Pakistan J. Zool., pp 1-9, 2022. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20210403040454

Expression, Purification and Enzymatic Activity of α-1,2 Mannosidase I Derived from *Trichoderma reesei* in *Pichia pastoris*

Siqiang Li[1,2], Tiantian Wang[1], Peng Sun[1], Airong Gao[1], Xin Gong[1], Yuanhong Xu[2], Baogen Wang[2], Jun Wu[1]* and Bo Liu[1]*

[1]*Department of Microorganism Engineering, Beijing Institute of Biotechnology, 20 Dongdajie Street, Fengtai District, Beijing 100071, China.*
[2]*School of Biological and Food Processing Engineering, Huanghuai University, 76 Kaiyuan Road, Zhumadian 463000, China.*

Siqiang Li and Tiantian Wang contributed equally to this article.

Article Information
Received 03 April 2021
Revised 11 May 2021
Accepted 13 June 2021
Available online 23 December 2022 (early access)

Authors' Contribution
JW, BL and SL designed the experiments. TW, AG, PS and XG performed experiments and provided intellectual input. YX and BW contributed intellectual input and generated critical plasmid and strain. SL and TW supervised the study, interpreted the data, and wrote the manuscript.

Key words
α-1,2 mannosidase MDS I, Fermentable expression, *Pichia pastoris*, Enzymatic activity, *Trichoderma reesei*

ABSTRACT

α-1,2 mannosidase I (MDS I) is a desired tool enzyme to modify oligosaccharides and their analogues in structurally homogeneous and defined forms *in vitro*. This study was aimed to explore the acquisition of an effective MDS I *in vitro*. For this purpose a *Pichia pastoris* strain GS115 harboring a recombinant MDS I derived from *Trichoderma reesei* was constructed via conventional molecular cloning methods, and expressed in a 5-liter fermentation tank. The target protein was purified in three-step purification and identified by peptide mass of fingerprint. The enzymatic activity and optimal reaction conditions of MDS I were detected using DNA sequencer-assisted fluorophore-assisted carbohydrate electrophoresis. We obtained MDS I with a purity exceeding 90% in gram scales, which was capable of digesting α-1,2 linked mannose residues in high selectivity. The highest enzymatic activity of MDS I occurred at a pH of 7.0 and a temperature of 42°C. Enzymatic activity of MDS I was also influenced by metal ions, which were increased to 22% and 17%, respectively, by Co^{2+} and Cu^{2+} (2 mmol/L each), while were inhibited to some extent by Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}. This study has laid the foundation for the application of MDS I in future glycol-engineering research.

INTRODUCTION

The methylotrophic yeast *Pichia pastoris* has been widely used to produce various recombinant heterologous proteins due to several advantages (Higgins and Cregg, 1998), for example, it has a strong and inducible promoter system that is capable of producing proteins at high yields in relatively inexpensive growth media (Bretthauer and Castellino, 1999). However, N-glycosylation of *P. pastoris* expressed proteins are significantly different from mammalian proteins, which led to the limitations of *P. pastoris*. N-glycosylation is one of the most frequent post-translational modifications, which forms the high-mannose-type N-glycans in *P. pastoris* that differs dramatically from those complex and hybrid types N-glycans in humans (Khan *et al*., 2017; Hamilton and Zha, 2015). The high-mannose-type N-glycans probably hamper downstream processing of secreted glycoproteins and result in protein-based therapeutic agents rapidly cleared from blood (Zhou and Qiu, 2019). Moreover, naked mannoses also lead to a short half-life of protein-based therapeutic agents *in vivo*. That is, poor pharmacokinetic behavior and (or) adverse immune response are generally more likely produced, when glycoproteins with high-mannose-type N-glycans were developed as therapeutic agents in humans (Zhou and Qiu, 2019). Thus, the high-mannose-type N-glycans in *P. pastoris* is necessary to be engineered into complex and hybrid types in *vivo/vitro*.

Biosynthesis of N-glycans begins in the endoplasmic reticulum (ER) by forming a highly conserved $Man_8GlcNAc_2$ intermediate (Hamilton and Zha, 2015; Khan *et al*., 2017). Then $Man_8GlcNAc_2$ are modified via two different enzymes and finally formed significance different N-glycans in Golgi apparatus from humans to yeast. Specifically, three α-1,2 mannose residues are removed from $Man_8GlcNAc_2$ to form $Man_5GlcNAc_2$ intermediate that serves as a precursor for complex and hybrid N-glycans in humans, whereas an α-1,6 mannose residue is added to $Man_8GlcNAc_2$ intermediate to form $Man_9GlcNAc_2$ by α-1,6 mannosyl transferase and further increased to a high-mannose type N-glycans in yeast

* Corresponding author: junwu1969@163.com; liubo7095173@163.com
0030-9923/2021/0004-0001 $ 9.00/0

(Hamilton and Zha, 2015; Khan *et al*., 2017). Therefore, to block the high-mannose type and facilitate the formation of human-like N-glycans in yeast, it is necessary to obtain $Man_5GlcNAc_2$ intermediate via elimination of α-1,2 linkage from $Man_8GlcNAc_2$. α-1,2 mannosidase I (MDS I) catalyzes the removal of α-1,2 mannose residues from $Man_8GlcNAc_2$ and produces the precursor of human-like N-glycans (Khan *et al*., 2017; Hamilton and Zha, 2015). Thus, MDS I is more likely to be developed as a potential tool to obtain $Man_5GlcNAc_2$ intermediate in yeast. Furthermore, MDS I is also a potential tool enzyme for preparation of the oligosaccharides and their analogues in structurally homogeneous and defined forms *in vitro*, which will be highly significant to study the structure-function relationship of natural and modified N-glycans (Aikawa *et al*., 2014). For example, N-glycans derived from bovine ribonuclease B (Rnase B) were prepared in structurally homogeneous and defined forms, which were used as a standard substance for detecting N-glycans (Hamilton *et al*., 2003). Consequently, the studies on MDS I are beneficial to not only the formation of human-like N-glycans in yeast, but also the preparation of oligosaccharides in structurally homogeneous and defined forms *in vitro*.

