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Research article

Identification and characterization of *Lacticaseibacillus rhamnosus* HP-B1083-derived β-glucuronidase and its application for baicalin biotransformation

Xiao-Lei Ji [a], Yi-Nuo Xiao [c], Rui-Min Sun [b], Zhi-Wen Tan [b], Ya-Qi Zhu [b], Xue-Ling Li [b], Lan-Fang Li [b,**], Shao-Yang Hou [b,*]

[a] *Xinjiang Agricultural Vocational and Technical College, Changji, 831100, PR China*
[b] *College of Pharmacy, Heze University, Heze, 274015, PR China*
[c] *Jining Medical University, Jining, 272000, PR China*

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ABSTRACT

Baicalein, showing higher bioavailability and stronger pharmacological activity, can be obtained via a β-glucuronidase (GUS)-catalyzed transformation of baicalein 7-O-β-D-glucuronide (baicalin). Recently, we have found that the fermentation broth of *Lacticaseibacillus rhamnosus* HP-B1083 can efficiently convert baicalin to baicalein. In this study, the *L. rhamnosus* HP-B1083-derived enzyme involved in baicalin biotransformation was identified and characterized. First, the *LruidA* gene, encoding the responsible enzyme, was cloned and sequenced. Sequence analysis revealed that the deduced enzyme (designated as LrUidA) belonged to the glycosyl hydrolase family 2. The recombinant LrUidA was expressed and purified for characterization. LrUidA had a molecular weight of 70 kDa, with an optimal temperature of 50 °C and pH 4.5. Although LrUidA was susceptible to temperature, it possessed a relative pH stability. Its Michaelis–Menten constant, maximum reaction velocity and catalytic constant values were 9.710 mM, 13.08 mM/min/mg, and 14.95 s^{-1}, respectively. Site-directed mutagenesis experiment results demonstrated that the enzyme reaction uses side chains of E509 and E415 to hydrolyze the glycosidic bond of baicalin and involves three negatively charged residues, E450, D451, and D452, respectively. Surprisingly, biotransformation was performed under optimized reaction conditions by incubating the purified enzyme with 0.1 % baicalin for 4 h, resulting in a considerable conversion ratio of 99 %. Altogether, our findings provide insights into the properties of *L. rhamnosus* HP-B1083-derived enzyme and expand our understanding regarding using GUS for the industrial production of baicalein.

1. Introduction

Scutellaria baicalensis Georgi, also named Baical skullcap root or Huang Qin in China, has a long history of being used in traditional Chinese medicine [1]. *S. baicalensis* contains various active compounds, including flavonoids, polyphenols, and volatile oils, and this herb has wide applicability in the pharmaceutical, flavor, and food additive industries [2]. Of all the active ingredients, baicalin and

* Corresponding author. College of Pharmacy, Heze University, Heze, 274015, PR China.
** Corresponding author. College of Pharmacy, Heze University, Heze, 274015, PR China.
E-mail addresses: 654780547@qq.com (L.-F. Li), xhou1994@163.com, hsy@hezeu.edu.cn (S.-Y. Hou).

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baicalein have garnered the most attention owing to their strong pharmacological activities [3]. Baicalin is a flavonoid glycoside and is structurally created by the conjugated binding of β-D-glucuronic acid and baicalein (Fig. 1). The effects of both flavonoid compounds against inflammation [4], malignancies [5], cardiovascular diseases [6], neurodegenerative conditions [7], microbial infections [8], and diabetes mellitus [9] have been studied. The contents of Baicalin and baicalein in *S. baicalensis* roots are approximately 101.1 and 54.1 mg/g (dry weight) [10].

The human body poorly absorbs Baicalin until it undergoes a hydrolytic reaction by β-glucuronidase-producing intestinal bacteria or their enzymes, whereupon its aglycone, baicalein, can be absorbed [11]. Studies have shown that when used as a botanical drug, baicalein often exhibits stronger physiological activities than baicalin [12,13]. Baicalein, for example, has been reported to show more antiglycation, anti-radical, and anti-α-glucosidase activity, whereas baicalin cannot be used as an anti-α-glucosidase agent although exhibits similar activities [14]. Therefore, baicalin should be transformed from a glycoside-dominated component to an aglycone-dominated component for easier absorption in the human body and for showing stronger pharmacological activity, when used as medicine and food. Conventionally, the conversion is achieved through acid hydrolysis, which may unexpectedly cause an unsatisfactory conversion rate, unsafe impurities, and serious environmental pollution [15].

