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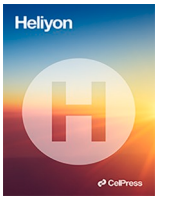

Expression and characterization of a protease-resistant β-D-fructofuranosidase BbFFase9 gene suitable for preparing invert sugars from soybean meal

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ABSTRACT

A novel gene (*BbFFase9*), with an ORF of 1557 bp that encodes β-D-fructofuranosidase from *Bifidobacteriaceae bacterium*, was cloned and expressed in *Escherichia coli*. The recombinant protein (BbFFase9) was successfully purified and showed a single band with a molecular mass of 66.2 kDa. This was confirmed as a β-D-fructofuranosidase and exhibited a high specific activity of 209.2 U/mg. Although BbFFase9 was a soluble protein, it exhibited excellent tolerance to proteases such as pepsin, trypsin, acidic protease, neutral protease and Flavourzyme®, indicating its potential applicability in different fields. BbFFase9 exhibited typical invertase activity, and highly catalyzed the hydrolysis of the α1↔2β glycosidic linkage in molecules containing fructosyl moieties but with no detectable fructosyltransferase activity. It was optimally active at pH 6.5 and 50 °C and stable between pH 6.0 and 9.0 at a temperature of up to 45 °C for 30 min BbFFase9 could also effectively hydrolyze galacto-oligosaccharides, which are a flatulence factor in soybean meal, thus releasing new types of product such as melibiose and mannotriose, or degrading them into invert sugars, the sweeter fructose and glucose. This study is the first to report the application of this type of β-D-fructofuranosidase.

1. Introduction

Invert sugar, a mixture of glucose and fructose, is widely used in the food industry as syrup, an ingredient in jams, and in candy drops and sweets, because of its sweetening properties, superior to those of sucrose, greater resistance to crystallization, and higher solubility [1]. The process most often used to produce invert sugar is acid hydrolysis, but this can make it susceptible to changes in color and flavor that affect its shelf life. Using this technique also generates products of undesirable color as well as forming hydroxymethyl-furfural, a product with high carcinogenic potential for humans [2]. In comparison, enzymatic catalysis, with its milder processing conditions and higher quality products, is becoming the preferred process for producing invert sugar.

β-D-fructofuranosidase (EC 3.2.1.26), also known as invertase, is a highly polymorphic protein classified into the family of 32 glycoside hydrolases (GH). It can strongly catalyze the hydrolysis of the α1↔2β glycosidic linkage in molecules containing fructosyl moieties such as sucrose, to produce an equimolar mixture of D-glucose and D-fructose. β-D-fructofuranosidase can also efficiently act on the α1↔2β bonds of raffinose and stachyose then invert them into melibiose and mannotriose, known as inverted sugar. These novel

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invert sugars have gained considerable attention because they can improve the nutritional values of products. For example, they can promote calcium absorption in the intestines and help cure atopic dermatitis. They are also a novel autophagy-inducing small molecule that may inhibit aggregation-mediated neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, as well as diseases mediated by polyglutamine [3]. Overall, direct hydrolysis by β-D-fructofuranosidase offers the most potential for application in the invert sugar processing industry.

Although β-D-fructofuranosidase has been detected in a wide range of living organisms, it is mainly produced by microorganisms, such as yeasts, molds and some bacteria. Most studies have focused on β-D-fructofuranosidases from yeasts and molds, such as *Saccharomyces cerevisiae* and *Aspergillus* spp. For example, several food industries have used these strains extensively as potent β-D-fructofuranosidase producers, as they are known not to be pathogenic or toxicogenic [4]. However, studies on bacterial β-D-fructofuranosidases are very limited thus identifying novel strains of bacteria with the potential to produce β-D-fructofuranosidase is required. Extensive studies have shown that *Bifidobacterium* spp., categorized as GRAS (generally regarded as safe), have the potential to produce β-D-fructofuranosidase. These include *B. longum* [5], *B. adolescentis* [6], *B. breve* [7], *B. lactis* [8] and *B. infantis* [9]. Other aspects of these β-D-fructofuranosidases have also been studied, including enzyme production [6], enzyme purification [9], biochemical characterization [7,10] and the cloning and expression of β-D-fructofuranosidase genes [8]. These preliminary exploratory studies have shown this type of β-D-fructofuranosidase has good prospects for use in various industries, including the production of invert sugar. However, these studies have all focused on *Bifidobacterium* species and their genes for producing β-D-fructofuranosidase. The hydrolyzation of raffinose and stachyose using these characterized β-D-fructofuranosidases from *Bifidobacterium* spp. has rarely been useful because of their limited resources.

For a better understanding, many more potential resources and the identification of novel properties of β-D-fructofuranosidase from *Bifidobacterium* spp. are required. The present study aimed to reveal novel β-D-fructofuranosidase genes from *Bifidobacteriaceae bacterium*, and to describe their heterologous expression, purification and characterization and to evaluate their ability to produce invert sugars using a novel recombinant β-D-fructofuranosidase.

2. Materials and methods

2.1. Reagents

Chelating Sepharose (Ni-IDA) resin matrix was purchased from GE Life Sciences (Pittsburgh, PA, USA), a glucose-oxidase kit from Applygen Technologies Inc. (Beijing, China), *p*NP substrates (*p*NP-α-D-galactopyranoside, *o*NP-β-D-galactopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-α-L-arabinopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-α-D-mannopyranoside), melibiose, raffinose and stachyose from Sigma (St. Louis, MO, USA) and soybean meal from a local market. All other chemicals were of analytical grade, unless stated otherwise.

2.2. Gene cloning and expression of the β-D-fructofuranosidase gene

A novel GH 32 β-fructofuranosidase gene (GenBank No: GDY89989.1) was isolated from the gene bank of NCBI. To identify the potential catalytic function of this registered gene and explore the potential application of the recombinant protein, the codon optimized gene encoding this gene was chemically synthesized by Synbio Technologies (China). The gene fragment was further amplified by PCR using two primers which contain *Nde*I and *Xho*I sites (underlined): BbFFase9-up (ATTCTACATATGATGACTGGCTTCACTCCGGAT) and BbFFase9-down (ATTCCGCTCGAGCTACTCCAGTCCGATGGACTTCAT). The purified PCR products were then digested with *Nde*I and *Xho*I, followed by subcloning into the pET-28a(+) vector. All the recombinant plasmids used in the process were sequenced and verified.