In this paper, MDS I from *Trichoderma reesei* was heterologously expressed via wildtype *P. pastoris* GS115 (*his4*$^-$) in a five liters fermenter. The enzyme was nearly homogeneous purified and its reaction conditions were optimized, which made a foundation for development and application of MDS I in future.

MATERIALS AND METHODS

Bacterial strains, yeast strains, plasmids and other materials

Plasmid pAO815α-M harboring synonymous mutations of *mds I* gene (Genbank: AF212153, the 944th G to A) from *T. reesei* was prepared in our lab (Zhan, 2004). *P. pastoris* GS115 (*his4*$^-$) was obtained from Invitrogen, *E. coli* DH5α from Cwbiotech; yeast extract, agar and tryptone from OXOID; yeast nitrogen base without amino acids (YNB) from Difco; T4 DNA ligase, DNA ladder (500-15000 bp), restriction endonuclease, and DNA polymerase from New England Biolabs (NEB); low molecular weight protein marker (17-0446-01) from GE, DNA extraction kit from Cwbiotech were purchased, respectively. Other reagents were obtained from Sangonbiotech unless stated otherwise.

Expression vector construction

The DNA sequence of complete *mds I* gene was derived from the plasmid pAO815α-M and employed to design primers for polymerase chain reaction (PCR) amplifications. The primers were synthesized by Sangonbiotech. PCR were performed with a Biometra PCR Amplifier using 2.5 unit of Prime STAR HS DNA polymerase, 10 μg/mL of template DNA (pAO815α-M plasmid), 0.4 mmol/L of each deoxynucleotide triphosphate (dNTP), and 0.4 μmol/L of corresponding synthetic nucleotide primers (forward primer: AAGAGAGGCTGAAGCTTATCCAAAGCCGG GCGCCACAAAAC and reverse primer: *GAATTC*TAA*CAACTCGTCGTG*AGCAAGGTGGCCGC CCCGTCG, *Xho* I and *EcoR* I were marking with underline). The amplified DNA fragments were digested with *Xho* I and *EcoR* I and inserted into pPIC9 plasmid that contained signal sequence of α-mating factor from *Saccharomyces cerevisiae*, which digested with the same restriction endonucleases. Then recombinant molecules were transformed into the *E. coli* DH5α competent cell. The transformants were selected on Luria-Bertani/Ampicillin (LB/Amp) plates (1% tryptone, 0.5% yeast extract, 1% sodium chloride, 1.5% agar and 0.01% Amp). The *mds I* gene was verified by digestion with restriction enzymes and DNA sequencing. The resulting plasmid was named pPIC9-α-M.

Transformation

The *P. pastoris* strain GS115 (*his4*$^-$) was cultured overnight to the early exponential phase in YPD medium (2% glucose, 1% yeast extract, 2% peptone). The harvested cells were thoroughly washed with ice-cold water twice and then resuspended in ice-cold 1 mol/L of sorbitol. The recombinant plasmid pPIC9-α-MDS I was digested by *Sac* I to directly integrate into chromosome of the *P. pastoris* by electroporation with Gene Pulser (Invitrogen). The transformed cells were plated on a screening plate MD/UR (1.5% agar, 1% YNB, 1% glucose, 4×10^{-5}% biotin, 0.1% uracil and 0.01% arginine) after incubated for 1 h in 700 μL 1mol/L of sorbitol at 25°C. 5 days later, single colonies were isolated from selected plates and were confirmed by PCR.

Expression of MDS I

The recombinant yeasts were seeded into YPD medium (3 mL) with shaking in glass tubes at 300 rpm and 25°C for 48 h. Then 1 mL cultures from this YPD medium were transferred into 100 mL YPD initial volume (at a 1% volumetric ratio) and incubated at 300 rpm and 25°C. After 24 h, the seed flasks obtained property optical density (OD_{600} = 10) to ensure that cells were growing exponentially upon transfer.

Cultivation was further carried out in a 5 L glass bench-top Biostat-B bioreactor (Sartorius-Stedim). Specifically, 3 L of initial media (BSM) with 10% volumetric ratio of seeds were inoculated in bioreactor.

Cultivations were performed in fed batch mode under the following conditions: 24°C, pH=6.0 (controlled by addition of 30% ammonium hydroxide), dissolved oxygen (DO) >20% (maintained by adjusting the agitation speed from 300 to 1,200 rpm), and constant cascading agitation and aeration. After the depletion of initial glycerol (40 g/L) in BSM medium (indicated by a spike in DO), a glycerol feed was initiated to increase the cell biomass under limiting conditions. Glycerol was added as a 50% solution containing 12 mL/L of PTM1 salts (6.0 g/L of $CuSO_4$•5 H_2O, 3.0 g/L of $MnSO_4$•H_2O, 65 g/L of $FeSO_4$•7 H_2O, 20 g/L of $ZnSO_4$•7 H_2O, 0.5 g/L of $CoCl_2$·6 H_2O, 0.2 g/L of $NaMoO_4$·2 H_2O, 0.1 g/L of KI, 5 mL/L of H_2SO_4) and 2 mL/L of biotin until wet cells weight reached about 230 g/L, and the feed rate was set to 48 mL/h. The whole glycerol fed-batch phase was lasted for 5 hours. In methanol induction phase, a solution of 100% methanol contained 12 ml/L of PTM1 salts and 2 mL/L of biotin, was added initially after a 30 min starvation phase when methanol was fed exponentially from 2.4 mL/h until reached the maximum rate 12 mL/h. Then the entire induction phase was conducted by methanol pulse with 0.5% methanol. After DO decreased to <10% the first shot of methanol was delivered. All subsequent methanol shots were triggered by rapid increases in DO that indicated methanol depletion. The entire induction process was lasted for 120 h. Samples of fermentation supernatant were taken every 12 h and *P. pastoris* biomass analysis was determined by measurement of OD_{600}.

