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# Characterization of lactase-conjugated magnetic nanoparticles

Joey N. Talbert, Julie M. Goddard*

*Department of Food Science, University of Massachusetts, 102 Holdsworth Way, Amherst, MA 01003, USA*

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ABSTRACT

Conjugation of lactase to magnetic nanoparticles is of interest in biosensor and ingredient processing applications that require high enzyme concentration and catalyst separation from the reaction stream. However, little is known about the effects of these materials on the physicochemical attributes of conjugated lactase. Lactase (*Aspergillus oryzae*) was covalently attached by carbodiimide chemistry to carboxylic-acid functionalized magnetic particles having a hydrodynamic radius of 18 nm. The resulting enzyme–nanoparticle conjugates were characterized with regard to particle size, zeta potential, enzyme kinetics, temperature and pH stability, catalyst recovery, and secondary structure changes. Following attachment, the materials retained colloidal stability and individual particle characteristics with a zeta potential of −33 mV compared to −46 mV for the native particle. The conjugated enzyme showed no changes in secondary structure and exhibited significant catalytic activity with a catalytic efficiency of $2.8 \times 10^3\ M^{-1}\ s^{-1}$ compared to $2.5 \times 10^3\ M^{-1}\ s^{-1}$ for the native enzyme. Relative to the free enzyme, the conjugated enzyme was recovered for repeated use with 78% activity retained after five cycles. This work demonstrates that carboxylic-acid functionalized magnetic nanoparticles can be utilized as a means of producing a simple and effective conjugated-lactase system that achieves both particle and enzyme stability.



## 1. Introduction

Enzymes conjugated to materials represent an area of interest for applications that require biocatalyst separation, enzyme recycling, and/or enzyme stabilization [1–3]. Applications that employ enzyme-conjugated materials include pharmaceuticals, textiles, fine chemical and food processing, bioactive materials and packaging, and biosensors. Though a number of materials have been utilized to retain the activity of enzyme, induce stabilization, or promote reusability, knowledge of the physicochemical interactions between materials and enzymes is often unclear.

Enzyme-conjugated nanomaterials are used to produce novel value-added products, reduce waste, and enable diagnostics [4–9]. Nanomaterials are of interest for enzyme immobilization application due to the unique properties of the conjugates that are exhibited when the carrier material is reduced to the nanoscale. Compared to macroscale and microscale materials, nanomaterials offer the advantage of increased enzyme loading per unit volume, enhanced mobility of the conjugated catalysis, and reduced barriers to substrate diffusion [10–13]. Additionally, decreasing the size of the support material to the nanoscale has been shown to preserve the activity retention of conjugated enzymes better than that of macroscale and microscale materials [14–19].

Reducing the size of a carrier can promote increased activity retention of a conjugated enzyme due to decreased protein–protein lateral interactions [18]. Protein–protein lateral interactions are reduced with increasing curvature of the support, which can be significant at the nanoscale [18]. Increased particle curvature has also shown to enhance enzyme activity retention due to reduced protein–surface interactions [15–17]. Additionally, reducing the size of carrier material from the macroscale to the nanoscale has demonstrated to result in increased mobility of the enzyme and a corresponding increase in enzyme activity due to enhanced substrate collisions [19].

Magnetic particles are of special interest for enzyme immobilization due to the ability of these materials to be separated from the reaction stream [20,21]. Unlike centrifugation, which requires high speeds or materials with a high density compared to the reaction solution, magnetic particles can be readily removed using an external magnet. Likewise magnetic particles do not require filtration units that may, due to the size of particles, limit product flux. These properties are advantageous for microfluidic reactions and processing applications (e.g. chemical/ingredient manufacturing, synthesis of pharmaceutical intermediates, and conversion of food products) where external separation is desired [22–24].

Lactase (β-galactosidase; EC 3.2.1.23) is an enzyme commercially derived from *Kluyveromyces lactis* and *Aspergillus oryzae* that

* Corresponding author at: Department of Food Science, 344 Chenoweth Labs, 102 Holdsworth Way, Amherst, MA 01003, USA. Tel.: +1 413 545 2275; fax: +1 413 545 1262.
*E-mail addresses:* jtalbert@foodsci.umass.edu (J.N. Talbert), goddard@foodsci.umass.edu (J.M. Goddard).