Compared with the chemical hydrolysis method, biotransformation studies on baicalin into baicalein using β-glucuronidases (GUSs) have been more popular over recent years owing to their specificity, high efficiency, and environmental friendliness. Recent studies have confirmed that several high-purity and commercially available enzymes derived from *Escherichia coli* exhibit a low baicalein yield of 70–80 % [16–18]. Additionally, baicalein bioproduction has been investigated in *Aspergillus niger* [19], *White-rot fungi* [20], and *Lacticaseibacillus brevis* [21]. However, most of these GUSs producing microbes are potentially pathogenic or non-generally recognized as safe (GRAS) microbes. Therefore, the screening and optimization of enzyme production for food-grade microorganisms are still being pursued.

Lacticaseibacillus rhamnosus is a heterofermentative lactic acid-producing bacterium species that can be isolated from many different environments, including human intestine, vagina, mouth, and fermented foods [22]. Strains of this species are known for their potential benefits to human health; therefore, investigating flavonoid metabolism in *L. rhamnosus* spp. can be insightful [23]. It was discovered that a newly isolated, *L. rhamnosus* HP-B1083, can spontaneously convert baicalin into baicalein. Therefore, the main object of this study is to survey the responsible enzyme through genetic sequence analysis, enzymic biochemical characterization, and site-directed mutagenesis analysis to reveal its potential in baicalein production.

2. Materials and methods

2.1. Bacterial strains, plasmids, culture conditions, and reagents

The bacterial strains and plasmids are listed in Table S1. *L. rhamnosus* HP-B1083 was obtained from pickled Chinese cabbage in the town of Heishan in Jinzhou, Liaoning, China. The strain was identified by 16S ribosomal RNA gene sequencing (National Center for Biotechnology Information [NCBI] GenBank accession number OQ608447.1). The pure bacterial culture was maintained anaerobically in the De Man–Rogosa–Sharpe medium (BD, MD, U.S.A.) at 37 °C for 24 h. All *E. coli* strains were cultured in the Luria–Bertani medium containing 20 % agar at 37 °C (HiMedia, Mumbai, India) without special instructions, and kanamycin (50 μg/mL) was added when required. Baicalin and baicalein were purchased from Sigma (St. Louis, MO). All other chemicals and solvents were of analytical reagent grade and obtained from standard commercial sources.

2.2. Gene cloning and sequence analysis of GUS from L. rhamnosus HP-B1083

Genomic DNA from *L. rhamnosus* HP-B1083 was extracted as previously described [24]. The L. *rhamnosus* β-glucuronidase gene (*LruidA*), encoding GUS, was amplified directly from the genomic DNA of *L. rhamnosus* HP-B1083 with the primer pair LruidA-F/LruidA-R. The obtained DNA fragments contain the coding region of *LruidA* and the homology arms of the plasmid vector. Then, the fragments were recombined using *E. coli* GB05-dir to generate the pET28a(+)-*LruidA* expression plasmid using the Red/ET cloning technology. The resultant expression plasmid pET28a(+)-*LruidA* was transformed into *E. coli* BL21(DE3) for heterologous expression. The cloned gene was sequenced with a pair of universal primer pair T7/T7-term. The *LruidA* sequence was deposited in the GenBank database under the accession number OR576892. The primers used in this study are listed in Table S2.

The Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and DNAMAN software were used for comparison. A phylogenetic tree was constructed following the method of Marchler-Bauer et al. [25] with a neighbor-joining

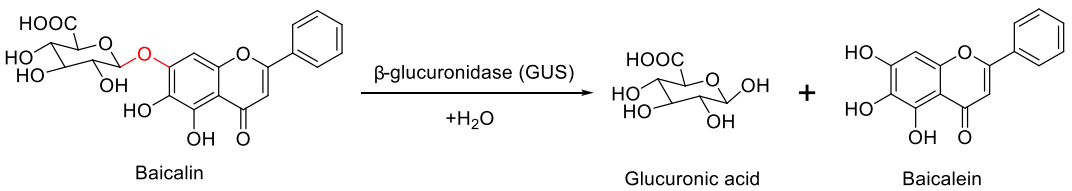

Fig. 1. Reaction scheme for converting baicalin to baicalein catalyzed by the β-glucuronidase (GUS). The glucuronide bond attacked by GUS is highlighted in red color.

algorithm using MEGA version 7.0.26.