Purification of MDS I

The fermentation broth was separated by centrifugation (20 min, 15000 g, 4°C) and deposits were removed. After the addition of ammonium sulfate to 80% saturation into supernatant, the total proteins were purified via centrifuged at 10000 g for 20 min. The total proteins were dissolved in 1 mol/L of ammonium sulfate at pH 7.5, and further purification by a Phenyl-HP column (1.6 cm×20 cm). The Phenyl-HP column was pre-equilibrated with solution A (1 mol/L of ammonium sulfate solution containing 20 m mol/L of Tris-HCl pH 7.5) and eluted by solution B (20 m mol/L of Tris-HCl, pH 7.5). Finally, desalting was performed at 4°C via Sephadex G25 (2.5 cm×30 cm) column. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and peptide mass fingerprinting (PMF, target protein was digested by trypsin and analyzed by MALDI-TOF-MS) were performed to confirm the purified proteins. Besides, the purity of protein was analyzed via SDS-PAGE. The integral optic density (IOD) of each protein band was measured via an Image pro plus 6.0. With the IOD ratios of the MDS I band to the total protein bands in the lane, the relative purity of the target protein was calculated.

Enzymatic activity assays of MDS I

For the release of N-glycans, 550 μL of RNase B (0.5 mg) containing 0.5% SDS and 1% β-mercaptoethanol were incubated in boiling water for 5 min, after cooling at room temperature, NP-40, sodium phosphate (pH 7.0) and PNGase F were added at final concentration of 1%, 50 mmol/L and 30 U/mL, respectively, and then incubated at 37 ℃ for 18 h. The reaction solution was precipitated with precooled acetone (-20°C) by centrifugation (12,000 g, 10 min). The supernatant was discarded and the precipitate was extracted twice with pre-cooled methanol. The sugar chains were freeze-dried in vacuum.

For the label of N-glycans, N-glycans and 2 μL of labeling buffer (20 mmol/L of 8-amino-1,3,6-pyrenetrisulfoacid, APTS was dissolved in DMSO which mixed with equal volumes of 1.2 mol/L of citric acid and 1 mol/L of $NaCNBH_3$, prepare fresh) were mixed and incubated at 37°C for 18 h. The labeled N-glycans were purified by Sephadex G10 in water.

For the enzymatic activity assays of MDS I, 4 μL of reaction mixtures containing of 0.4 μL sodium phosphate buffer (50 mmol/L, pH=7.0), 2.6 μL of N-glycans (from 0.05 mg of RNase B) and 1 μL of MDS I (8 μg/mL). Reaction mixtures were carried out at 37°C for 1h before quenching. Negative controls were conducted under the same conditions except for adding heat-deactivated MDS I in replacement of the native enzyme. In the study of pH influence on the enzymatic reaction, all N-glycans prepared from 0.05 mg RNase B, 8 μg/mL of MDS I, 100 μg/mL BSA were added into reaction buffers which were 50 mmol/L of citric acid/sodium citrate at pH 3-5, sodium phosphate at pH 7-9 and glycine/NaOH at pH 11, respectively. The reaction was carried out at 37°C for 1h before quenching. In the study of temperature influence, the sodium phosphate buffer (pH 7.0) with all N-glycans from 0.05 mg of RNase B and 8 μg/mL of MDSI, were incubated at 16, 37, 42 and 60 °C, respectively, for 1 h before quenching. To evaluate the influence of metal ions on the activity of MDS I, the reaction was carried out in sodium phosphate buffer (pH 7.0) containing all N-glycans from 0.05 mg of RNase B, 8 μg/mL of MDSI, with 10 mmol/L of various cations, respectively, including EDTA, Cu^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+} and Ca^{2+}. The reaction mixture was incubated at 37 °C for 1 h before quenching. All results were repeated three times.

The reactions were monitored by DNA sequencer-assisted fluorophore-assisted carbohydrate electrophoresis (DSA-FACE) with an ABI DNA sequencer (Liu *et al*., 2009). Specifically, 1 μL of prepared samples, 8 μL of deionized formamides and 1 μL of the ROX-labeled

Genescan™ 500 standards were added into reactions. Alternatively, a mixture containing a rhodamine-labeled 6-,30-meric oligonucleotide (consisting of 5'-TAC-3' basic sequence repeats, which were synthesized and PAGE-purified by Life Technologies) was also used as internal reference of sequencer. Analyses were performed using the Genescan 3.7 software.

RESULTS AND DISCUSSION

MDS I was existed widely in plenty of cells and proposed to involve in biosynthesis of hybrid and complex N-glycans (Amann *et al*., 2019). The data obtained from *T. reesei* (Maras *et al*., 1999), *P. citrinum* (Yoshida *et al*., 1993), mammalian (Amann *et al*., 2019) and insect (Moremen *et al*., 1994) made it clear that MDS I catalyzed the reactions of removal of three α-1,2 mannose residues from $Man_8GlcNAc_2$ to form $Man_5GlcNAc_2$ intermediate. Due to the unique enzymatic function of MDS I, it was desirable to develop to be a tool enzyme in glycobiology and glycol-chemistry fields. In present study, an effective expressive vector, fermentation expressive, purification and enzymatic activity assays of MDS I were performed, which will be discussed next.