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is used in the food industry to reduce lactose in whey and fluid milk, for production of oligosaccharide prebiotics, and as a component in the analysis of lactose [25]. Lactase has been shown to retain high activity on zinc oxide nanoparticles and chitosan-coated magnetic nanoparticles [26,27]. Zinc oxide nanoparticles, however, require separation by centrifugation which is not practical for biosensor and processing applications. Though chitosan-coated and chitosan-tethered materials are advantageous for retaining the enzymatic activity of lactase from *A. oryzae*, colloidal stability of these materials can be a hurdle to their implementation [28,29]. Carboxylic acid functionalized magnetic nanoparticles are of interest for covalent lactase conjugation due to the colloidal stability of the materials, commercial availability, low cost, and specificity of conjugation chemistries. However, the activity of lactase has been shown to be adversely affected by surface carboxylic acid groups [28,30].

Given the advantageous colloidal and economic characteristics of carboxylic acid functionalized materials, it would be of value to identify means to retain enzymatic activity of lactase after immobilization to these materials. Reducing the size of the material to the nanoscale may provide a means to reduce interactions between the material and the enzyme so as to retain enzymatic activity of these functionalized supports. As such, we have evaluated the physicochemical characterization of lactase (*A. oryzae*) covalently immobilized to carboxylic acid functionalized superparamagnetic nanoparticles using carbodiimide chemistry. Immobilized conjugates were evaluated as a function of kinetics, colloidal stability, particle size, secondary structure changes, environmental stability, and reusability.

## 2. Materials and methods

### 2.1. Materials

Superparamagnetic nanoparticles with iron oxide cores and surface carboxylic acid groups derived from oleic acid (SHP) were purchased Ocean Nanotech (Springdale, Arkansas, U.S.A.). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from ProteoChem (Denver, CO, U.S.A.). 2-Nitrophenol (99%) was purchased from Acros Organics (Geel, Antwerp, Belgium). O-nitrophenol-β-D-galactopyranoside (ONPG), sulfo-N-hydroxysuccinimide (Sulfo-NHS), bicinchoninic acid (BCA) assay reagents, and bovine serum albumin were purchased from Thermo Scientific (Rockford, IL, U.S.A.). Amicon Ultra centrifugal filter devices (50 K MWCO) were purchased from Millipore Ireland (Carrigtwohill, Co. Cork, Ireland). Syringe filters (0.22 μm) were purchased from Fisher (Fairlawn, NJ, U.S.A.). Dried lactase preparation from *A. oryzae* was kindly donated by Enzyme Development Corporation (New York, NY, U.S.A.) and purified as described below. All other chemicals and reagents were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.) and used as received.

### 2.2. Lactase preparation

Commercial lactase was purified by preparing a solution in phosphate buffer (10 mM; pH 7.0) that was syringe filtered through a 0.22 μm filter, followed by centrifugal filtration in 50 kDa MW centrifugal filter units (5000 × *g*; 22 °C). The lactase solution was recovered in phosphate buffer (10 mM; pH 5.5) and stored at 4 °C until use. Protein concentration of the purified lactase preparation was determined using the BCA assay as described elsewhere [31].

### 2.3. Nanoparticle separation and recovery

Nanoparticles were removed from reaction solutions using a high gradient magnetic separator (Ocean Nanotech: SuperMag Separator). The extent of nanoparticle recovery was assessed by measuring the absorbance of the recovered nanoparticle solution at 400 nm and comparing to a standard curve developed through serial dilution of a known concentration of nanoparticles.

### 2.4. Core particle size

Transmission electron microscopy (TEM) was employed to evaluate core nanoparticle size. Samples were diluted to a concentration of ca. $4 \times 10^{13}$ particles/ml in nanopure water. Samples were sonicated for 30 s and 5 μl was placed on a CF-400-Cu carbon film square mesh copper grid (Electron Microscopy Science; Hatfield, PA). The water was allowed to evaporate and the sample was analyzed using a JEOL CX-100 kV TEM. Particle size was determined using Image J software.