2.3. Heterologous expression and purification of recombinant enzyme (designated as LrUidA) and site-directed mutants

The recombinant *E. coli* BL21 was cultured at 37 °C until the optical density at 600 nm (OD_{600}) was 0.6. Then, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and low incubation temperature (16 °C) were used for the expression of recombinant LrUidA. The cells were harvested after 24 h.

To purify the enzyme, 3 L of culture was harvested and resuspended in 70 mL of citrate buffer (pH 4.5). The nano homogenizer (ATS Engineering Inc.; AH100B) was used to lyse the bacterium. The centrifugation at 12,500 rpm for 40 min was used to discard insoluble lysate. Ni resin chromatography (GE Healthcare, USA) was used to purify the soluble LrUidA in the supernatant. The dialysis bag (MD34; 8,000–14,000 kDa; Viskase, USA) was used to remove salts from the protein solution.

The purified enzyme was analyzed using 12 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The purified enzyme was stored in 20 % glycerol at −80 °C until further experiments. Protein concentrations were examined using Bradford assays with bovine serum albumin as a standard.

The site-directed mutagenesis experiments of LrUidA were carried out using the Fast Mutagenesis Kit (TransGen Biotech, Beijing, China).

2.4. Enzyme activity assay

The enzymatic activity was determined by the capability of GUSs to catalyze the substrate baicalin at 50 °C and pH 4.5 for 30 min. The reaction mixture contained 10 mg LrUidA and 0.1 % (w/v) substrate baicalin in a volume of 100 μL. The isovolumetric ethyl acetate was added for the quenching reaction, and then the products obtained were analyzed using high-performance liquid chromatography (HPLC). One unit (1 U) is defined as the amount of enzyme that hydrolyzes 1 nM baicalin for 1 min. All average data were from three replicates.

The effects of pH and temperature on the activity of purified LrUidA were examined. The optimal temperature was determined by measuring the residual activity of LrUidA after incubating the reaction mixture containing 50 mM citrate buffer (pH 4.5) at different temperatures while keeping other factors constant. The optimal pH value was tested by measuring the residual activity of LrUidA after overnight dialysis at 50 °C in different buffers (50 mM), namely citrate buffer (pH 3.0–6.0), potassium phosphate buffer (pH 6.0–7.5), and Tris–hydrochloric acid buffer (pH 7.5–9.0), while keeping other factors constant. The temperature and pH stabilities were determined by measuring the residual activities after incubating the enzyme in 50 mM citrate buffer (pH 4.5) for 12 h at various temperatures and after dialyzing the enzyme for 12 h against various 50 mM buffers at 4 °C, respectively. Upon determining the optimal pH and temperature for the enzyme, velocities were determined for multiple substrate concentrations at the enzyme's optimal pH and temperature, and the Michaelis–Menten kinetics module in SigmaPlot was used to calculate Michaelis–Menten constant (K_m) and catalytic constant (k_{cat}).

2.5. Baicalin biotransformation by the recombinant LrUidA

Baicalin biotransformation in vitro was performed by incubating the reaction mixture in 250-mL laboratory bottles at 100 rpm for 12 h under optimal reaction conditions (pH 4.5 and 50 °C). The reaction mixture contained 25 mL of baicalin solution with a variable concentration in citrate buffer and purified enzyme (9 U/mL). The samples were taken at each time point. The products were extracted by repeatedly adding an equal volume of ethyl acetate three times, concentrated by vacuum rotary evaporation, and eventually resuspended in 500 μL of methanol for HPLC analysis.

2.6. HPLC analysis

HPLC analysis was conducted using an Agilent 1260 series (Agilent Technologies, USA) with a C_{18} RP-column (Agilent ODS-C_{18} 5 μm, 4.6 × 250 mm) at 280 nm, with 0.1 % formic acid, 20 % acetonitrile, and 30 % methanol in H_2O over 25 min. The injected volume of the sample was 10 μL, and the flow rate was set at 1 mL/min. The column temperature was maintained at 30 °C. The substrate baicalin was prepared at a concentration of 0.4 mg/mL in methanol, whereas baicalein (product) was prepared at 0.5 mg/mL in 0.1 % formic acid, 20 % acetonitrile, and 30 % methanol in H_2O for HPLC analysis.