Cloning, expression and purification of MDS I

Vector construction

To express *mds I* gene derived from *T. reesei* in *P. pastoris* and illustrate the function of the gene product, the expression vector was constructed in this study by considering the following factors: 1) the *mds I* gene was inserted into *P. pastoris* secretory expression vector pPIC9 under a AOX promoter (Fig. 1A), to control expression of *mds I* gene product; 2) α-mating factor was utilized as signal peptide to facilitate the secrete of heterologous proteins (Richter *et al*., 2006). The 1.5 kb of *mds I* gene was amplified from pAO815α-M (Fig. 1b) via PCR and inserted into pPIC9 (Fig. 1A); 3) when the plasmid pAO815α-M was constructed in previous study, a synonymous mutation of natural *mds I* gene that the 944th base of G had been changed into A by site directed mutation, to promote the use of *Xho* I restriction enzyme (Zhan, 2004), besides, *mds I* gene in present study was from pAO815α-M, deservedly harboring this synonymous mutation. The entire gene of *mds I* was confirmed by restriction enzyme digestion (Fig. 1C) and DNA sequencing that the desired gene was made and no other mutations. The desired vector pPIC9-α-M was linearized with *Sac* I and transformed into *P. pastoris* strain GS115 (*his4*-) via electroporation.

Expression of MDS I

The transformants were selected via expression of *his4* by vector pPIC9-α-M, determined by PCR and then expressed in 5 mL of BMGY medium. For expression of MDS I, high expression potential transformant was selected from dozens of transformants via shake flask culture (data not shown), which was further incubated in a 5-L glass bioreactor. The fermentation conditions probably affected protein expression levels. For example, the maximum oxygen uptake rate and methanol concentration which determined OD and induction expression of strain were most likely influenced protein expression level (Berdichevsky *et al*., 2011). Although fermentation conditions were not optimized further in this study, the production was increased approximately 30-fold compared to previous report (Maras *et al*., 1999). The expression levels of MDS I accumulated progressively during the first 108 h after methanol induction (Fig. 2). Notably, MDS I had already expressed after 12 h induction, the expression levels were improved with the prolonging of time. In addition, OD values of fermentation broth were adjusted the same level to improve reliable of the protein production via SDS gels.

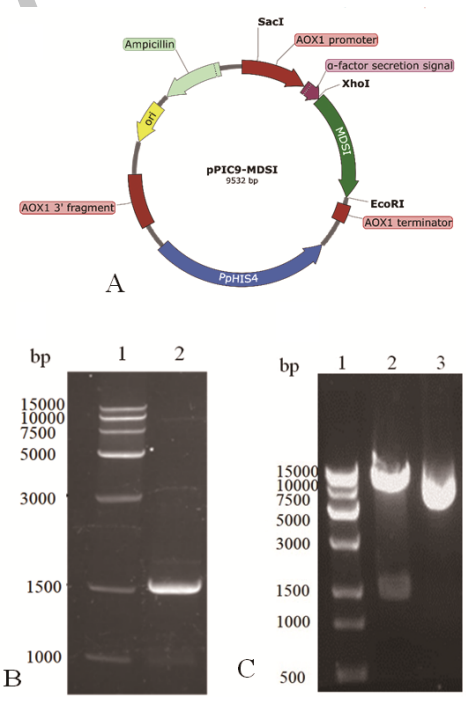

Fig. 1. Construction of the expression vector pPIC9-α-M, A) schematic diagram of the expression vector pPIC9-α-M; B) PCR amplification of *mds I* gene from pAO815α-M, molecular marker (lane 1), *mds I* gene (lane 2); C) dentification of pPIC9-α-M by enzyme digestion, molecular marker (lane 1), pPIC9-α-M digested by EcoR I and XhoI (lane 2) and circle plasmid pPIC9-α-M as control (lane 3).

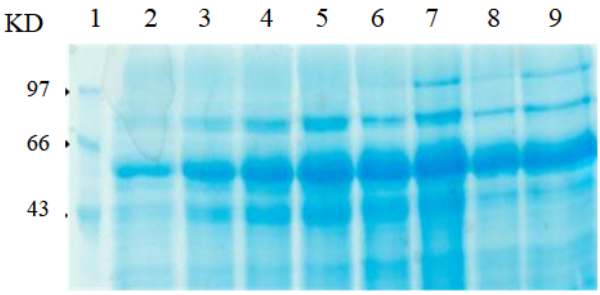

Fig. 2. Expression level analysis of the MDS I in the 5-liter fermentation culture supernatant at different time points by SDS-PAGE, molecular marker (lane 1), 12 h (lane 2), 24 h (lane 3), 48 h (lane 4), 60 h (lane 5), 72 h (lane 6), 84 h (lane 7), 96 h (lane 8), 108 h (lane 9).

Purification of MDS I

The secreted MDS I in the supernatant was purified using a three-step purification protocol as described in methods. Due to the amounts of fermentation broth was too large (3L), total proteins were firstly precipitated with ammonium sulfate. About 80% total proteins were precipitated for further purification (Table I). SDS-PAGE studies of purified proteins showed that the molecular weights (56 kD) agreed with the predicted molecular weights of MDS I (Fig. 3). After Phenyl-HP column purification, the protein was essentially homogeneous (Fig. 3). After the protein concentrations of each purification step were detected and summarized in Table I. About Sephadex G25 purification, 60 mg of the target product with a purity of 90% from 100 mL of the supernatant was obtained.

