Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 54 (2008) 83–89
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

# Characterization of lysine-tagged *Bacillus stearothermophilus* leucine aminopeptidase II immobilized onto carboxylated gold nanoparticles

Chien-Li Wu [a], Yi-Pu Chen [a], Jia-Ci Yang [a], Huei-Fen Lo [b,*], Long-Liu Lin [a,**]

[a] *Department of Applied Chemistry, National Chiayi University, 300 University Road, Chiayi 60083, Taiwan*
[b] *Department of Food and Nutrition, Hungkuang University, Taichung 433, Taiwan*

Received 18 July 2007; received in revised form 10 December 2007; accepted 22 December 2007
Available online 4 January 2008

**Abstract**

*Bacillus stearothermophilus* leucine aminopeptidase II tagged C-terminally with either tri- or nona-lysine (BsLAPII-$Lys_{3/9}$) was constructed and over-expressed in *Escherichia coli* M15 (pRep4). The recombinant enzymes were purified to homogeneity by nickel-chelate chromatography and their molecular masses were determined to be approximately 45 kDa by SDS/PAGE. Surface modification of colloidal gold with 16-mercaptohexadecanoic acid was employed to generate the carboxylated nanoparticles. BsLAPII-$Lys_9$ was efficiently immobilized onto the carboxylated gold nanoparticles (AuNP-COOH) and the obtained bioconjugate showed excellent biocatalytic activity in the immobilized form. Additionally, the bioconjugate material exhibited a significant enhancement in temperature stability and could be reused over 5 successive cycles.


*Keywords:* *Bacillus stearothermophilus*; Leucine aminopeptidase; *Escherichia coli*; Lysine tag; Gold nanoparticles; Immobilization

## 1. Introduction

There is growing interest in the use of nanoparticles modified with biomolecules for the rational design of nanostructured functional materials [1–5]. Metallic and semiconductor nanoparticles can now be synthesized from a wide range of materials, showing fascinating properties due to their small dimensions [6,7]. Gold nanoparticles (AuNPs) have attracted much more attention in recent years especially because of their biocompatibility to a range of biomolecules such as amino acids [8,9], proteins/enzymes [10–14], and DNA [15,16]. They can provide an environment that is similar to the nature for enzyme immobilization [17]. However, the grafting of proteins onto metal nanoparticels is especially complicated since the conformation of a protein is liable to change through nonspecific interactions, leading to lose its unique biocatalytic activities [18]. Among methods that specifically anchor and separate a concerned protein, recombinant protein synthesis with an affinity ligand at the N- or C-terminal might be a breakthrough to avoid nonspecific interactions. A surface modified by $Co^{2+}$-nitrilotriacetic acid, for instance, has been used for the specific binding of histidine-tagged proteins [19]. Ethylene glycol monolayer has also been reported to considerably suppress the nonspecific adsorption and the subsequent denaturation of proteins [20,21].

Aminopeptidases are widely distributed exopeptidases that selectively remove the N-terminal amino acid residues from peptides and proteins. They are shown to be essential for protein maturation, degradation of nonhormonal and hormonal peptides, and determination of protein stability [22,23]. With respect to the relative efficiency on which residues are removed, leucine aminopeptidase (LAP) removes most effectively Leu and other hydrophobic residues from peptide substrates [22]. From the view point of commercial applications, LAP is useful for improving the bitter off-taste of protein hydrolysates [24] and for biotransforming L-homophenylalanyl amide to L-homophenylalanine, the versatile intermediate for a class of angiotension I-converting enzyme inhibitors [25]. Earlier, we have cloned and over-expressed the soluble form of *Bacillus stearothermophilus* LAP II (BsLAPII) in recombinant *Escherichia coli* [26]. BsLAPII consists of two identical 44.5-kDa subunits and shows a marked preference for leucine-*p*-nitroanilide (Leu-*p*-NA). The recombinant enzyme is sensitive to oxidative damage by $H_2O_2$, leading to the disassociation of the

* Corresponding author. Tel.: +886 4 26318652.
** Corresponding author. Fax: +886 5 2717901.
*E-mail addresses:* hflo@sunrise.hk.edu.tw (H.-F. Lo), llin@mail.ncyu.edu.tw (L.-L. Lin).

1381-1177/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2007.12.024

dimeric structure [27]. To identify the histidine residues essential for the catalytic activity of BsLAPII, site-directed mutagenesis was performed on the four conserved His residues of the enzyme [28]. Since the Leu substitutions of His-345 and His-378 made the enzyme inactive, we proposed these two residues to be important for the proper function of the enzyme. In this study, we demonstrate the manufacture of a highly water dispersive gold nanoparticles that can efficiently immobilize the lysine-tagged BsLAPII. BsLAPII-$Lys_9$ binds strongly to the gold nanoparticles and retains a considerable enzymatic activity.

## 2. Materials and methods

### 2.1. Materials, bacterial strains, and growth conditions

$HAuCl_4 \cdot 3H_2O$ (99.9%), citric acid (trisodium salt, 99%), and 16-mercaptohexadecanoic acid (MHA, 90%) were obtained from Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA). The oligonucleotides used were synthesized by Mission Biotechnology Inc. (Taipei, Taiwan). Restriction enzymes were purchased from Promega Life Sciences (Madison, WI, USA). Nickel nitrilotriacetate ($Ni^{2+}$-NTA) resin was acquired from Qiagen Inc. (Valencia, CA, USA). Reagents for polyacrylamide electrophoresis including acrylamide, bisacrylamide, ammonium persulfate, and TEMED were the products of Bio-Rad Laboratories (Hercules, CA, USA). All the solvents otherwise specified were reagent grade, and triply distilled water of resitivity greater than 18.0 MΩ cm was used in making solutions.