3. Results

3.1. Gene cloning and sequence analysis of GUS from L. rhamnosus HP-B1083

According to the annotated GUS gene sequence data of *L. brevis* AB820700 [21], the primer pair targeting the gene in *L. rhamnosus* HP-B1083 was designed. Thus, the DNA fragment, *LruidA* (1,812 bp), was PCR-amplified and sequenced. A BLAST search result on this sequence showed that the gene sequence contained 100 % identical amino acids with three GUSs from *Lacticaseibacillus rhamnosus* spp. (NCBI GenBank accession number: 6ECA.A, ASY48914.1, and WP_005687546.1) (Fig. 2). A conserved domain search showed that *LruidA* belongs to glycosyl hydrolase (GH) family 2 containing the following two conserved domains: a TIM barrel domain (amino acid: 279–593), and a sugar-binding domain (amino acid: 17–183). The highly conserved amino acid residues were also found using

multiple alignments (Fig. S1). Islam et al. [26] demonstrated that Glu540 and Glu451 in the human GUS play a catalytic role in the reaction as the nucleophile residue and the acid-base residue respectively. Likewise, Glu509 and Glu415 were found to be conserved in the GUS of *L. rhamnosus* HP-B1083. The phylogenetic tree was constructed using GUS protein sequences across different bacterial species to investigate their evolutionary relationship. Results of the phylogenetic tree showed that the GUS of *L. rhamnosus* HP-B1083 is more closely related to the one of *Bifidobacterium longum* sp., instead of the one (NCBI GenBank accession number: WP_270320310.1) of the same genus origin *L. brevis* sp., indicating horizontal gene transfer of GUSs may occur in the human gut microbiota.

3.2. Heterologous expression and purification of the recombinant LrUidA

The SDS–PAGE analysis of purified recombinant enzyme revealed the presence of a single band of approximately 70 kDa, indicating that most of the other proteins were removed (Fig. 3).

3.3. The biochemical property of the recombinant LrUidA

The optimum temperature of the recombinant LruidA was 50 °C (Fig. 4A). Less than 30 % of activity was lost within the range of 40–55 °C. The enzymic activity decreased to nearly 60 % at 37 °C. The enzyme was susceptible to temperature since only approximately 40 % of activity was retained at 60 °C after 12 h and less than 10 % at 65 °C (Fig. 4B). The optimum pH of the recombinant LruidA was 4.5 (Fig. 4C). Less than 30 % of activity was lost within the range of pH 3.5 to 5.5. The activity decreased to 61 % and 40 % of the highest values at pH 3 and pH 6.5, separately. The enzyme possessed relative pH stability since less than 10 % of activity was lost after 12 h within the range of pH 4.5–7.5 (Fig. 4D). Furthermore, the K_m, V_{max}, k_{cat} values of the recombinant LrUidA at 50 °C and pH 4.5 were 9.710 mM, 13.08 mM/min/mg, and 14.95 s^{-1}, respectively (Fig. S2).

3.4. Site-directed mutagenesis of enzyme

To identify the key residues of LrUidA, E509 and E415 were each converted to alanine, respectively. This mutation was inspired by amino acid sequence alignment results mentioned above. We hypothesized that E509 and E415 in LrUidA participated in the classical Koshland retaining mechanism, as the nucleophile and general acid/base, respectively [27]. The resultant E509A LrUidA and E415A LrUidA variants were overexpressed in *E. coli* BL21(DE3), purified (Fig. 5A), and employed in enzymatic assays. Not surprisingly, neither E509A LrUidA nor E415A LrUidA displayed any activity (Fig. 5B). These results indicate that E509 and E415 play essential roles in the enzymatic conversions as reported for class GUSs of the GH2 family [28].