Table I. Purification of MDS I from 100 mL supernatant.

Purification steps	Total volum (mL)	Total protein (mg)	Purity (%)
Supernatant	100	516±65	27±3
Ammonium sulfate precipitation	100	481±32	30±4
Phenyl-HP	25	71±8	77±8
Sephadex G25	38	60±5	90±3

PMF analysis of expressive product

Initially, the expressive product was identified as MDS I by detecting molecular mass via SDS-PAGE. 12% SDS-PAGE showed a major band of about 56 KDa, which corresponded to the theoretical molecular weight of MDS I that consisted of 523 amino acids (Fig. 3). To further analysis of expressive product, the desired band was digested by trypsin and analyzed by MALDI-TOF-MS. Approximately 32% of the peptide sequence was matched well with an identified MDS I from *T. reesei* (Fig. 4), in addition to protein scores greater than 74 are significant (P <0.05), provided defined evidence that the obtained product was MDS I. Thus, nearly homogeneous and gram levels of MDS I had been obtained in our lab, whose enzymatic properties were investigated and would be discussed next.

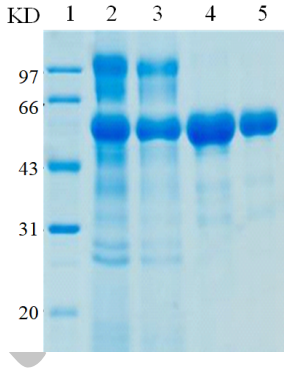

Fig. 3. SDS-PAGE analysis of the purified MDS I, molecular marker (lane 1), 10 μL MDS I from raw liquid (lane 2) and each elution after purification from ammonium sulphate precipitation (lane 3), Phenyl-HP (lane 4), Sephadex G25 (lane 5) was subjected to the 12% SDS-PAGE and stained by Coomassie blue.

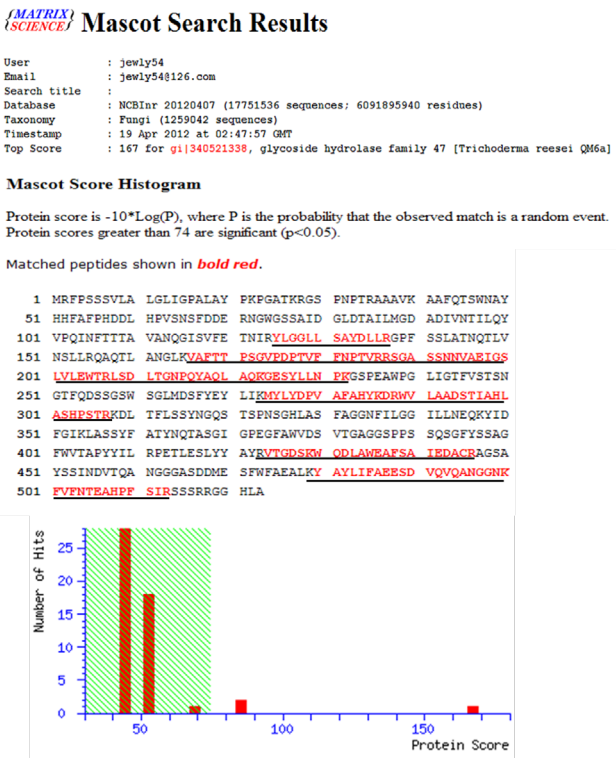

Fig. 4. PMF analysis of the expressive product.

Enzymatic properties of MDS I

One of the most difficulties in studying the enzymatic properties of glycosyltransferases or glycosidases was preparation of their substrates *in vitro* (Kang *et al*., 2019; Wang *et al*., 2019; Li *et al*., 2016). In this study, N-glycans of RNase B was selected as substrates of MDS I mainly because: 1) RNase B is a kind of commercially available glycoproteins; 2) RNase B is slightly heterogeneous ranged from 14.9 kDa to 15.5 kDa due to oligomannose-type N-glycans that are comprised of a family of several glycoforms, however, each protein molecular contains only one N-glycan; 3) the difference of glycoforms denoted as $Man_9GlcNAc_2$ through $Man_5GlcNAc_2$ was caused by α-1,2 mannose that can be removed via MDS I (Fig. 5) (Tarelli *et al*., 2000); 4) analysis of all five glycoforms from $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ is a mature technology in our lab (Yang *et al*., 2011; Liu *et al*., 2009). However, due to the acquisition of N-glycans from RNase B was too little, besides, the purification and labeling process of N-glycans were relatively complex and expensive, the quantification of N-glycans was not easy to access. Thus, the studies on enzymatic properties of MDS I are extremely limited, such as V_{max} and K_m.

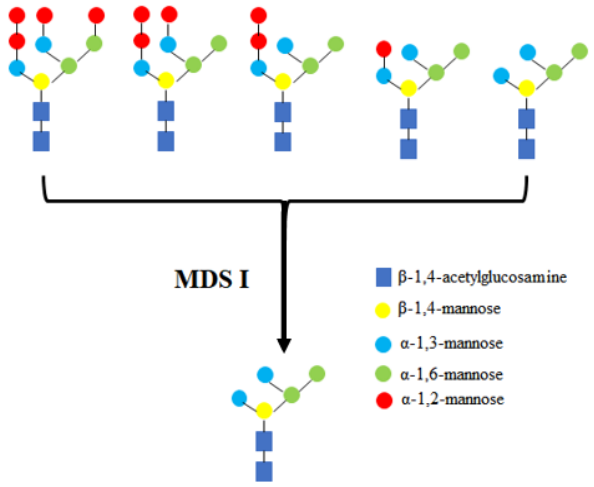

Fig. 5. Five glycoforms of RNase B and MDS I catalyze reaction.