The recombinant plasmids were then transformed into *E. coli* BL21 (DE3)-competent cells for gene expression. A native colony of *E. coli* BL21 (DE3) harboring *BbFFase9* in pET28a (+) was inoculated into LB medium containing kanamycin (50 μg/mL) then incubated at 37 °C until the optical density reached 0.6–0.8. IPTG was used to induce expression at a final concentration of 1 mM, followed by further culture at 20 °C for 12 h.

2.3. Purification of recombinant β-D-fructofuranosidase and SDS-PAGE analysis

The harvested cells (original cultured volume was 1 L) were centrifuged at 24, 200 *g* and 4 °C for 10 min, then resuspended in lysis buffer A (50 mM pH 7.4 Tris-HCl) and disrupted by sonication. The supernatant was collected by centrifugation and loaded onto an Ni-IDA column (0.8 × 10 cm) equilibrated with buffer B (buffer A containing 20 mM imidazole). After binding, buffer C (buffer A containing 50 mM imidazole) was used to wash the prepared column at a flow rate of 1.0 mL/min. Finally, the eluting buffer (buffer A containing 200 mM imidazole) was used to elute the target protein. The purified protein as a single peak was pooled, then its activity checked and further monitored by SDS-PAGE.

The purified BbFFase9 was monitored using 12.5% SDS-PAGE at 110 V running for 70 min, with a crude protein concentration of 30 μg, purified protein 10 μg, and the protein bands were stained with Coomassie brilliant blue R-250. Its molecular mass was determined using a low molecular mass standard containing rabbit phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), rabbit actin (43.0 kDa), bovine carbonic anhydrase (31.0 kDa), trypsin inhibitor (20.1 kDa) and hen egg white lysozyme (14.4 kDa) [11].

2.4. Enzyme assay and protein determination

20 μL of properly diluted BbFFase9 with final amount of protein varied from 1.5 μg to 5.0 μg were mixed with 180 μL of sucrose (200 mg/mL) in 50 mM sodium phosphate buffer (pH 6.5). The mixture was incubated at 50 °C for 10 min. The β-D-Fructofuranosidase activity was assayed by measuring the release of glucose using the Glucose-oxidase kit. One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmol of glucose/min. The protein concentrations were measured by the Lowry method with BSA as standard [12].

2.5. pH and temperature properties

0.5 mg/mL of protein was used to study the pH and temperature properties of the β-D-fructofuranosidase. In details, the optimal pH for activity was determined in various buffers (50 mM): MES (pH 5.0–6.5), sodium phosphate (pH 6.0–8.0), MOPS (pH 6.5–8.0), Tris-HCl (pH 7.5–9.0) and glycine-NaOH (pH 9.0–10.5). The stability of BbFFase9 at these pH values was further analyzed by incubating the protein at 40 °C for 30 min, then rapidly cooling the treated enzyme at 0 °C, followed by checking the residual activity using the enzyme assay.

The optimal temperature was determined from enzyme assays at temperatures ranging from 25 to 70 °C in sodium phosphate buffer (50 mM, pH 6.5). The thermostability of BbFFase9 at the different temperatures was then checked by assessing the residual enzyme activity after treatment for 30 min.

2.6. Influence of different salts on enzyme activity

The influence of different salts ($CaCl_2$、 KCl、 $MgCl_2$、 $FeCl_3$、$MnCl_2$、$CoCl_2$、$ZnCl_2$、$AlCl_3$、$CuCl_2$、$FeCl_2$) on BbFFase9 was also determined. BbFFase9 was incubated with various salts which final concentration was 1 mM at 40 °C in a 50 mM sodium phosphate buffer (pH 6.5) for 30 min then their residual activity was measured using the standard assay.

2.7. Resistance of BbFFase9 to proteases

The potential resistance of BbFFase9 to various proteases was also tested. BbFFase9 was incubated in the solutions (1 mg/mL) of various proteases in various buffers (50 mM), including trypsin (pH 8.0, Tris-HCl), pepsin (pH 5.0, MES), Proteinase K (pH 7.0, MOPS), acidic protease (pH 5.0, MES), neutral protease (pH 6.5, MOPS), alkaline proteinase (pH 9.0, Tris-HCl) and Flavourzyme® (pH 6.5, MOPS) at 37 °C for 30 min or 60 min, respectively. In each case, the residual activity of the β-D-fructofuranosidase and the control (untreated) was measured using the standard assay. Specifically, the enzyme activities of these control under different condition were 83.9 U/mL (pH 8.0, Tris-HCl), 17.3 U/mL (pH 9.0, Tris-HCl), 19.9 U/mL (pH 5.0, MES), 193.7 U/mL (pH 7.0, MOPS), 166.2 U/mL (pH 6.5, MOPS), respectively.

2.8. Substrate specificity and action mode of BbFFase9

The substrate specificity of BbFFase9 towards sucrose, raffinose, stachyose, different polysaccharides (glucan, xylan, arabinan and sodium carboxymethyl cellulose) and various *p*NP-derivatives (*p*NP-α-D-galactopyranoside, *o*NP-β-D-galactopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-α-L-arabinopyranoside, *p*NP-α-L-arabinofuranoside and *p*NP-α-D-mannopyranoside) was determined. The enzyme activity towards polysaccharides was assayed at an enzyme concentration of 5–10 mg/mL by the DNS method [13] and towards *p*NP-derivatives by the rate of *p*NP released during hydrolysis from 5 mM of the substrate at 50 °C in 50 mM sodium phosphate buffer (pH 6.5).