Biernat et al. [29] elucidated the crystal structure of the enzyme of GenBank accession number 6ECA. A (100 % identity with

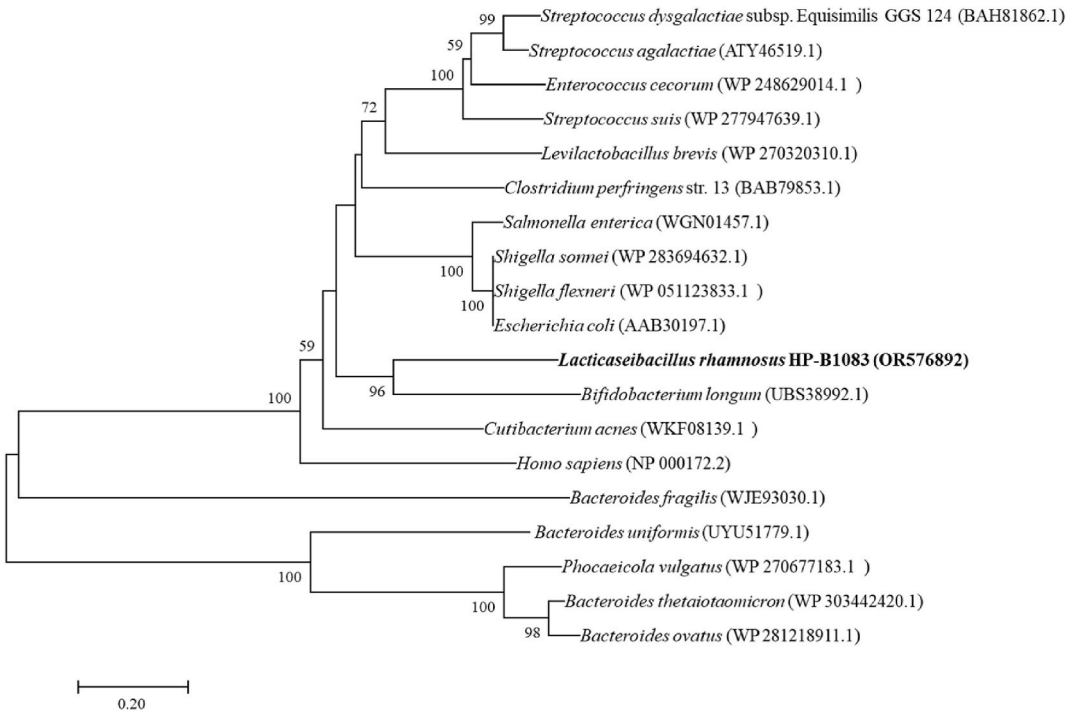

Fig. 2. Neighbor-joining phylogenetic tree based on β-glucuronidase (GUSs) amino acid sequence. Bootstrap values are shown as percentages of 1000 replicates, with those above 50 % shown at the branch points. Bar, 0.20 substitutions per nucleotide position.

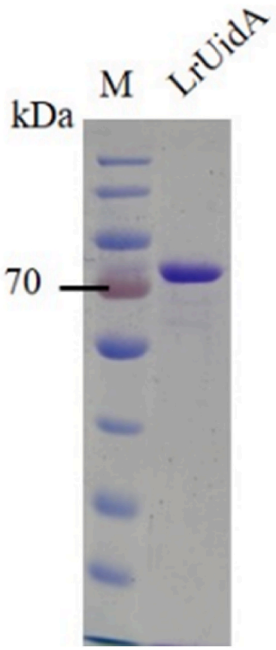

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of purified LrUidA. Coomassie Brilliant Blue-stained 12 % SDS–polyacrylamide gels are shown. Lane M, molecular weight markers (Thermo, cot. 26616). Uncropped and unadjusted version was shown in Fig. S3.