*E. coli* Novablue (Novagen Inc., Madison, WI, USA) was used for the preparation and construction of recombinant plasmids. *E. coli* M15 (pRep4) from Qiagen was used for T5 RNA polymerase-mediated expression of wild-type and Lys-tagged BsLAPIIs. The *E. coli* cells harboring plasmids were grown aerobically at 37 or 28 °C in Luria-Bertani (LB) medium supplemented with 100 μg ampicillin/ml for Novablue strain or 100 μg ampicillin/ml and 25 μg kanamycin/ml for M15 (pRep4) strain.

### 2.2. Construction, expression and purification of BsLAPII-$Lys_{3/9}$

To construct plasmids encoding BsLAPII fused with Lys tags at the C-terminal end, PCR amplification reactions were performed with 1 U of *Taq* DNA polymerase, 0.25 μM of each synthetic primer, 10 μM of each deoxynucleotide triphosphate, and the buffer recommended by the manufacturer. These amplifications were done in a PerkinElmer thermal cycler (GeneAmp PCR system 2400). The *lap* gene was amplified from pQE-LAPII [26] with the forward primer LAPIIf (5′-GAGGATCCGTTGGGAGAAGGAA-3′), and the reverse primers LAPIIr-$Lys_3$ (5′-AAGCTTTTACTTCTTCTTCGCCAGTTCGAATGCCCAGTT-3′) and LAPIIr-$Lys_9$ (5′-AAGCTTTTACTTCTTCTTCTTCTTCTTCTTCTTCGCCAGTTCGAATGCCCAGTT-3′). The PCR amplification was initiated at 94 °C for 3 min followed by 30 cycles of denaturation at 94 °C for 2 min, annealing at 52 °C for 1.5 min, and extension at 74 °C for 2 min, with a final extension at 74 °C for 10 min. The PCR products were analyzed on 1% agarose gel and purified using the DNA extraction kit (Viogene, Sunnyvale, CA, USA). The recovered products were cloned as the *Bam*HI-*Hin*dIII fragments into the corresponding sites of pQE-30 to yield pQE-LAPII-$Lys_3$ and pQE-LAPII-$Lys_9$, respectively.

For high-level expression of BsLAPII-$Lys_3$ and BsLAPII-$Lys_9$, *E. coli* M15 (pRep4) harboring either pQE-LAPII-$Lys_3$ or pQE-LAPII-$Lys_9$ was grown at 37 °C in 100 ml of LB medium supplemented with the above-mentioned antibiotics to an optical density at 600 nm of approximately 1.0. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the cultivation proceeded at 28 °C for 12 h. The cells were harvested by centrifugation, resuspended in 3 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris–HCl; pH 7.9), and disrupted by sonication. The crude extracts were clarified by centrifugation and the total soluble proteins were mixed with $Ni^{2+}$-NTA resin pre-equilibrated with the binding buffer. Then, the columns were extensively washed with 20 mM Tris–HCl buffer (pH 7.9) containing 50 mM imadazole and 0.5 M NaCl, until no protein was detected in the eluate by measuring the $A_{280}$. The $His_6$-tagged enzymes were eluted from the resin by a buffer containing 0.5 M imidazole, 0.5 M NaCl and 20 mM Tris–HCl (pH 7.9).

### 2.3. Electrophoresis and determination of protein concentrations

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS/PAGE) with 4% polyacrylamide stacking and 10% polyacrylamide separating gels was performed with the Bio-Rad Mini-Protean III using the Laemmli buffer system [29]. Protein bands were stained with 0.25% Coomassie brilliant blue dissolved in 50% methanol–10% acetic acid, and destined in a 30% methanol–10% acetic acid solution.

Protein concentrations were determined by the Bradford method [30] with the Bio-Rad protein assay reagent, and bovine serum albumin was used as the reference standard.

### 2.4. Enzyme assay

LAP activity was assayed spectrophotometrically by monitoring the hydrolysis of Leu-*p*-NA [31]. The reaction mixture contained 2.0 mM Leu-*p*-NA, 50 mM Tris–HCl buffer (pH 8.0), 1.0 mM $CoCl_2$, and appropriate amount of the purified enzyme in a final volume of 500 μl. The mixture was incubated at 60 °C for 10 min and the reaction was terminated by the addition of 500 μl of 30% (v/v) acetic acid. The extent of hydrolysis was measured by determining the absorbance at 405 nm and reaction mixtures in the absence of enzyme was used as blanks. One unit of LAP activity is defined as the amount of enzyme that releases 1 μmol of *p*-nitroaniline (*p*-NA) per min at 60 °C.

The $K_m$ and $k_{cat}$ values were estimated by measuring *p*-NA production in 0.5 ml reaction mixtures containing various concentration of the substrate (0.3–2.0 $K_m$) in 50 mM Tris–HCl buffer, pH 8.0, and a suitable amount of enzyme. Samples were incubated for 10 min at 60 °C. The $K_m$ and $k_{cat}$ values were calculated from the rate *p*-NA production using Michaelis–Menten equation.