BbFFase9 at a concentration of 5 U/mL was used to check its potential hydrolysis abilities towards several oligosaccharides including sucrose, raffinose and stachyose. Substrates at a concentration of 20 mg/mL were incubated with the purified BbFFase9 in a reaction volume of 1 mL at 40 °C in 50 mM sodium phosphate buffer (pH 6.5) for 1, 3 and 12 h. The enzyme in the sample was then denatured and samples were spotted on a silica gel plate (Silica Gel 60F 254, Merck KGaA, Darmstadt, Germany) and developed twice in a solvent system containing N-butanol: acetic acid: water at 5: 3: 2 (v/v/v). All saccharides could be detected by heating in an oven after spraying the plates with a mixture of methanol and sulfuric acid (95: 5, v/v).

2.9. Application of BbFFase9 on the hydrolysis of oligosaccharides in soybean meal (SMOs)

To explore the potential application of BbFFase9 for the hydrolysis of SMOs, a mixture containing 5 U/mL of β-D-fructofuranosidase and 10% substrate was reacted at 40 °C in 50 mM sodium phosphate buffer (pH 6.5) for 12 h. Aliquots were then taken and heated to 100 °C for 5 min to denature the enzyme. The end-products were monitored and analyzed by thin layer chromatography. Finally, to fully hydrolyze all the oligosaccharides detected, another commercial α-galactosidase (G304917, Aladdin-e.com) was used for the synergistic hydrolysis of SMOs, using the same reaction assay and detection methods as above. HPLC was performed using a YMC-Pack ODS-AQ column (4.6 mm × 250 mm). The sugars were eluted using a mobile phase of 100% water at a flow-rate of 0.5 mL/min. Ten microliters of sample was injected onto the column and sugars were detected with a Refractive Index Detector (RID) (Agilent).

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   1 ATGACTGGCTTCACTCCGGATGCACCCGTCCTCCACGAAATCAAGAACCACAGCGAAGCT
     M  T  G  F  T  P  D  A  P  V  L  H  E  I  K  N  H  S  E  A
  61 CTGGCACAGGCGGAAGCAGGCGTTGCAGCCATGGCCGCCGAACGCAACAACCGCTGGTAC
     L  A  Q  A  E  A  G  V  A  A  M  A  A  E  R  N  N  R  W  Y
 121 CCGAAGTTCCACATCGCATCCAACGGCGGTTGGATCAACGATCCGAACGGCCTGTGCTTC
     P  K  F  H  I  A  S  N  G  G  W  I  N  D  P  N  G  L  C  F
 181 TATAAGGGCCGCTGGCACGTGTTCTACCAGCTGCACCCGTACGGCACCCAGTGGGGTCCG
     Y  K  G  R  W  H  V  F  Y  Q  L  H  P  Y  G  T  Q  W  G  P
 241 ATGCACTGGGGACACGTCTCCTCCACCGACATGCTCAACTGGAAGCGTGAGCCAATCATG
     M  H  W  G  H  V  S  S  T  D  M  L  N  W  K  R  E  P  I  M
 301 TTCGCCCCGACCCTTGAAGAGGAGAAGGACGGCGTATTCTCCGGTTCCGCAGTAATCGGC
     F  A  P  T  L  E  E  E  K  D  G  V  F  S  G  S  A  V  I  G
 361 GACGATGGCAAGCTCAAGTTCTACTACACCGGCCACCGTTGGGCCAACGGCCACGACAAC
     D  D  G  K  L  K  F  Y  Y  T  G  H  R  W  A  N  G  H  D  N
 421 ACCGGCGGCGACTGGCAGGTGCAGATGACCGCGCTGCCGGACAATGACGAGCTCACCTCC
     T  G  G  D  W  Q  V  Q  M  T  A  L  P  D  N  D  E  L  T  S
 481 GCCACCAAGCAGGGCATGATCATCGACTGCCCGACCGACAAGGTCGACCATCACTACCGC
     A  T  K  Q  G  M  I  I  D  C  P  T  D  K  V  D  H  H  Y  R
 541 GACCCGAAGGTCTGGAAGACCGGCGACACCTGGTACATGACCTTCGGTGTCTCCTCTGCG
     D  P  K  V  W  K  T  G  D  T  W  Y  M  T  F  G  V  S  S  A
 601 GATAAGCGCGGCCAGATGTGGCTGTTCTCCTCCAAGGACATGGTCCGCTGGGAATACGAG
     D  K  R  G  Q  M  W  L  F  S  S  K  D  M  V  R  W  E  Y  E
 661 CGCGTGCTCTTCCAGCACCCGGATCCGGATGTGTTCATGCTCGAATGCCCCGACTTCTTC
     R  V  L  F  Q  H  P  D  P  D  V  F  M  L  E  C  P  D  F  F
 721 CCCATCAAAGACAAGGACGGCAACGAGAAGTGGATCATTGGCTTCTCCGCCATGGGGTCC
     P  I  K  D  K  D  G  N  E  K  W  I  I  G  F  S  A  M  G  S
 781 AAGCCCAGCGGCTTCATGAACCGCAACGTCAACAACGCCGGCTACATGATCGGCACGTGG
     K  P  S  G  F  M  N  R  N  V  N  N  A  G  Y  M  I  G  T  W
 841 GAGCCGGGCGGCGAATTCAAGCCGGAAACCGAGTTCCGCCTGTGGGATTGCGGCCACAAC
     E  P  G  G  E  F  K  P  E  T  E  F  R  L  W  D  C  G  H  N
 901 TACTACGCCCCGCAGTCCTTCAATGTGGACGGCCGTCAGATCGTCTATGGCTGGATGAGC
     Y  Y  A  P  Q  S  F  N  V  D  G  R  Q  I  V  Y  G  W  M  S
 961 CCGTTCGTGCAGCCGATTCCCATGGAGGATGACGGCTGGTGTGGCCAGCTCACCCTGCCG
     P  F  V  Q  P  I  P  M  E  D  D  G  W  C  G  Q  L  T  L  P
1021 CGCGAGATCACCCTTGGCGATGACGGCGACGTGGTCACCGCGCCGGTCGCCGAAATGGAA
     R  E  I  T  L  G  D  D  G  D  V  V  T  A  P  V  A  E  M  E
1081 GGCCTGCGCGAGGATACCCTCGACCATGGTTCGATCACACTCGACATGGATGGGGAGCAG
     G  L  R  E  D  T  L  D  H  G  S  I  T  L  D  M  D  G  E  Q
1141 GTCATCGCCGATGACTCCGAGGCCGTGGAAATCGAAATGACCATCGACTTGGCTGCCTCC
     V  I  A  D  D  S  E  A  V  E  I  E  M  T  I  D  L  A  A  S
1201 ACTGCCGAGCGTGCGGGCCTGAAGATTCATGCCACTGAGGACGGTGCCTACACCTACGTG
     T  A  E  R  A  G  L  K  I  H  A  T  E  D  G  A  Y  T  Y  V
1261 GCTTATGACGACCAGATCGGCCGCGTGGTGGTGGATCGTCAGGCCATGGCCAACGGCGAT
     A  Y  D  D  Q  I  G  R  V  V  V  D  R  Q  A  M  A  N  G  D
1321 CGCGGCTACCGCGCCGCCCCACTGACCGACACCGAACTGGCCTCCGGCAAGCTGGACCTG
     R  G  Y  R  A  A  P  L  T  D  T  E  L  A  S  G  K  L  D  L
1381 CGCGTGTTCGTGGATCGCGGCTCCGTGGAGGTCTACGTCAACGGTGGCCACCAGGTGCTG
     R  V  F  V  D  R  G  S  V  E  V  Y  V  N  G  G  H  Q  V  L
1441 AGCTCCTACTCCTACGCCTCCGAAGGCCCGCGCGCCATCAAGCTCGCCGCCGAGTCCGGC
     S  S  Y  S  Y  A  S  E  G  P  R  A  I  K  L  A  A  E  S  G
1501 TCCCTCAAGGTGGATTCCCTGAAGCTCCACCACATGAAGTCCATCGGACTGGAGTAG
     S  L  K  V  D  S  L  K  L  H  H  M  K  S  I  G  L  E  *
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Fig. 1. Nucleotide and deduced amino acid sequences of the full-length cDNAs and flanking regions of BbFFase9.