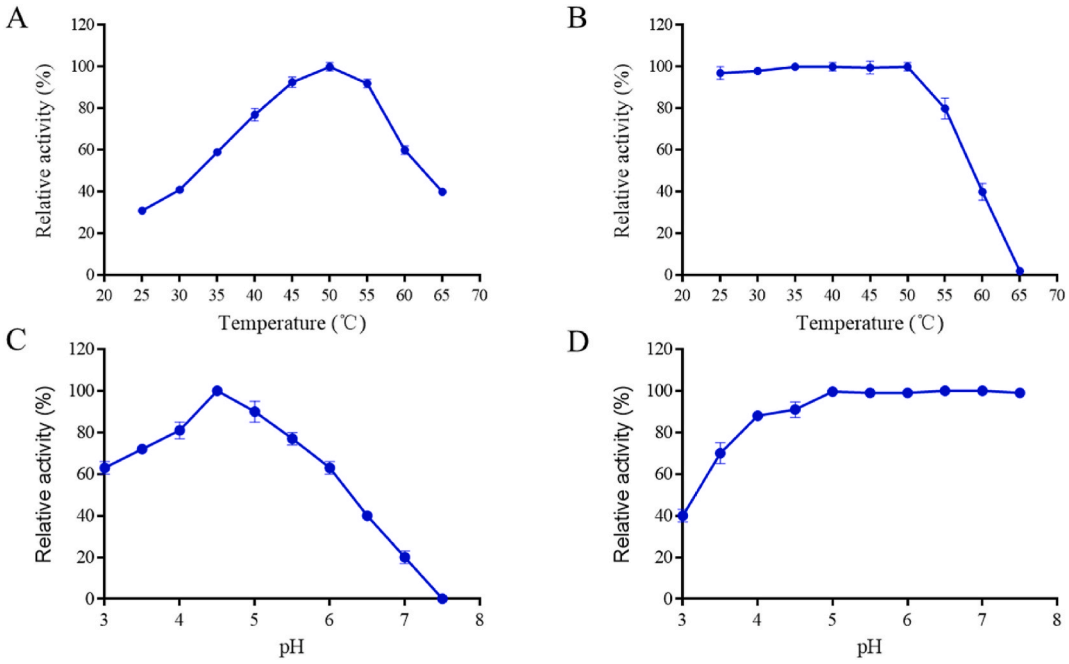

Fig. 4. Characterization of the recombinant LrUidA. A, Optimal temperature. The value at 50 °C was employed as 100 %. B, Thermal stability. The values were tested by measuring the residual activities after incubating the enzyme at pH 4.5 and various temperatures for 12 h. The value 12 h ago was employed as 100 %. C, Optimal pH value. The value at pH 4.5 was employed as 100 %. D, pH stability. The values were tested by measuring the residual activities after dialysis at 4 °C and various pH values for 12 h. The value 12 h ago was employed as 100 %.

LrUidA) and proposed the enzyme contains a unique patch of negatively charged residues, E450, D451, and D452, termed as the "EDD" motif, within their active sites. Further, the EDD motif was converted to the corresponding non-polar residues AAA of small size. The resultant AAA LrUidA variant was overexpressed, purified, and employed in enzymatic assays using baicalin as substrates. The result of

