Influence of different parameters on reverse micelle extraction combined with acetone precipitation to purify sn-1,3 extracellular lipase from *Aspergillus niger* GZUF36

Cuicui Chen^{1,2} · Hua Tian³ · Shuqi Xing^{1,2} · Cuiqin Li⁴ · Xuefeng Zeng^{1,2} · Laping He^{1,2,5}

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Abstract There are few reports on the feasibility of combined reverse micelle extraction and acetone precipitation to obtain electrophoretic pure enzymes. We aimed to purify a sn-1,3 extracellular lipase from a novel *Aspergillus niger* GZUF36 through this combination in this work. This lipase preliminarily purified by controlling the volume ratio (1:2.5) of crude enzyme solution and acetone. Then, we studied effects of different parameters on reverse micelle extraction. The suitable surfactant, pH, salt and cosolvent and extraction time for forward extraction were 125 mM cetyl trimethylammonium bromide (CTAB), 9.0, 0.075 M NaCl, 10% *n*-hexanol and 30 min, respectively. Under these conditions, the forward extraction rate reached $90.3\% \pm 3.2\%$. The suitable salt, pH, extraction time and short chain alcohol for backward extraction were consecutively 1.5 M KCl, 6.5, 60 min and 10% ethanol. Adding 10% ethanol shows a significant advantage of improvement the extraction rate. Under these optimal conditions, the total extraction rate and purification factor of lipase reached 76.8% and 10.14, respectively. SDS-PAGE showed that molecular weight of the pure protein was

42.7 kDa and TLC exhibited sn-1,3 selectivity of this lipase. LC–MS/MS analysis revealed that the lipase had 297 amino acid residues and was likely to glycosylate. Through the study of different parameters, it demonstrated that the new and simple combination of reverse micelle extraction using CTAB as surfactant and *n*-hexanol as cosolvent for forward extraction and adding ethanol for backward extraction and acetone precipitation is a promising method to get almost an electrophoretically pure sn-1,3 lipase.

Keywords *Aspergillus niger* · sn-1,3 Extracellular lipase · Reverse micelle extraction · Acetone precipitation

Introduction

1,3-Diacylglycerol (1,3-DAG) is a kind of oil that can be converted into energy in the digestive without store, which can lessen the incidence of obesity (Rudkowska et al. 2012; Takase 2007). Preparation of 1,3-DAG by lipase is a green and effective method. Enzymatic method for preparing 1,3-DAG includes ester synthesis (Duan et al. 2010) and glycerolysis (Li et al. 2018; Yamane et al. 1994). Compared with ester synthesis, glycerolysis is a promising method for preparing 1,3-DAG, because it has advantages of cheaper raw materials and no need to removing water during reaction. In our previous work, we screened a novel *Aspergillus niger* strain GZUF36 with two high sn-1,3-position selectivity of lipases including an intracellular lipase (Li et al. 2018) and an extracellular one (data report in this work). The intracellular lipase used as whole-cell lipase to synthesize 1,3-DAG and its sn-1,3 selective catalytic properties further discussed (Li et al. 2018). However, extracellular lipase properties from *A. niger* GZUF36

✉ Laping He
helaping@163.com; lphe@gzu.edu.cn

¹ Key Laboratory of Agricultural and Animal Products Store & Processing of Guizhou Province, Guiyang 550025, China

² College of Liquor and Food Engineering, Guizhou University, Guiyang 550025, China

³ College of Life Science, Xinyang Normal University, Xinyang 464000, Henan, China

⁴ School of Chemistry and Chemical Engineering, Guizhou University, Guiyang 550025, China

⁵ Guizhou Pork Products Research Center of Engineering Technology, Guiyang 550018, China

were still unclear except sn-1,3 selectivity and organic solvent tolerance. Therefore, if extracellular lipase can isolate and purify from fermentation, it would lay the groundwork for further studies on enzymatic properties, improving enzymatic properties and application in food industry such as synthesis of structural lipids.