3. Results and discussion

3.1. Cloning and expressing of the β-D-fructofuranosidase gene from B. bacterium

Genes or protein sequences registered in the NCBI are a valuable resource for scientists studying catalytic enzymes. A novel β-D-fructofuranosidase gene (*BbFFase9*) was obtained from this database, and its nucleotide and deduced amino acid sequences are listed in Fig. 1. The full-length *BbFFase9* gene had an open reading frame (ORF) of 1557 bp encoding 518 amino acids with a predicted molecular mass of 58.0 kDa and a deduced *p*I value of 4.65. Based on a homology search of the deduced amino acids of BbFFase9 by BLAST, its sequence exhibited the highest similarity (>90%) with several sequences submitted for β-D-fructofuranosidases from *Bifidobacterium* spp., such as *B. breve* (GenBank No: AUD87604.1) and *B. longum* (GenBank No: 3PIG_A) but cloning has not been further discussed.

Microorganisms, such as *Aspergillus* spp. [14], *Candida* spp. [10], *Bacillus* spp [15]. and *Bifidobacterium* spp. [5], are good candidates for producing β-D-fructofuranosidase. Recently, more physiological functions of *Bifidobacterium* spp. have been discovered, making them potentially one of the most prolific producers of enzymes such as β-D-fructofuranosidase. Consequently, investigating β-D-fructofuranosidases from a wider range of *Bifidobacteriaceae* spp. has expanded to explore their potential use in various industries. The novel β-D-fructofuranosidase gene cloned from *Bifidobacteriaceae bacterium* was successfully expressed in the recombinant protein BbFFase9. The particular enzymatic properties of BbFFase9 and potential for hydrolysis to produce invert sugar were then characterized.

3.2. Purification of the recombinant β-D-fructofuranosidase (BbFFase9)

The recombinant β-D-fructofuranosidase (BbFFase9) was successfully expressed in *E. coli*, and could be purified in a soluble band by one step of Ni-IDA affinity chromatography (Fig. 2). The purification process is summarized in Table 1. After purification, a pure enzyme solution with a volume of 16.5 mL was obtained and the purified BbFFase9 exhibited a single, homogenous protein band on SDS-PAGE corresponding to 66.2 kDa. After purification, the specific activity of the recombinant BbFFase9 increased from 145.4 to 209.2 U/mg, a 1.4-fold increase in purification.

The molecular mass of BbFFase9 was similar to those mostly reported for β-D-fructofuranosidases from *Bifidobacteriaceae* spp. in a range of 59–75 kDa, such as those from *B. longum* subsp. *infantis* ATCC 15697 (75 kDa), *B. breve* UCC2003 (60 kDa) [7], *B. lactis* DSM10140^T (59.4 kDa) [8] and *B. infantis* ATCC 15697 (70 kDa) [9]. In comparison, other bacterial β-D-fructofuranosidases, such as those from *Microbacterium trichothecenolyticum* (nearly 66.2 kDa) [3] and *Bacillus subtilis* LYN12 (66 kDa) [15] also exhibited a similar

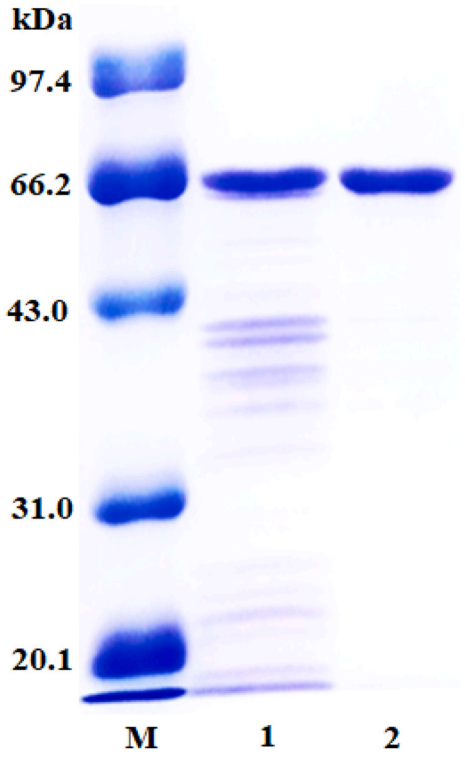

Fig. 2. SDS-PAGE analysis of purified BbFFase9. Lane M, low molecular weight standard protein markers; lane 1, crude lysate; lane 2, purified BbFFase9.

