University of Chicago Cancer Research Center DNA sequencing facility.

2.3. Expression and purification of recombinant PtNHase

The *Pt*-His_6/pET28a$^+$ and *Pt*-activator/pET21a$^+$plasmids were co-transformed into *E. coli* BL21(DE3) competent cells (Stratagene) for expression. A 100 mL LB-Miller starter culture was inoculated from a single colony with 50 μg/mL and 100 μg/mL of kanamycin and ampicillin, respectively. A 6 L culture was inoculated from this starter culture using 7 mL per liter and grown at 37 °C until an $OD_{600\,nm}$ of 0.6–0.8 was reached. The culture was cooled to 20 °C, induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), supplemented with 0.25 mM $CoCl_2$ [28,29], and expressed at the same temperature for 16–18 h. Cells were harvested by centrifugation at 6370 × *g* 4 °C, for 5 min. The cells were resuspended at 3 mL per gram of buffer A (50 mM NaH_2PO_4, pH 7.5, 500 mM NaCl, 10 mM imidazole) then lysed by sonication on ice for 8 min (30 s on, 45 s off) using a 21W Misonex sonicator 3000. Cell debris was removed by two rounds of centrifugation at 31,000 × *g*, 4 °C, for 20 min. The protein was purified by immobilized metal affinity chromatography (IMAC) using Ni-NTA (nickel-nitrilotriacetic acid) Superflow Cartridges (Qiagen). The column was equilibrated with buffer A and the crude extract was loaded onto the column. Unbound protein was washed with 15 column volumes (CV) of buffer A with 25 mM imidazole. The protein was eluted with a linear gradient (0–100%) of buffer B (buffer A with 500 mM imidazole) over 20 CVs at a flow rate of 2 mL/min. Peak fractions were pooled, resuspended in buffer C (50 mM Tris–HCl pH 7.5, 300 mM NaCl) and concentrated with an Amicon Ultra-15 centrifugal filter device with a molecular weight cutoff (MWCO) of 30,000 (Millipore). The purity of *Pt*NHase was analyzed by using 12.5% SDS-PAGE. The protein concentration was determined by UV absorbance at 280 nm using a calculated molar extinction coefficient of 174, 640 $M^{-1}\,cm^{-1}$ and the Coomassie (Bradford) Protein Assay Kit (Thermo Scientific Pierce). The calculated molecular mass of the heterotetramer was 101 kDa. Theoretical molecular mass and protein extinction coefficients were calculated with the ExPASy ProtParam tool [30].

2.4. Kinetic characterization of PtNHase

The activity of purified *Pt*NHase was determined by measuring the hydration of 100 mM acrylonitrile to acrylamide (225 nm, $\varepsilon = 2.9\,mM^{-1}\,cm^{-1}$) in 50 mM Tris–HCl, pH 7.5 at 35 °C. Assays were performed in a 1 mL quartz cuvette in triplicate on a Shimadzu UV-2450 PC spectrophotometer equipped with a TCC temperature controller. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the production of 1 μmol of acrylamide per minute at 35 °C.