Reverse micelle extraction has been applied to purify proteins and attracted much attention in the food industry for its high selectivity and easy preparation (Mohd-Setapar et al. 2014; Zhao et al. 2018). Reverse micelles are thermodynamically stable nanoscale aggregates in which a surfactant is dissolved in an organic solvent, and the concentration exceeds the critical micelle concentration (Pérez et al. 2014; Tonova and Lazarova 2008). In the reverse micelle, the polar end of the surfactant is outside and in contact with a nonpolar organic solvent. The polar head organizes to form a polar nucleus. This nucleus dissolves in water and forms a “pool” that dissolves the bioactive substances, such as enzymes, proteins, and nucleic acids. This avoids direct contact of active substances with organic solvents outside the micelles. Thus, biological inactivation avoids (Chen et al. 2017; Gaikawai et al. 2012b). Many proteins have successfully extracted by reverse micelles and well preserved for the properties of biomolecules. Zhao et al. (2019) purified peanut proteins by sodium di [2-ethylhexyl] sulfosuccinate (AOT) reverse micelles, and analyzed its surface and volatility characteristics.

It is clear from the discussion that advantages of reverse micelle extraction are evident. However, the protocol including industrial application and mechanism of reverse micelle extraction remains a challenge. Because the reverse micelle relatively complicates, forming and stabilizing reverse micelles were affected by many factors, such as the concentration and species of ions, pH, selection of organic solvent and surfactant species (Ding et al. 2016; Fragosio et al. 2012). Moreover, after the reverse micelle formation, the strong electrostatic between the polar head of the ionic surfactant and the protein may also lead to protein inactivation. So, the influence of different parameters on the relationship between preparation of reverse micelles and enzyme recovery needs to investigate. In recent years, many researchers have also focused on combining reverse micelles with other technologies to achieve the goals (Hebbbar et al. 2012; Zhang et al. 2016). However, there are few reports on the feasibility of reverse micelle extraction combined with acetone precipitation to obtain electrophoretic pure enzymes.

To this end, the present work aimed to purify sn-1,3 extracellular lipase by a new and simple protocol of reverse micelle extraction combined with acetone precipitation. Then, the molecular weight of the pure protein was also measured by SDS-PAGE and the amino acid sequence was analyzed by LC-MS/MS.

Materials and methods

Materials

Aspergillus niger GZUF36 was isolated by our laboratory and deposited at the China Center for Type Culture Collection (CCTCC) under the CCTCC Preservation No. M2012538, which free access. 1,3-Diolein, 1,2-diolein, and triolein (purity > 99%) as standards, analytical-grade triolein (purity > 65%), AOT (purity > 99%, TLC grade, Porcine Pancreatic lipase (PPL, 1000 U/g) were purchased from Sigma-Aldrich CO. (USA). Acetonitrile and Isopropanol (HPLC grade, purity > 99.99%) were procured from AppliChem Co. (Germany). CTAB (purity > 99.0%) was obtained from Solarbio Science and Technology Co. (Beijing, China). Bovine serum albumin (Biochemical reagent) and Coomassie Brilliant Blue G-250 (Ultra-pure) acquired from Biotopped Com. (Beijing, China). Isooctane, *n*-butanol, *n*-hexanol, and anhydrous ethanol were from Fuyu Co. (Tianjin, China). Acetone (analytical grade) was from Chuandong Chemical Co. (Chongqing, China). Silica gel plates (10 × 20 cm) were from Qingdao Ocean Chemical Co., Ltd, China. Gauze was from Guangxi Nanning Boenkang Biotechnology Co., Ltd, China. All of the other chemicals were of analytical grade and commercially available.

Microorganism growth and lipase production

Seed and fermentation medium for *A. niger* GZUF36 were prepared according to the previous report (Li et al. 2018). Two-ring spores of *A. niger* GZUF36 from slant were inoculated to 250 mL—flasks containing 50 mL of seed medium and incubated at 30 °C and 180 rpm for 24 h. Then, 2% (v/v) seed culture was inoculated to 250 mL—flasks containing 50 mL of the fermentation medium for incubation in a shaker at 30 °C and 180 rpm for 48 h (Li et al. 2018). After incubation, the fermentation broth filtered with eight-layer gauze to remove the *A. niger* GZUF36 mycelium. Then, the supernatant was collected by centrifugation (centrifuge GL-20G-H, Shanghai Anting Scientific Instrument Factory, China, the same below) at 10,000 rpm and 4 °C for 10 min. The crude enzyme solution of lipase was purified further as described below.