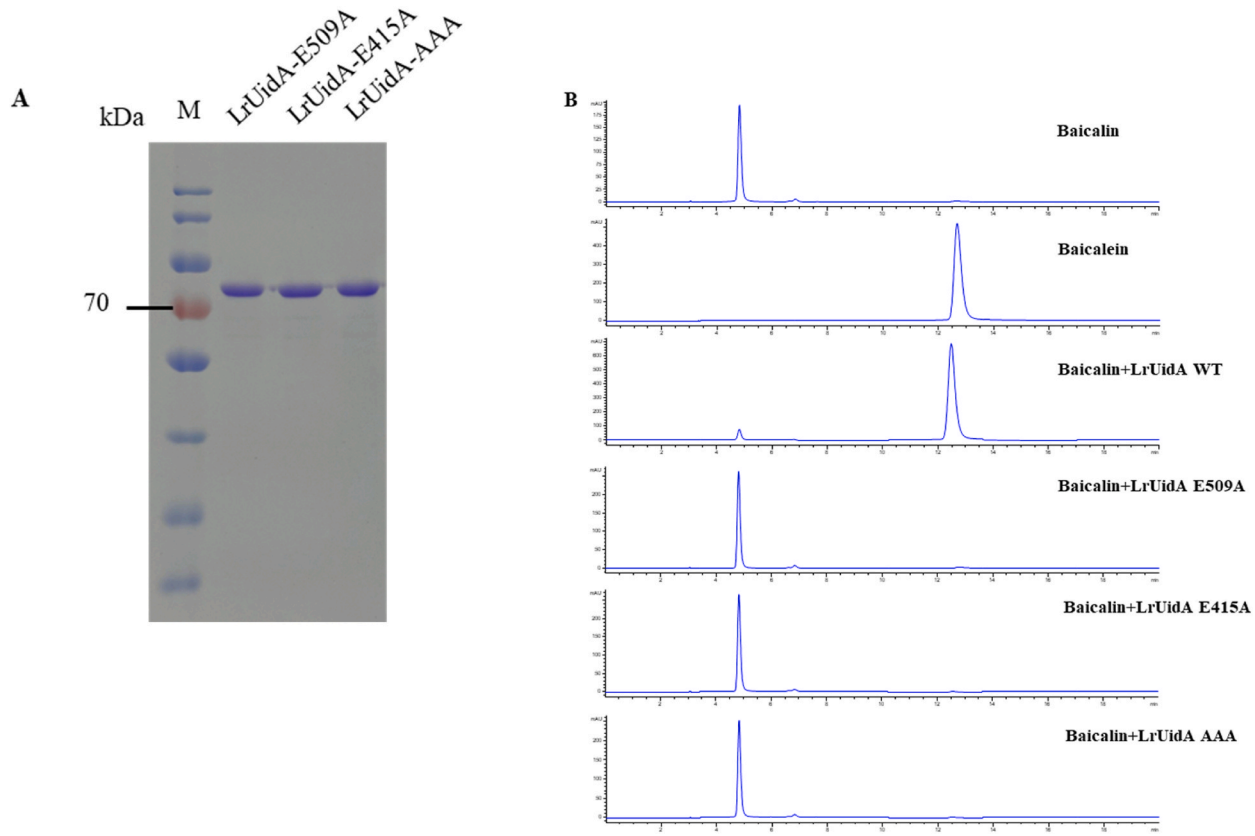

Fig. 5. A, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of purified LrUidA mutants. Coomassie Brilliant Blue-stained 12 % SDS–polyacrylamide gels are shown. Lane M, molecular weight markers. B, HPLC analysis of the LrUidA and its mutants-catalyzed conversion of baicalin.

the HPLC analysis showed that the mutant did not display any activity, indicating the EDD motif is required for the processing of small glucuronide rather than enzyme-substrate binding.

3.5. Biotransformation of baicalin by the recombinant LrUidA

To determine the ability of the purified enzyme to produce baicalein, various concentrations (0.05, 0.1, 0.15, and 0.2 %) of baicalin were incubated with the purified enzyme, and the enzymatic conversion results were analyzed by HPLC. The capability of the GUS to produce baicalein in vitro was considerable but inhibited by higher substrate concentration (Fig. 6). It reached its highest values of 64 g/L of baicalein after 10 and 12 h of incubation with a 0.2 % initial baicalin concentration, resulting in a lower yield of 88 %. After the 4-h incubation, 26.7 ± 0.27 g/L baicalein was obtained with 0.1 % baicalin, resulting in a yield of 99 %. This value of product concentration was not only considerably higher than the value for 0.05 % of baicalin at the same time point, but it also reached baicalein peak 1 h earlier. Furthermore, the reaction rate was limited by higher substrate concentrations. At 0.15 % and 0.2 % baicalin, the baicalein peak was obtained after 8 h or even 10 h, respectively.

4. Discussion

The GUSs from *Lacticaseibacillus rhamnosus* are thought as a useful tool for the formation of the safe bioconversion strategy of prodrugs including baicalin, because *Lacticaseibacillus rhamnosus* strains are regarded as GRAS microbes [30]. This study was devoted to the sequencing of the gene encoding the GUS in *L. rhamnosus* HP-B1083, heterologous expression, biochemical characterization and investigation of active sites of the enzyme, and to reveal the efficiency of an untested biocatalyst source for biotransformation of baicalin. The LrUidA was classified into GH family 2 via sequence analysis. Based on the CAZy database [31], family 2 GUSs (EC 3.2.1.31) are retaining enzymes and follow a classical Koshland double-displacement mechanism. Reaction occurs with acid/base and nucleophilic assistance provided by two amino acid side chains, typically glutamate or aspartate. By BLAST analysis, we revealed that E509 and E415 were the putative catalytic sites. To further confirm catalytic residues, E509 and E415 were replaced by non-nucleophilic residues. Alanine is preferred as a replacement residue due to its small size. We successfully determined the catalytic sites through the kinetic analysis of mutants. Furthermore, we showed that the EDD motif is required for the enzyme activity via the

