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Formation of cross-linked nitrile hydratase aggregates in the pores of tannic-acid-templated magnetic mesoporous silica: Characterization and catalytic application

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ABSTRACT

Tannic-acid-templated magnetic mesoporous silica nanoparticles (TA-MMSNs) were synthesized for the first time. The TA-MMSNs were monodisperse spherical particles with a diameter of around 250 nm and a magnetization saturation value of 35.26 emu/g. The specific surface area of TA-MMSNs was 423.4 m^2/g, and the diameter and cumulative volume of the pores were 9.349 nm and 1.071 cm^3/g, respectively. The TA-MMSNs were used to prepare immobilized NHase (CLNHAs@TA-MMSNs) by forming cross-linked nitrile hydratase aggregates (CLNHAs) in pores of TA-MMSNs using glutaraldehyde as a cross-linker. CLNHAs@TA-MMSNs and free NHase had the same optimum pH (pH 7), and the optimum temperature of CLNHAs@TA-MMSNs (40 °C) was higher than that of free NHase (30 °C). Compared with free NHase, CLNHAs@TA-MMSNs exhibited improved thermal, pH, mechanical and storage stability. Furthermore, CLNHAs@TA-MMSNs was applied in production of nicotinamide, and yield of nicotinamide could reach more than 98%. The tolerance of CLNHAs@TA-MMSNs to high concentration of substrate was better than that of free NHase, and yield of nicotinamide could still reach 29.74% after seven cycles of reaction. The kinetic parameters were investigated and the results indicated a lower substrate affinity and catalytic efficiency of CLNHAs@TA-MMSNs in comparison with free NHase. This work demonstrated that TA-MMSNs could be efficiently employed as supports for enzyme immobilization.

1. Introduction

Nicotinamide (NAM) is one of the important forms of vitamin B_3, has a wide range of applications in the medicinal and food industries [1–3]. The most common preparation method for NAM is hydrolysis of 3-cyanopyridine catalyzed by inorganic acid, but it is difficult to keep this reaction in the amidation stage without hydrolysis in concentrated sulfuric acid. Therefore, the production of NAM requires harsh reaction conditions and high-standard equipment, resulting in higher manufacturing costs. In contrast, as an improved method, the biotransformation of 3-cyanopyridine to NAM using nitrile hydratase (NHase, EC 4.2.1.84) has attracted increasing attention owing to the enzyme's high activity and high selectivity under mild reaction conditions [3,4].

NHase, consisted of two subunit, α- and β-subunit, is a class of multimeric metalloenzyme involved in microbial nitrile metabolism [5,6]. Simultaneously, NHase is an important industrial enzyme that can efficiently catalyze the hydration of nitriles to produce corresponding amides and has been successfully used in the industrial production of acrylamide (AM), 5-cyanovaleramide, etc [7]. NHase can also be used to catalyze the degradation of toxic nitrile wastes for environmental remediation [15]. However, the poor stabilities of free NHase including poor thermal, pH and mechanical stability under the reaction conditions restrict its industrial application [6,8,9]. Another major issue is that it is difficult to separate free NHase from the reaction mixture [10].

Immobilization of NHase on a suitable support provided a promising strategy to solve these problems [11]. For example, Maksimova et al. immobilized NHase on unmodified aluminum oxides and carbon-containing adsorbents. The obtained immobilized NHase displayed the high operational stability in acrylonitrile (AN) hydration [12]. They also immobilized NHase on benzoquinone-activated chitosan by covalent cross-linking and investigated the

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Nomenclatures

AN	Acrylonitrile
AM	Acrylamide
TA	Tannic acid
GA	Glutaraldehyde
NAM	Nicotinamide
SEM	Scanning electron microscopic
TEM	Transmission electron microscopic
BET	Brunauer-Emmett-Teller
BJH	Barrett-Joyner-Halenda
TGA	Thermogravimetric analysis
RSM	Response surface methodology
CCD	Central composite design
PBS	Phosphate buffered solution
DAD	Diode array detector
HPLC	High-performance liquid chromatography
TEOS	Tetraethylorthosilicate
FT-IR	Fourier-transform infrared
MSNs	Mesoporous silica nanoparticles
MMSNs	Magnetic mesoporous silica nanoparticles
NHase	Nitrile hydratase
CLEAs	Cross-linked enzyme aggregates
CLNHAs	Cross-linked nitrile hydratase aggregates
TA-MMSNs	Tannic-acid-templated magnetic mesoporous silica nanoparticles
NHase@TA-MMSNs	NHase-containing TA-MMSNs
CLNHAs@TA-MMSNs	CLNHAs-containing TA-MMSNs