Acetone precipitation

Acetone precipitation was referred to the report from Ewelina's report Ewelina et al. (2010) with some modifications. The crude enzyme solution (40 mL) precipitated by the same volume of precooled acetone. The mixture of crude enzyme and acetone was placed in a refrigerator at

– 18 °C for 4 h. The mixture was then centrifuged at 10,000 rpm and 4 °C for 15 min to remove the supernatant. The precipitates naturally dried for 20 min to remove the residual acetone. The precipitates were resuspended in 20 mL of Tris–HCl buffer (pH 7.0) and centrifuged at 10,000 rpm and 4 °C for 15 min. The supernatant was the initial purified lipase.

Forward extraction

Forward extraction and backward extraction were referred to the report from Xin et al. (2015) with some modifications. NaCl dissolved in the initial purified lipase. Its concentration kept at 0.1 mol/L unless mentioned otherwise. Then, the pH of the solution set to 7.0 by 0.05 M Tris–HCl and used as the aqueous phase of forward extraction. Reverse micelles prepared from known amounts of isooctane, hexanol, butanol, surfactant and water. Cetyl trimethylammonium bromide (CTAB) and AOT were investigated as a surfactant to probe into the effect of a surfactant on the reverse micelles. CTAB was dissolved in the mixed organic solvent containing isooctane, hexanol and butanol. AOT dissolved in isooctane. The aqueous and organic phases mixed at a volume ratio of 1:1, and the mixture shaken at 200 rpm and at 30 °C for some time. The mixture was centrifuged at 4000 rpm and 4 °C for 15 min to separate the organic phase from the mixture. The organic phase used for the backward extraction.

Backward extraction

The aqueous phase was 0.05 M Tris–HCl buffer (pH7.0) containing 1.0 mol/L KCl. The organic phase of the forward extraction and the aqueous phase were mixed at a volume ratio of 1:1 and shaken at 200 rpm and 30 °C for some time for backward extraction. The mixture centrifuged at 4 °C and 4000 rpm for 15 min, and the aqueous phase separated.

Enzymatic reaction

Enzymatic reaction was carried out according to the literature with some slight modification (Yamamoto and Fujiwara 1995). The reaction mixtures consisted of 0.1 mL of 65% triolein or 99% triolein, 1 mL of 25 mM sodium phosphate buffer (pH 6.4), and 1 mL of enzyme solution (10 U/mL extracellular sn-1,3 lipase from *A. niger* GZUF36 or PPL). As a control, the enzyme solution was changed to the same volume of the buffer solution. The reaction mixtures placed in 50-mL Erlenmeyer flasks with a glass-stopper and incubated at 30 °C and 500 rpm for 30 min. Then, 2 mL of n-hexane added to the reaction

mixtures and extracted the hydrolyzate for 30 min. The upper solution was used for TLC analysis.

Analysis

Lipase activity was determined through olive oil emulsification (Ramani et al. 2010). One unit of enzyme activity (U) was defined as the amount of enzyme required to hydrolyzing 1 μmol of fatty acid per min under given assay conditions. The sn-1,3 selectivity was analyzed by TLC, which conducted at 0.2-mm silica gel plates (10 × 20 cm). Plates developed with hexane/ether/acetic acid (v/v/v, 80:20:2), air-dried, and colored by I₂ vapor. Protein content determined by Bradford (1976) method to optimize the acetone precipitation conditions. The reverse micelle solution of CTAB/isooctane/hexanol/butanol may affect the protein content determined by Bradford method. Thus, protein content was indirectly determined through UV absorption spectrometry (Noble 2014) during optimizing extraction conditions. The water content in the organic phase was monitored by the Karl Fischer method (SKF1, Zibo Kulun, Shandong, China). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed using an electrophoresis apparatus (DYY-6C, Liuyi, China). The amino acid sequence of lipase was determined via LC–MS/MS by Wuhan Jin Kairui Bio-engineering Co., Ltd. (China).