Table 1
Summary of the recombinant β-D-fructofuranosidase (BbFFase9) expressed in *E. coli.*

Purification step	Total activity (U)[a]	Protein (mg)[b]	Specific activity (U/mg)	Purification factor (-fold)	Recovery (%)
crude supernatant	20667.7	142.1	145.4	1.0	100.0%
Ni-IDA	8582.2	41.0	209.2	1.4	41.5%

[a] Activity was measured in 50 mM phosphate buffer (pH 6.5) at 50 °C using 20% sucrose.
[b] The protein was measured by the Lowry method, using BSA as the standard.

molecular mass to that of BbFFase9. However, the molecular mass was dissimilar to those of β-D-fructofuranosidases from fungi, such as *A. oryzae* S719 (95 kDa) [14], *A. niger* (116 kDa) [16], *A. sojae* JU12 (35 kDa) [17] and *A. terreus* (32 kDa) [18].

A high specific activity is a key property for every glycoside hydrolase, because it makes it possible to enhance catalytic efficiency. This novel β-D-fructofuranosidase, BbFFase9, exhibited a higher specific activity (209.2 U/mg) than other β-D-fructofuranosidases extracted from the same genus previously reported, such as *B. adolescentis* G1 (101 U/mg) [19], *B. longum* KN29.1 (106.1 U/mg) [20] and *B. adolescentis* G1(86 U/mg) [21], but a lower specific activity than that from a new species, such as *B. longum* subsp. *infantis* ATCC 15697 (400.5 U/mg) [5].

3.3. Biochemical characterization of BbFFase9

The biochemical properties of BbFFase9 were first characterized: its maximum β-D-fructofuranosidase activity was exhibited at pH 6.5 (Fig. 3a) and at a temperature 50 °C (Fig. 3c). The highest enzyme activity reached to 520.0 U/mL and the relative enzyme activity is calculated based on this value as 100%. Additionally, this recombinant β-D-fructofuranosidase was stable within a pH range of

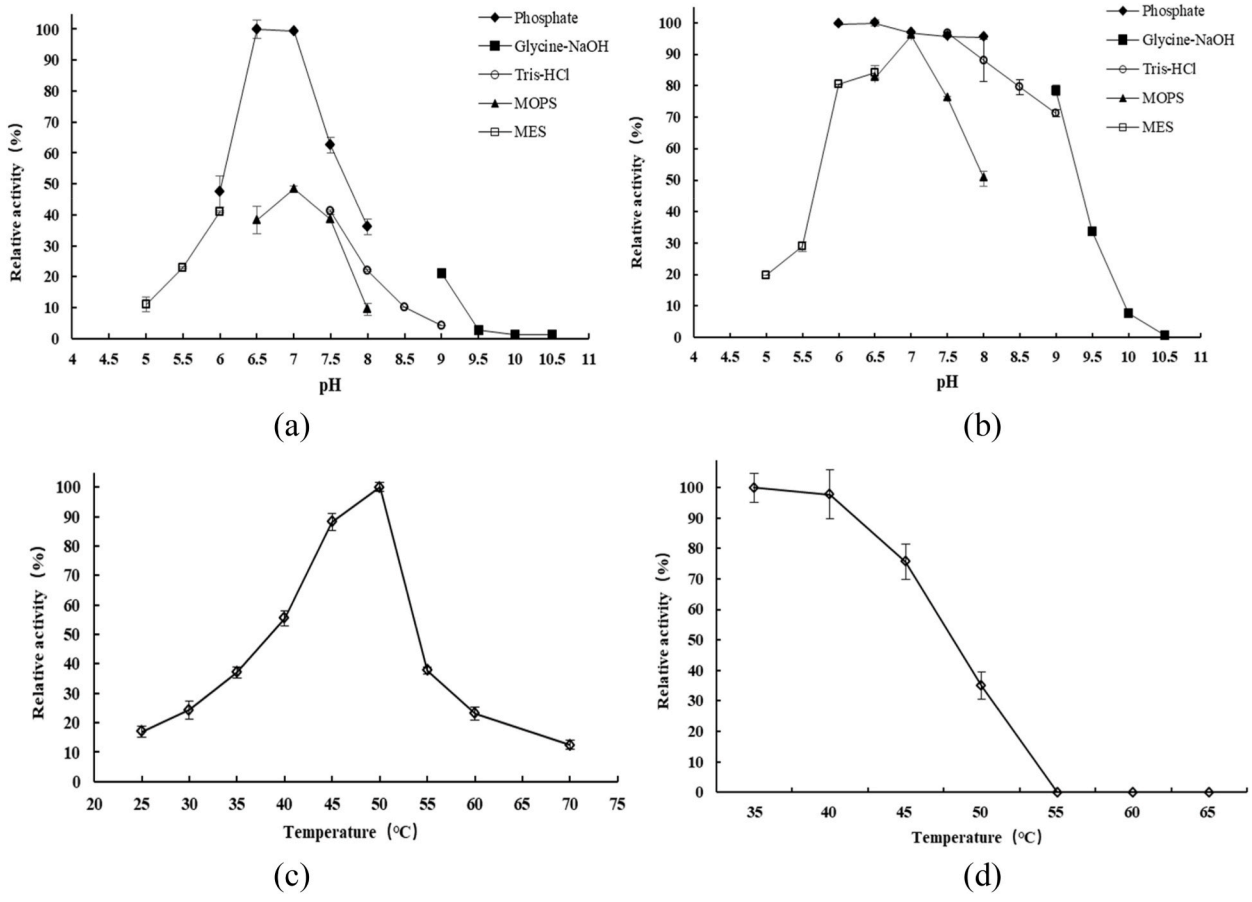

Fig. 3. pH and temperature profiles of BbFFase9. Effect of pH on the activity (a) and stability (b) was performed at 40 °C in 50 mM different buffers: MES (□), sodium phosphate (●), MOPS (▲), Tris-HCl (◆), glycine-NaOH (■). The remaining activities were measured after incubation for 30 min at 40 °C over various pH ranges. Effect of temperature on the activity (c) and thermostability (d) of BbFFase9 were determined at temperatures ranging from 25 °C to 70 °C in 50 mM sodium phosphate buffer (pH 6.5).