### 2.5. Nanoparticle activation

The scheme of the overall nanoparticle activation and enzyme conjugation pathway is illustrated in Fig. 1. Carboxylic acid groups on the surface of the nanoparticles were activated by addition of a volume of particles to achieve an available surface area of $1.3 \times 10^{16}$ nm$^2$/ml in 0.1 M borate buffer (pH 5.5). EDC was added to a final concentration of $4 \times 10^{-4}$ M and sulfo-N-hydroxysuccimide (sulfo-NHS) was added to a concentration of $6 \times 10^{-3}$ M. The reaction was allowed to proceed for 30 min at room temperature (ca. 20 °C) to yield an activated succinimidyl ester on the surface of the nanoparticles. At the completion of the reaction time, 2-mercaptoethanol was added to a concentration of $9 \times 10^{-4}$ M and allowed to react for 10 min to quench unreacted EDC [32].

### 2.6. Enzyme conjugation to nanoparticles

Purified lactase was added to the solution of activated nanoparticles in conjugation buffer to a concentration of 0.27 mg/ml and allowed to react for 2 h at room temperature (ca. 20 °C) under rotation at 15 rpm. At the completion of the reaction time, samples were magnetically separated from the reaction buffer at room temperature (ca. 20 °C) for 17 h. After completion of the separation time, the reaction buffer was removed and the particles were washed two additional times with the storage buffer (10 mM phosphate buffer; pH 5.5) prior to final dilution in the storage buffer.

### 2.7. Protein loading on nanoparticles

The protein concentration of soluble and immobilized enzyme was determined using an enhanced bicinchoninic acid (BCA) assay [31,33]. After completion of the BCA reaction period, the solution was passed through a 0.22 μm syringe filter to exclude aggregated particles. The absorbance of the filtered solution was read at 562 nm and compared to a bovine serum albumin (BSA) standard curve with protein-free nanoparticles as a negative control.

### 2.8. Surface chemistry

ATR-FTIR spectra of native nanoparticles and enzyme-conjugated particles were collected using an IR Prestige 21 spectrometer (Shimadzu Corporation, Kyoto, Japan) with a diamond ATR crystal. The software for collecting and viewing spectra was IRsolution (v. 1.3, Shimadzu Corp.). Each absorbance spectrum represents 32 scans at 4.0 cm$^{-1}$ resolution using Happ-Genzel apodization, using a clean ATR crystal exposed to the ambient atmosphere as a background. Magnetically separated samples were diluted in ethanol and dried on the ATR crystal. KnowItAll software (v. 8.1, Biorad Laboratories, Inc., Philadelphia, PA, U.S.A.) aided in spectra visualization and analysis.

### 2.9. Enzyme activity

For routine analysis, the activity of lactase was determined based on the Food Chemicals Codex method for the determination of acid lactase units [34]. An amount of the native or immobilized enzyme consisting of 1 μg of protein was added to 2.5 ml of a buffer solution containing 9.6 mM solution of ortho-nitrophenyl-β-galactopyranoside (ONPG), and allowed to react under shaking for 15 min at the 50 °C and pH 5.0 (0.1 M acetate buffer). At the completion of the time period, 2.5 ml of 10% sodium carbonate was added to stop the reaction. The solution was diluted to 25 ml with deionized water, absorbance read at 420 nm, and activity calculated from the extinction coefficient of ortho-nitrophenol (ONP). Activity retention was expressed as percentage activity relative to the maximum activity of each treatment under optimum pH and temperature conditions. Soluble and immobilized enzyme activity was tested at temperatures of 50–75 °C at constant pH (pH 5.0; 0.1 M acetate

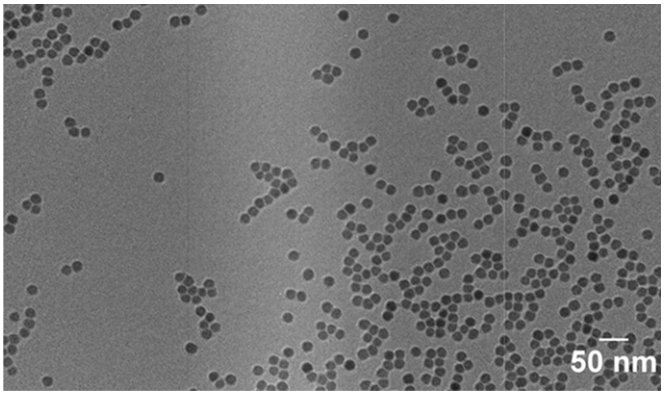


**Fig. 1.** Transmission electron microscopy images of native nanoparticles.

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