The purification of acetone precipitation was evaluated by estimating the enzyme specific activity (SA, U/mg protein), recovered activity (RA), and the purification factor (PF; fold) by using Eqs. (1)–(3). The SA of the enzyme is defined as the unit activity of the enzyme in the unit weight of the protein.

$$SA = EA/PC \quad (1)$$

$$RA = E_1/E_0 \quad (2)$$

$$PF = SA_1/SA_0 \quad (3)$$

where EA is the enzyme activity, PC is the total protein concentration (mg/ml), E_1 is the lipase activity after acetone purification, E_0 is the enzymatic activity of crude lipase solution from *A. niger* GZUF36, SA_1 is the specific activity after acetone purification, SA_0 is the specific activity of the crude lipase solution.

The forward extraction rate (FER), backward extraction rate (BER), and the purification factor (PF1) were calculated on the basis of the enzymatic activity in aqueous phases after forward extraction and backward extraction. Their formulas were shown as follows Eqs. (4)–(6).

$$FER = (E_2 - E_3)/E_2 \quad (4)$$

$$BER = E_4/(E_2 - E_3) \quad (5)$$

$$PF1 = SA_3/SA_2 \quad (6)$$

where E_2 is the lipase activity before reverse micelle extraction, E_3 is the activity of lipase in aqueous phase during the forward extraction, E_4 is the activity of lipase in the aqueous phase during the backward extraction, SA_2 is the SA in aqueous before reverse micelle extraction, SA_3 is the SA in the aqueous phase during the backward extraction.

All experiments repeated at least thrice. Data are shown as mean $\pm$ standard deviation (SD). ANOVA performed using SPSS 17.0 (SPSS, Chicago, IL, USA), and differences determined via Tukey's HSD test and considered as statistically significant at $p < 0.05$.

Results and discussion

Effects of acetone precipitation on the RA and PF of the lipase activity

The effect of the volume ratio of fermentation broth to acetone on the RA and PF of *A. niger* lipase activity was investigated at -18°C for 4 h. And the acetone-precipitated lipase dissolved in 0.05 M Tris-HCl to determine the enzymatic activity. The results are shown in Fig. 1. The volume ratio of acetone to fermentation broth significantly affected RA and PF ($p < 0.05$). When the volume ratio was less than 1.0, the RA and PF of the lipase activity were low. These characteristics indicated that the amount of acetone was too low, and lipase hydration layer was not destroyed. This resulted in the lipase to remain in the solution. The volume ratio of acetone to fermentation broth was 2.5, and the RA (71.23%) and PF (2.04) of the enzymatic activity reached the maximum, which significantly higher

($p < 0.05$) than that by the other volume ratio. These indicated that the lipase almost precipitated. However, the excessive acetone content destroyed the enzyme, resulting in the partial inactivation of the enzyme. Thus, the volume ratio of 2.5 selected as the volume ratio for the next experiment.

Forward extraction

Effect of surfactant on forward extraction

The surfactant affects the size of the reverse micelle particles formed (Michor et al. 2016). In the present work, the surfactant evaluated. The surfactant significantly affected RA and PF ($p < 0.05$). Under the same conditions, the RA of the enzymatic activity in the CTAB/isooctane/*n*-hexanol/*n*-butanol ($33.96\% \pm 2.36\%$) reverse micelles was thrice that of the AOT/isooctane ($10.33\% \pm 1.89\%$), indicating that CTAB was more favorable than the other system for the enzymatic activities (Fig. 2a). This may be the result of the large molecular weight of the lipase, which is unsuitable for AOT/isooctane reverse micelles. Although AOT/isooctane has been mainly used as a surfactant in reverse micelles, it is mostly used to extract of lipase with a low molecular weight. When the molecular size and molecular weight of the protein are large, a large aqueous polar nucleus needs to dissolve the protein. Therefore, CTAB selected as the best surfactant to prepare the reverse micelle. Guo et al. (2018) used reverse micelles from different Gemini surfactants to extract bromelain from pineapple peel and found that different surfactants significantly affected on the extraction rate.