6.0–8.0 (Fig. 3b) and up to 40 °C (Fig. 3d), retaining more than 90% of its initial activity.

Generally, β-D-fructofuranosidases from different genera exhibit different optimal pH values, which make them valuable in particular applications. Overall, fungal β-D-fructofuranosidases have an optimal pH in the range of 5.0–6.5 while those from bacteria are in a range from 6.0 to 8.0. BbFFase9, with an optimal pH of 6.5, was similar to that of other bifidobacterial β-fructofuranosidases [3,5], but lower than that of many other bacterial β-fructofuranosidases [22–24], and higher than most fungal β-D-fructofuranosidases from *Candida guilliermondii* [10], *Gongronella* sp. w5 [25] and *Aspergillus* spp. [18,26], which have optimal pH values in the range 5.0–6.0. Despite its optimal acid pH, BbFFase9 exhibited good pH stability between 6.0 and 9.0, making it useful for industrial applications. In contrast, two other reported bifidobacterial β-fructofuranosidases were only stable in a narrow range of pH values from 5.5 to 7.5 [7,8]. Different types of buffer liquid systems have a slight impact on enzyme activity, under the same pH conditions. For example, BbFFase9 can maintain higher stability in both of phosphate and Tris-HCl buffer liquid systems. Additionally, in these two systems, there is less interference with protein activity, making the protein more stable and more conducive to maintaining enzyme activity stability.

Most bifidobacterial β-D-fructofuranosidases previously described have optimal temperatures in a range from 35 °C to 45 °C [7–9,20]. In contrast, BbFFase9 was found to have an optimal temperature of 50 °C, equal to those from *B. longum* [5,20] and *B. adolescentis* [21]. Its optimal temperature was higher than many other bacterial β-D-fructofuranosidases previously reported, such as those from *B. subtilis* (40 °C) [15], *Microbacterium trichothecenolyticum* (35 °C) [3] and *Synechocystis* spp. (30 °C) [22]. BbFFase9 was stable up to 45 °C, similar to that of recombinant β-D-fructofuranosidase from *B. longum* [5] and a little higher than that of several fungal β-D-fructofuranosidase from *Gongronella* sp. w5 [25]. Overall, pH and thermal stability are important as commercially profitable features of an enzyme given that the operation of enzyme-catalyzed reactions at a moderate temperature and weak acidity can reduce energy usage and equipment costs [27].

The effects of various salts on the activity of BbFFase9 β-D-fructofuranosidase were checked (Fig. 4). Among them, the enzyme activity of the untreated blank group is 514.8 U/mL and the relative enzyme activity is calculated based on this value as 100%. Of the salts tested, Cu^{2+} was the only one that completely inhibited the activity of BbFFase9 β-D-fructofuranosidase, while Zn^{2+} ions only moderately inhibited its activity (65.8%). In comparison, the recombinant enzyme could be slightly activated by K^{+} (108.2%), Ca^{2+} (104.8%) and Mg^{2+} (104.6%), but less so by Fe^{3+} (101.5%), Co^{2+} (98.5%), Fe^{2+} (97.4%), Mn^{2+} (96.7%) and Al^{3+} (95.6%).

Different salts have been reported to usually be an important factor influencing the active sites and/or the substrate binding site of various enzymes [13]. In particular, many of the cations mentioned above have been found to inhibit or activate different β-D-fructofuranosidases. Cu^{2+}, shown to inhibit the activity of BbFFase9 β-D-fructofuranosidase, also exhibited a similar inhibitory effect on many other β-D-fructofuranosidases such as InvDz13 from *Microbacterium trichothecenolyticum* [3], GspInv from *Gongronella* sp. w5 [25] and β-D-fructofuranosidase from *B. breve* UCC2003 [7]. A reason for the inhibitory effect of Cu^{2+} ions is that they are known to catalyze the auto-oxidation of cysteines to form intra-molecular disulfide bridges or the formation of sulfenic acid [28]. However, several fungal β-D-fructofuranosidases from *A. sojae* and *A. thermomutatus* have also been reported to be slightly affected by Cu^{2+} ions [17,29]. Zn^{2+} ions can also inhibit the activity of other bifidobacterial β-D-fructofuranosidases from *B. breve* UCC2003 [7], *B. lactis* DSM10140^{T} [8] and *B. infantis* ATCC 15697 [9]. In comparison, the other salts all slightly affected the activity of BbFFase9 β-D-fructofuranosidase with some of them also found to be able to inhibit or activate enzyme activity: Mn^{2+} ions can strongly increase the activity of InvDz13 by up to 432.6% [3]; both Ca^{2+} and Mg^{2+} ions have been found to improve the activity of enzyme from *A. thermomutatus* (122.8%) [29] and *B. subtilis* (127.2%) [15]; Fe^{2+} ions completely inhibited the activity of FTase [14]; and K^{+} ions even inhibited the activity of β-D-fructofuranosidase from *B. subtilis* (66.4%) [15]. Overall, the sensitivity of different β-D-fructofuranosidases to different salts can vary greatly so can have a practical significance on the enzymatic hydrolysis of biomasses that contain particular salts.