catalytic properties. It was found that immobilized NHase could remain active at pH 3.0–4.0, whereas the free NHase was inactivated under the same conditions [13]. Holz and co-workers encapsulated the cobalt-type NHase in a silica-derived sol-gel material to produce immobilized NHase. The immobilized NHase retained 50% of its activity after being used for 13 consecutive reactions for the conversion of AN to AM. The thermostability and long-term storage stability of the immobilized NHase were also substantially improved [10]. Yadav and co-workers prepared poly(vinyl alcohol)/chitosan-glutaraldehyde cross-linked NHase and the immobilized NHase was found to be reusable up to nine successive batch reactions. The immobilized NHase showed higher retention of activity in methanol compared to its free counterpart [14]. In our previous report, cross-linked NHase aggregates were prepared in mesoporous onion-like silica by using macromolecular dextran polyaldehyde as a cross-linker through a carrier-bound cross-linked enzyme aggregates (CLEAs) method. The stabilities of immobilized NHase were improved significantly compared to the free NHase [15]. This carrier-bound CLEAs method combining the advantages of physical adsorption with CLEAs provides a most promising method for enzyme immobilization. However, there are limited reports on NHase immobilization using this method.

To prepare immobilized enzyme, host materials play an important role for the improvement of enzyme properties. Mesoporous silica has been widely accepted as an ideal support for enzyme immobilization owing to its high specific surface area, tunable pore size, large pore volume, good biocompatibility, high chemical stability and easy surface modification [15–19]. Currently, surfactants are the most commonly used templating agents to produce the mesopores within silica materials [20]. For example, mesoporous silica materials were usually prepared with ionic surfactants and nonionic surfactants as pore-forming agents [21]. Fluorocarbon surfactants were also used as pore-forming agent to synthesize mesoporous silica [22]. Nevertheless, the surfactants used in the traditional synthesis of mesoporous materials are not only expensive but also toxic, which are harmful to the environment and human health [23–27]. To solve these problems, a new, low-cost, and environmentally friendly non-surfactant templating route to mesoporous silica is desired. Tannic acid (TA), a glucoside polymer of gallic acid with multiple phenolic hydroxyl groups that can be found in many plants, was used as pore-forming agent for the synthesis of mesoporous silica. Compared to surfactants [15], TA was a cheap and environmentally harmless pore-forming agent. Moreover, it can be removed easily by water or ethanol extraction [28,29,60]. However, in spite of these clear advantages, there are limited examples of using tannic-acid-templated mesoporous silica for enzyme immobilization so far.

The mesoporous silica is most commonly prepared in the form of particles, and pore diameter of mesoporous silica particles that attracts most interest is in the range of 2–15 nm. For enzyme immobilization, the enzyme molecules should be situated close to the openings of pores where they can meet the substrate molecules, and then they can fully exert their action. If the enzyme molecules are buried in the inner part of the pores where they will be less exposed to the substrate molecules, they will be less active. For these reasons, it is therefore advantageous that the silica particles are as small as possible, and have more shallow pores [30]. In this regard, mesoporous silica nanoparticles (MSNs) with a diameter of a few hundred nanometers or even small may be the suitable enzyme supports. However, the MSNs have drawbacks that they are difficult to be separated from reaction solution due to their small particle size. Fortunately, the magnetic performance can significantly simplify the entire process of separation [31]. Thus, magnetic mesoporous silica nanoparticles (MMSNs) which combines the advantages of mesoporous structure and magnetic properties are attracting more and more researchers' attention due to convenient manipulation [32,33].

Thus, in this work, tannic-acid-templated magnetic mesoporous silica nanoparticles (TA-MMSNs) were synthesized using a modified method according to the previous report [28]. The obtained TA-MMSNs were characterized by electron microscopy and N_2 adsorption-desorption. The magnetization hysteresis loops and FT-IR spectrum of TA-MMSNs were also analyzed. NHase was immobilized on TA-MMSNs through the carrier-bound CLEAs method [34,35]. The cross-linked NHase aggregates (CLNHAs) formed in the pores of TA-MMSNs were named CLNHAs@TA-MMSNs. The preparation conditions were optimized. The effects of pH and temperature on the activity of CLNHAs@TA-MMSNs were studied, and the thermal, pH, mechanical and storage stability of CLNHAs@TA-MMSNs were also investigated. Furthermore, CLNHAs@TA-MMSNs was applied in production of NAM, and the reaction conditions were also investigated.

2. Materials and methods

2.1. Materials

Nitrile hydratase (EC 4.2.1.84, 11.09 U/mg) from *Rhodococcus rhodochrous* strain that carried the cloned nitrile hydratase gene was purchased from Hangzhou Biosci Biotech Co., Ltd. (China). Tannic acid (ACS-grade) was purchased from Alfa Aesar (America). Tetraethylorthosilicate (TEOS, AR) was purchased from Tianjin Damao Chemical Factory (China). Fe_3O_4 was purchased from Beijing Deke Daojin Science And Technology Co., Ltd. (China). Ammonium hydroxide (25–28%) and ethanol absolute (AR) was purchased from Tianjin Fengchuan Chemical Reagent Co., Ltd. (China). Glutaraldehyde (GA, 50%) was purchased from Tianjin Bodi Chemical Co., Ltd. (China). Trypsin (bovine pancreas) was purchased from Shanghai Jinsui Bio-technology Co., Ltd. (China). Acrylonitrile (AR) was purchased from Tianjin Fuchen Chemical