To confirm the enzymatic activity of MDS I, its catalyzed reaction product was isolated, labeled and analyzed as described in methods. DSA-FACE showed the product was $Man_5GlcNAc_2$ due to the peak of product was agreed well with that of the ROX-labeled Genescan™ 500 standards. This study proved unambiguously evidence that MDS I could catalyze the elimination of α-1,2 linked mannose residues (Fig. 6). Although the substrates of MDS I

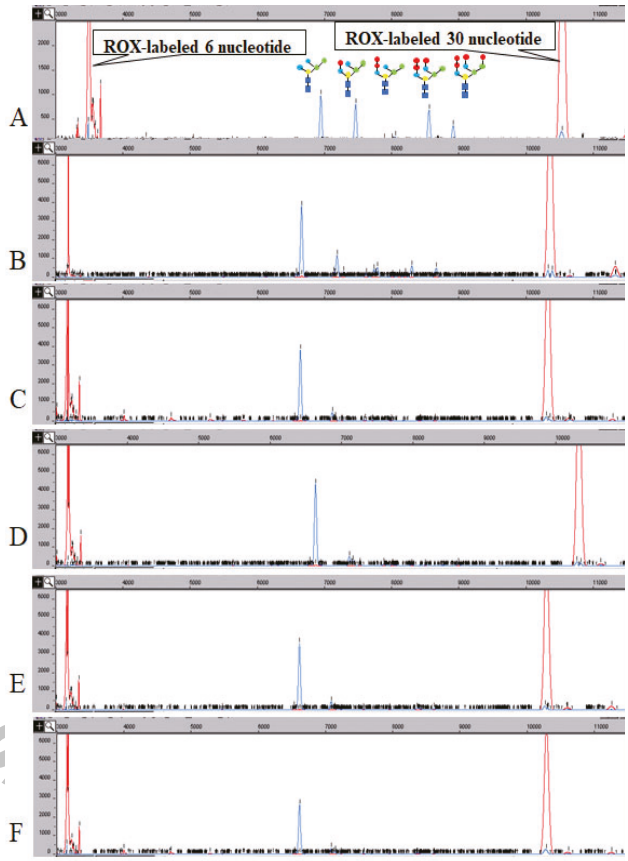

Fig. 6. The effect of pH on the activity of MDS I, control (A), pH 3.0 (B), pH 5.0 (C), pH 7.0 (D), pH 9.0 (E) and pH 11.0 (F).

contained α-1,2-, α-1,3-, α-1,6- and β-1,4- linked mannoses, only α-1, 2 linked mannoses were trimmed, suggested MDS I probably had strict selective activity to α-1,2 linked mannose. Besides, the reaction products were single $Man_5GlcNAc_2$ glycoform or five different glycoforms ($Man_5GlcNAc_2$, $Man_7GlcNAc_2$, $Man_7GlcNAc_2$, $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$), depended on the amounts of MDS I in reaction mixtures and reaction times. For example, the products of reactions were $Man_5GlcNAc_2$-$Man_9GlcNAc_2$ when mixtures contained 8 μg/mL of MDS I that were carried out for 1h, however, only $Man_5GlcNAc_2$ was produced when mixtures contained 16 μg/mL of MDS I that were carried out for 1h. To optimize the reaction conditions for MDS I, it was treated with RNaseB N-glycans at varied pH (Fig. 6), temperatures (Fig. 7), and metal ions (Fig. 8). The reactions were monitored using DSA-FACE. As depicted in Figure 9A, MDS I had maximal catalytic activity at pH 7.0. However, MDS I tolerated a relatively wide range of pH, as 50% of its activity was retained from 5.0 to 9.0. Besides,

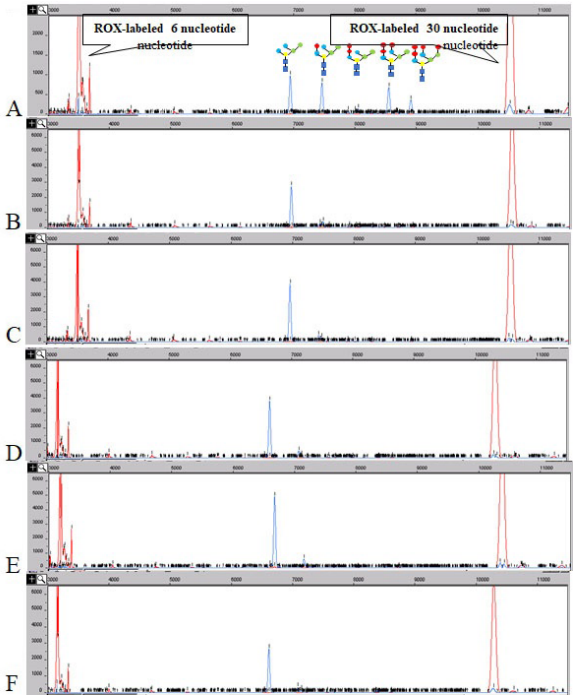

Fig. 7. The effect of temperature on the activity of MDS I, control (A), 16 ℃ (B), 25 ℃ (C), 37 ℃ (D), 42 ℃ (E) and 60 ℃ (F).