3.4. Resistance of BbFFase9 to protease

On testing its proteolysis, BbFFase9 exhibited an excellent tolerance to most of the proteases within 30 min: pepsin (98.4%), acidic

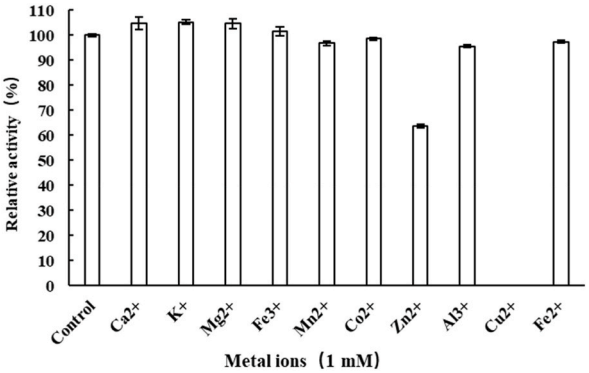

Fig. 4. Effect of different salts on the β-D-fructofuranosidase of BbFFase9.

protease (81.9%), Flavourzyme® (79.3%), trypsin (78.8%) and neutral protease (70.0%) (Fig. 5) but both alkaline proteinase and Proteinase K almost completely denatured the recombinant β-D-fructofuranosidase. After 60 min incubation, BbFFase9 was exposed to seven different proteases, each of which had varying effects on its activity. Acidic protease and pepsin had a mild impact on BbFFase9, with over 50% activity retained after 60 min of exposure, resulting in a relative enzyme activity of 52.5% and 62.7%, respectively. However, protease K and alkaline protease had strong inhibitory effects, with protease K completely inactivating BbFFase9 after 60 min of exposure, leaving only 0.7% of the relative enzyme activity of alkaline protease. Treatment with neutral protease led to a significant decrease in BbFFase9 activity, with the relative activity decreasing from 69.9% (30 min) to 4.0% (60 min) after 60 min of exposure. Flavourzyme® and trypsin also had varying degrees of influence on the activity of BbFFase9. Treatment with Flavourzyme® resulted in a decrease in BbFFase9 activity from 79.3% to 35.9%, while treatment with trypsin led to a decrease in activity from 78.8% to 49.9%.

These observations are significant because, in different food and feed industries, processed products are often supplemented with both proteases and glycoside hydrolases to make protein-rich materials more edible while increasing their nutritional value. However, the soluble enzymes used in these situations face the great challenge of hydrolysis by different proteases. Therefore, the ability of these enzymes to resist these hydrolytic proteases in the same application environment is a crucial factor. As β-D-Fructofuranosidase is a soluble protein, it also faces this challenge in its future applications, but, unfortunately, very few details are available from recent studies. The present study has found for the first time that BbFFase9 strongly tolerated most of the proteases tested. Herein, protease K and alkaline protease were found to affect the enzymatic activity of BbFFase9, attributing to the fact that BbFFase9 is a protein consisting of amino acids such as Ala, Cys, Thr, and Gly, probably. Protease K and alkaline protease have a specific ability to hydrolyze the aliphatic and alkaline amino acids, which may constitute the primary structure forming the enzyme's active site, thus leading to the inhibition of BbFFase9 activity. A similar property has also been found in previous studies on other kinds of glycoside hydrolases such as xylanase [13,30] and glucosidase [31].

3.5. Substrate specificity and action mode of BbFFase9

The substrate specificity of BbFFase9 was determined for potential substrates including various polysaccharides and *p*NP derivatives. The recombinant β-D-fructofuranosidase exhibited the highest specific activity for sucrose, followed by raffinose and stachyose (data not shown). However, it showed no detectable activity with melibiose, mannotriose and the other polysaccharides or *p*NP derivatives tested. To evaluate the potential application of BbFFase9, its enzymatic action on three oligosaccharides, sucrose, raffinose and stachyose, was analyzed (Fig. 6a & Fig. 6b). This showed that BbFFase9 could hydrolyze all the substrates tested to produce an equimolar mixture of fructose and glucose or melibiose or mannotriose, indicating that it had strongly catalyzed the hydrolysis of the α1↔2β glycosidic linkage in molecules containing fructosyl moieties.

The specific activity of BbFFase9 on various possible substrates, including polysaccharides and *p*NP-derivatives which represent different glycosidic linkages, was evaluated, but this β-D-fructofuranosidase was only activated on sucrose, raffinose and stachyose. Most microbial β-D-fructofuranosidases are known because of their high activity towards sucrose, but, unlike many bifidobacterial β-D-fructofuranosidases, information regarding their hydrolytic effect on raffinose or stachyose has been rarely mentioned [19–21]. Only a few bacterial β-D-fructofuranosidases have been reported to exhibit detectable activity towards raffinose, such as those from *M. trichothecenolyticum* [3], the recombinant GspInv from *Gongronella* sp. w5 [25] and *Leuconostoc mesenteroides* [32]. The activities of bacterial β-D-fructofuranosidases toward stachyose have been rarely reported [17]. Thus, the novel substrate specificity of BbFFase9 shows good potential for application in the future. The results on the enzymatic action of BbFFase9 based on testing several oligosaccharides have indicated that the α1↔2β linkage, which is abundant in sucrose, raffinose and stachyose, was exactly the type of bond that this β-D-fructofuranosidase could hydrolyze. During the hydrolysis of sucrose, raffinose and stachyose by BbFFase9, no new or larger oligosaccharides were produced, indicating that this β-D-fructofuranosidase exhibited a typical invertase activity but with no detectable fructosyltransferase activity. In comparison, several other β-D-fructofuranosidases such as those from *A. oryzae* S719 [14] and *A. thermomutatus* [29] exhibited a higher fructosyltransferase activity that could be used for processing fructooligosaccharides.

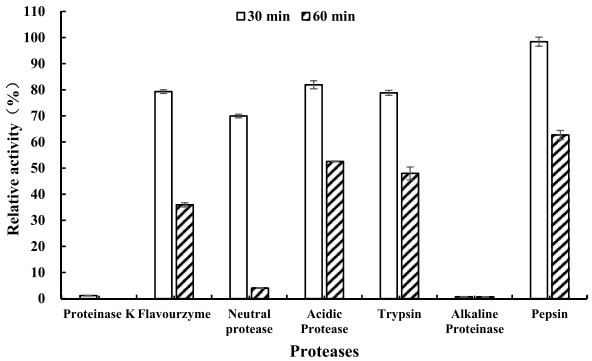

Fig. 5. The ability of β-D-fructofuranosidase BbFFase9 to resistance various proteases.