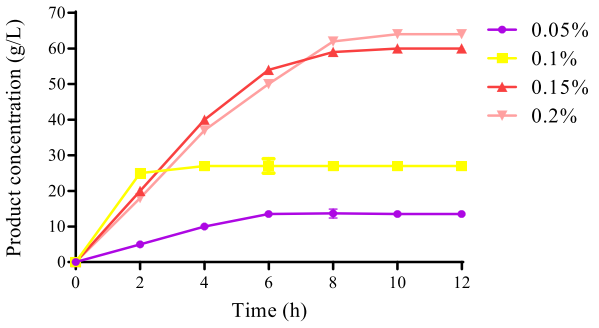

Fig. 6. Time profiles of baicalein production catalyzed by the purified LrUidA of *L. rhamnosus* HP-B1083 with different concentrations of baicalin. Three independent trials were performed.

same method. These sites are usually occupied by polar or hydrophobic amino acid residues in other GUSs, such as M447, F448, and C449 at the corresponding sites in the GUS of *E. coli* (Fig. S1). Altogether, these results expand our knowledge of the characteristics of the active site of LrUidA and provide support for further directed enzyme modification.

The biotransformation of baicalin to baicalein is strategically significant for cancer prevention and treatment along with the treatment of pathogenic bacterial infection and inflammation. As the purified LrUidA was used for bioconversion of baicalin, 0.05 % baicalin was converted completely within 6 h, and 0.1 % baicalin was converted with a 99 % conversion rate. Compared to all biotransformation studies of microbial β-glucuronidases in the current published literature (70%–90 %), the obtained yield here was almost 10 % higher (16–21). The reasons for the considerable yield may include the inherent properties of LrUidA, acidic media, and the high reaction temperature. The weakly acidic environment of the reaction system may facilitate the protonation of substrate molecules contributing to the dissociation of β-D-glucuronic acid residue from baicalin [32]. Protonation of substrate molecules can affect the interactions between substrate molecules and enzyme proteins, which can further affect the conversion efficiency of enzymes on substrate molecules [33]. Under weakly acidic reaction conditions, all baicalin ($pKa_1 = 7.6$) molecules are protonated. Furthermore, baicalin is most stable at pH 4.28, which is near our reaction pH [34]. In addition, our reaction temperature is relatively high, which is conducive to the dissolution of baicalin with low solubility. Thus, biocatalysis by LrUidA in acidic pH and high temperature may facilitate the advanced bioproduction of baicalein in this way.

For the enzymatic reaction, however, the high reaction temperature is a double-edged sword because although high temperature increases the solubility of the substrate, it can cause instability of the enzyme, leading to a baicalein conversion limit. The analysis of the enzyme stability indicated that more than half of the enzyme activity was lost after 12 h. Furthermore, even at the optimal pH, there was still a slight loss of enzyme activity. An enzyme inhibition phenomenon may affect conversion efficiency. As the substrate concentration increases, a decrease in yield rate and enzyme activity was observed. It has been demonstrated that the side product of the reaction, β-D-glucuronic acid, limits the enzymatic conversion as an inhibitor [35]. Although high substrate concentration could lead to enzyme inhibition, the advantage of LrUidA that enables almost complete conversion of 0.1 % baicalin is completely applicable to the production of baicalin [19]. Consequently, these results provide important insight into GUS from *Lacticaseibacillus rhamnosus,* which catalyzes the production of bacailien with more pharmacological activity and reveals new possibilities for biotool development.

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Data availability statement

Data associated with our study has been deposited into a publicly available repository. The GenBank accession number for HP-B1083 16S rRNA gene sequence and *LruidA* gene sequence are OQ608447.1 and OR576892.

CRediT authorship contribution statement

Xiao-Lei Ji: Writing – original draft, Funding acquisition. **Yi-Nuo Xiao:** Funding acquisition, Formal analysis. **Rui-Min Sun:** Formal analysis. **Zhi-Wen Tan:** Investigation, Data curation. **Ya-Qi Zhu:** Visualization. **Xue-Ling Li:** Methodology. **Lan-Fang Li:** Writing – original draft, Visualization, Methodology, Formal analysis. **Shao-Yang Hou:** Writing – review & editing, Supervision, Resources, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing

interests:Shao-Yang Hou reports financial support was provided by Shandong natural resources fund. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38028.

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