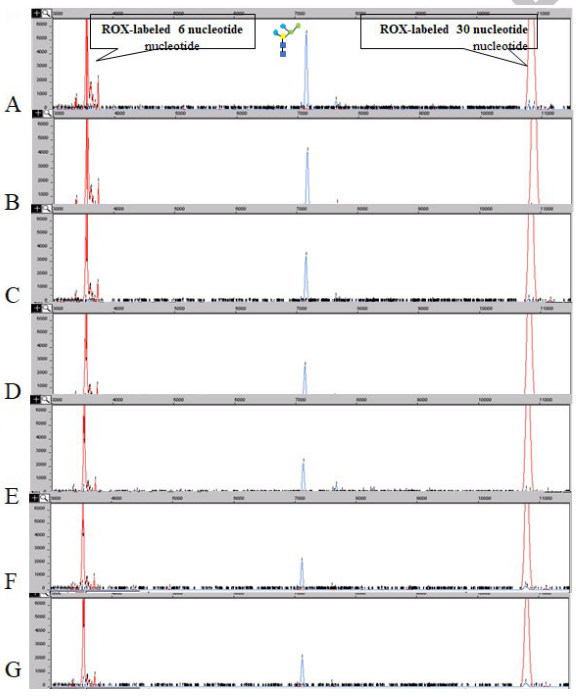

Fig. 8. The effect of metallic ions on the activity of MDS I, Cu^{2+} (A), Co^{2+} (B), Zn^{2+} (C), Mn^{2+} (D), control (E), Mg^{2+} (F) and Ca^{2+} (G).

the optimum temperature of MDS I was 42 ℃ (Fig. 9B) but retained 50% of activity from 25 to 60 ℃, which indicated this enzyme tolerated a relatively wide range of temperatures. The optimized pH and temperature were obtained for MDS I in this study, was valuable for application research of this enzyme in future. It was also found that divalent cations were not necessary for the biological activities of MDS I. However, the activities were increased to 22% and 17%, respectively, by Co^{2+} and Cu^{2+} (2 mmol/L each), while were inhibited to some extent by Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} (Fig. 9C). Although this is not the first report for research on MDS I derived from *T. reesei* in *P. pastoris*, our research also has some positive significance (Maras *et al*., 1999; Van Petegem *et al*., 2001). For example, this study has established a straightforward, efficient and economical strategy for acquisition of an effective MDS I by a 5-L fermentation expression, which laid a foundation for preparation of this enzyme in a large amount. Considering the high demands for tool enzymes in glycobiology and glycol-chemistry fields, fermentation expression studies of MDS I are significant. In addition, this study had provided optimum reaction conditions for MDS I, such as reaction temperature, pH, ions, were valuable for future study. Therefore, this paper on expression, purification and enzymatic activities of MDS I derived from *T. reesei* in *P. pastoris*, laid foundations for application of MDS I in future glycol-engineering research.

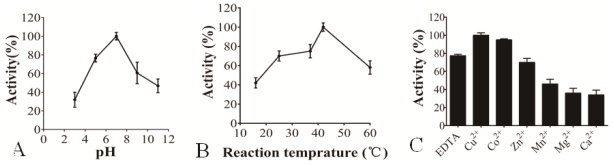

Fig. 9. The influence of pH (A), temperature (B), cation (C) on the reaction of RNase B N-glycans catalyzed by MDS I.

CONCLUSIONS

This study has established a straightforward, efficient and economical strategy for acquisition of an effective MDS I derived from *T. reesei*. For example, approximately gram levels of MDS I were obtained via a methanol induced expression vector pPIC9 with an α-mating factor contributed to the secretion of protein into the extracellular matrix, *P. pastoris* strain GS115 (*his4*$^-$) and 5-L fermentation expression in our lab. Moreover, MDS I was purified and examined in detail, which provided the direct biochemical evidence for the function of the enzyme and formed the foundation for more in-depth studies.

Overall, easy access to the high purity and effective of MDS I would promote applied and commercialize of the enzyme in the field of glycobiology and glycol-chemistry.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (NSFC) [No. 81673339 and No. 81773619], National Science and Technology Major Projects [2018ZX09J18105-003 and 2018ZX09101005-013-002], the Natural Science Foundation of Henan Province (212300410203), the Scientific Research Foundation for Advanced Talents of Huanghuai University (No.1000.12.01.1678) and the Preparation for the National Natural Science Foundation of Huanghuai University [No. 1000. 11.07.1678.1002].

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Aikawa, J., Takeda, Y., Matsuo, I. and Ito, Y., 2014. Trimming of glucosylated N-glycans by human ER alpha1,2-mannosidase I. *J. Biochem.,* **155:** 375-384. https://doi.org/10.1093/jb/mvu008

Amann, T., Hansen, A.H., Kol, S., Hansen, H.G., Arnsdorf, J., Nallapareddy, S., Voldborg, B., Lee, G.M., Andersen, M.R. and Kildegaard, H.F., 2019. Glyco-engineered CHO cell lines producing alpha-1-antitrypsin and C1 esterase inhibitor with fully humanized N-glycosylation profiles. *Metab. Eng.,* **52:** 143-152. https://doi.org/10.1016/j.ymben.2018.11.014

Berdichevsky, M., D'Anjou, M., Mallem, M.R., Shaikh, S.S. and Potgieter, T. I., 2011. Improved production of monoclonal antibodies through oxygen-limited cultivation of glycoengineered yeast. *J. Biotechnol.,* **155:** 217-224. https://doi.org/10.1016/j.jbiotec.2011.06.021

Bretthauer, R.K., Castellino, F.J., 1999. Glycosylation of *Pichia pastoris*-derived proteins. *Biotechnol. Appl. Biochem.,* **30:** 193-200. https://doi.org/10.1111/j.1470-8744.1999.tb00770.x

Hamilton, S.R. and Zha, D., 2015. Progress in yeast glycosylation engineering. *Meth. mol. Biol.,* **1321:** 73-90. https://doi.org/10.1007/978-1-4939-2760-9_6