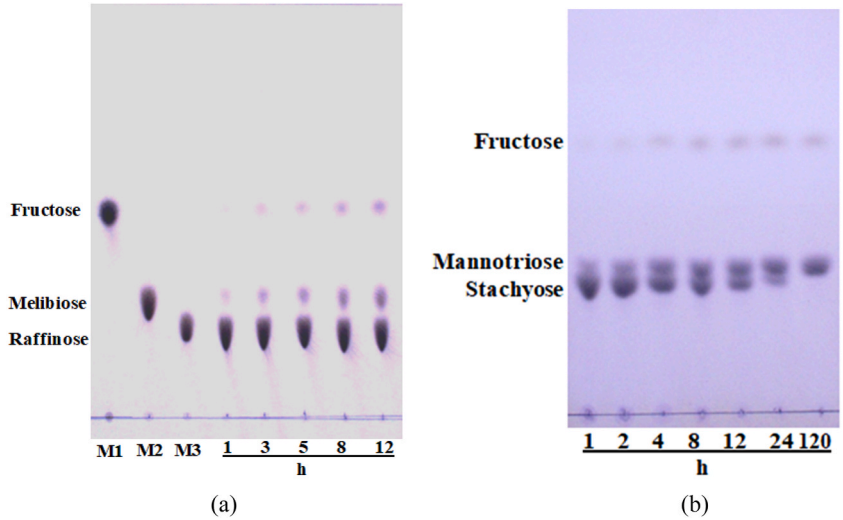

Fig. 6. Hydrolysis of raffinose (a) and stachyose (b) by BbFFase9. The reaction mixture containing 20 mg/mL of substrates in 50 mM sodium phosphate buffer (pH 6.5) and BbFFase9 (5 U/mL) was incubated at 40 °C. Lane M1 (a/b), fructose; Lane M2 (a/b), melibiose/mannotriose; Lane M3 (a/b), raffinose/stachyose.

3.6. Application of BbFFase9 on the hydrolysis of SMOs

The end-products from the hydrolysis of SMOs by BbFFase9 was further studied (Fig. 7) and showed that BbFFase9 could degrade the SMOs directly, leading to the production of mannotriose, melibiose, fructose and glucose. The SMOs could also be completely degraded after commercial α-galactosidase was used for their synergistic hydrolysis with BbFFase9 to produce fructose, glucose and galactose. Following HPLC analysis, the soybean meal treated with BbFFase9 exhibited a melibiose conversion rate of 25.79% and a mannose trisaccharide conversion rate of 14.01%. Notably, BbFFase9 demonstrated strong sucrose hydrolysis capabilities with a conversion rate of 84.24% for invert sugar.

Oligosaccharides, such as stachyose and raffinose form the highest concentration of galacto-oligosaccharides in various soybean products, and possibly lead to abdominal distension in monogastric animals caused by the metabolism of intestinal microorganisms [3]. Thus, the saccharification of these carbohydrates can be a significant factor in improving the nutritive and protein intake values of

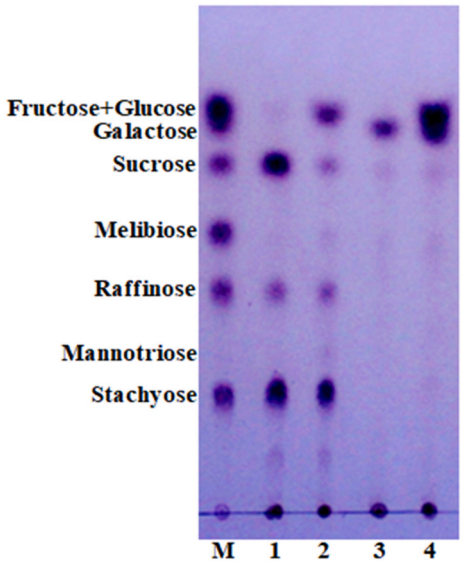

Fig. 7. Degradation of SMOs by BbFFase9. The reaction mixture containing 100 mg/mL of soybean meal in 50 mM sodium phosphate buffer (pH 6.5) and 5 U/mL of enzymes (BbFFase9; α-galactosidase; mixture of BbFFase9 and α-galactosidase) was incubated at 40 °C. Lane M, a mixture of fructose, glucose, galactose, sucrose, melibiose, raffinose, mannotriose and stachyose; Lane 1, crude SMOs; Lane 2, hydrolytic products from SMOs by BbFFase9; Lane 3, hydrolytic products from SMOs by α-galactosidase; Lane 4, hydrolytic products from SMOs by BbFFase9 and α-galactosidase.

different soybean products [31]. Different processing techniques, such as soaking, cooking, germination, and enzymatic hydrolyzation, have been used to remove these galacto-oligosaccharides [33]. For a long time, enzymatic hydrolysis using α-galactosidase to hydrolyze raffinose and stachyose was considered as a promising strategy, because the reactions can frequently be conducted with mild energy usage and hence, cost-saving conditions [34]. Many studies have also found that raffinose and stachyose are typical fructosyl derivatives with α1↔2β glycosidic linkages which can be hydrolyzed by β-D-fructofuranosidase, thus releasing the valuable products, melibiose and mannotriose. These new types of oligosaccharide in soybean have attracted considerable attention because of their beneficial attributes: their potential inhibition of aggregation-mediated neurodegenerative disorders and diseases mediated by polyglutamine [35,36]; their benefits as a high-value additive in human functional foods and pharmaceuticals which maintain and promote good health [37]; their promotion of calcium absorption in the intestines; and their use in curing atopic dermatitis. During the hydrolysis process by BbFFase9, melibiose and mannotriose were clearly detected, a novel advantage for BbFFase9, regarding its potential applications for improving the nutritive values of soybean products. The present study is also a first attempt to discover new applications for this type of β-D-fructofuranosidase, which was only rarely found in β-D-fructofuranosidases from other species [3]. On this basis, under the synergistic hydrolysis of β-D-fructofuranosidase with α-galactosidase, SMOs could be processed into invert sugar which can then be used as a syrup, an ingredient in jams, candy drops and sweets, because of its sweetening properties, superior to those of sucrose, its greater resistance to crystallization, and its higher solubility.

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Author contribution statement

Zhou Chen: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.
Yimei Shen: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Run Wang: Performed the experiments.
Siting Li: Contributed reagents, materials, analysis tools or data.
Yingmin Jia: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors 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.2023.e19889.

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