Hamilton, S.R., Bobrowicz, P., Bobrowicz, B., Davidson, R.C., Li, H., Mitchell, T., Nett, J.H., Rausch, S., Stadheim, T.A., Wischnewski, H., Wildt, S. and Gerngross, T.U., 2003. Production of complex human glycoproteins in yeast. *Science,* **301:** 1244-1246. https://doi.org/10.1126/science.1088166

Higgins, D.R. and Cregg, J.M., 1998. Introduction to *Pichia pastoris*. *Meth. mol. Biol.,* **103:** 1-15. https://doi.org/10.1385/0-89603-421-6:1

Kang, W., Ma, T., Liu, M., Qu, J., Liu, Z., Zhang, H., Shi, B., Fu, S., Ma, J., Lai, L., He, S., Qu, J., Wing-Ngor, A.S., Ho, K.B., Yu, L.W., Deng, Z., Xia, J. and Liu, T., 2019. Modular enzyme assembly for enhanced cascade biocatalysis and metabolic flux. *Nat. Commun.,* **10:** 42-48. https://doi.org/10.1038/s41467-019-12247-w

Khan, A.H., Bayat, H., Rajabibazl, M., Sabri, S. and Rahimpour, A., 2017. Humanizing glycosylation pathways in eukaryotic expression systems. *World. J. Microbiol. Biotechnol.,* **33:** 4. https://doi.org/10.1007/s11274-016-2172-7

Li, S., Wang, H., Ma, J., Gu, G., Chen, Z. and Guo, Z., 2016. One-pot four-enzyme synthesis of thymidinediphosphate l rhamnose. *Chem. Commun.,* **52:** 13995-13998. https://doi.org/10.1039/C6CC08366H

Liu, B., Gong, X., Chang, S., Yang, Y., Song, M., Duan, D., Wang, L., Ma, Q. and Wu, J., 2009. Disruption of the OCH1 and MNN1 genes decrease N-glycosylation on glycoprotein expressed in *Kluyveromyces lactis*. *J. Biotechnol.,* **143:** 95-102. https://doi.org/10.1016/j.jbiotec.2009.06.016

Maras, M., Callewaert, N., Piens, K., Claeyssens, M., Martinet, W., Dewaele, S., Contreras, H., Dewerte, I., Penttila, M. and Contreras, R.,2000. Molecular cloning and enzymatic characterization of a *Trichoderma reesei* 1,2-alpha-D-mannosidase. *J. Biotechnol*., **77:** 255-263. https://doi.org/10.1016/S0168-1656(99)00222-9

Moremen, K.W., Trimble, R.B. and Herscovics, A., 1994. Glycosidases of the asparagine-linked oligosaccharide processing pathway. *Glycobiology,* **4:** 113-125. https://doi.org/10.1093/glycob/4.2.113

Richter, S., Nieveler, J., Schulze, H., Bachmann, T.T. and Schmid, R.D., 2006. High yield production of a mutant *Nippostrongylus brasiliensis* acetylcholinesterase in *Pichia pastoris* and its purification. *Biotechnol. Bioeng.,* **93:** 1017-1022. https://doi.org/10.1002/bit.20705

Tarelli, E., Byers, H.L., Wilson, M., Roberts, G., Homer, K.A. and Beighton, D., 2000. Detecting mannosidase activities using ribonuclease B and matrix-assisted laser desorption/ionization-time of flight mass spectrometry. *Anal. Biochem.,* **282:** 165-172. https://doi.org/10.1006/abio.2000.4606

Van Petegem, F., Contreras, H., Contreras, R. and Van

Beeumen, J., 2001. *Trichoderma reesei* α-1,2-mannosidase: structural basis for the cleavage of four consecutive mannose residues. *J. mol. Biol.,* **312:** 157-165. https://doi.org/10.1006/jmbi.2001.4946

Wang, H., Li, S., Xiong, C., Jin, G., Chen, Z., Gu, G. and Guo, Z., 2019. Biochemical studies of a β-1,4-rhamnoslytransferase from *Streptococcus pneumonia* serotype 23F. *Org. Biomol. Chem.,* **17:** 1071-1075. https://doi.org/10.1039/C8OB02795A

Yang, X., Liu, B., Song, M., Gong, X., Chang, S., Xue, K. and Wu, J., 2011. Construction of yeast Pichia pastoris to produce Man5GlcNAc2 mammalian mannose-type glycoprotein. *Chinese J. Biotechnol.,* **27:** 108-117. Article in Chinese. https://kns.cnki.net/kcms/detail/detail.S8XSMqT4nRMm7l2fJYofQnLTezRMEft9p

Yoshida, T., Inoue, T. and Ichishima, E., 1993. 1,2-alpha-D-mannosidase from *Penicillium citrinum*: molecular and enzymic properties of two isoenzymes. *Biochem. J,* **290:** 349-354. https://doi.org/10.1042/bj2900349

Zhan, J., 2004. *Expression and activity assay of T. reesei α-1,2-mannosidase in Pichia yeast*. Master thesis, Beijing Institute of Biotechnology, Beijing, China. Article in Chinese. https://kns.cnki.net/kcms/detail/detaile=CMFD9904andfilename=2004097300.md2F7lm2BMgwRY8pLFq7EqMh9zzdHiR

Zhou, Q. and Qiu, H., 2019. The mechanistic impact of N-Glycosylation on stability, pharmacokinetics, and immunogenicity of therapeutic proteins. *J. Pharm. Sci.,* **108:** 1366-1377. https://doi.org/10.1016/j.xphs.2018.11.029