Effects of pH and CTAB concentration on forward extraction

pH controls the electrostatic between a protein and a surfactant by influencing the charge on a protein. Too high or too low pH not only causes protein denaturation, but also does not form a reverse micelle due to mixing the protein and the surfactant into an emulsion. Figure 2b shows the effects of pH on the forward extractions of lipase with CTAB reverse micelles. The FER of lipase increased rapidly ($p < 0.05$) as pH increased, and the maximum FER of 53.73% was reached at pH 9.0. This indicated that the electrostatic attraction between lipase and surfactants plays an important role in the transfer of lipase to reverse micelles. When pH exceeds 9, the FER showed a downward trend. This may be due to the competitive inhibition of OH^- ions on protein molecules, and then more surfactant polar heads are combined with OH^- ions (Chen et al. 2017; Gaikar and Kulkarni 2010).

Fig. 1 Effect of ratio of crude lipase solution to acetone on acetone precipitation. Means with different lower case letters on the curve are significantly different ($p < 0.05$); means with different uppercase letters are also significantly different ($p < 0.05$)

Fig. 2 Effect of different parameters on forward extraction: **a** surfactant types, **b** pH, **c** concentration of surfactant, **d1**, **d2** ionic strength, **e** co-solvent content, **f** extraction time. Means with different lower

case letters on the curve or bars are significantly different ($p < 0.05$); means with different uppercase letters on the bars are also significantly different ($p < 0.05$), the same below Fig. 3

Fig. 3 Effect of different parameters on back extraction: **a** ionic species and strength, **b** pH, **c** time, **d1** effect of absolute ethanol content on BER and purification fold, **d2** effect of absolute ethanol on extraction time

The effect of CTAB concentration on forward extraction was also evaluated (Fig. 2c). As the concentration of CTAB increased, the BER significantly ($p < 0.05$) increased first and then decreased. At low concentrations, the amount of reverse micelles dissolving water W_0 increased ($p < 0.05$). This indicated that the size of the reverse micelles kernel increased and the number of

reverse micelles did not reached the maximum. When the CTAB concentration was 75–125 M, W_0 and FER decreased ($p < 0.05$) because of the increase in the number of micelles. Although a single reverse micelle to extract protein reduced, the extent of the increase was greater than the degree of reduction in the single reverse micelle extraction. When the concentration of CTAB was greater

than 125 mM, W_0 almost did not change ($p > 0.05$). FER decreased because the number of aggregates of the reverse micelles has exceeded the optimal number. Increasing the number of reverse micelles reduced the interfacial area of the reverse micelles, and the collision between the reverse micelles changed the structure of the partially reverse micelles (Hebbar et al. 2008). These resulted in a decrease in the rate of the reverse micelle extraction. Hence, a CTAB concentration of 125 mM was chosen for the reverse micelles in the subsequent experiments. Chen et al. (2017) used reverse micelles from ammonium glucosamine (GA) and lactosyl ammonium (LA) surfactants to extract bovine serum albumin (BSA). The amount of the transferred BSA was dependent on the concentration of GA and LA surfactants.

Effect of salt and cosolvent on forward extraction

The transfer of lipase in the organic phase was also influenced by salt (Fig. 2d1, d2). Without salt, the FER was significantly low ($p < 0.05$), and the interface became clear when the salt content reached 0.05 M. However, a significantly highest FER ($p < 0.05$) was observed when the salt content was 0.075 M with CTAB reverse micelle (Fig. 2d1). The reason was that the repulsive force between the polar head groups of the surfactant at low concentrations plays a dominant role in the whole system. The low concentration of the salt solution produced a large amount of water in the inner core of the reverse micelles, and the stability of the reverse micelles deteriorated (Fig. 2d2). As the NaCl concentration increased, the electrostatic force between the protein and the surfactant gradually exhibited a dominant role. This facilitated the transfer of proteins into the reverse micelles of the kernel, and the extraction rate also increased. However, excessive NaCl concentration caused the electrostatic shielding effect of the extraction (Kinugasa et al. 2003). That is, the increase in electrostatic between the salt ion and the surfactant would cause the protein to fall out of the reverse micelle. The increase in salt concentration caused a significant decrease in W_0 ($p < 0.05$), thereby reducing the size of the reverse micelles and the “squeeze” of the protein from the reverse micelles (Krishna et al. 2002).

The effects of different concentrations of *n*-hexanol on the extraction of reverse micelles were investigated by maintaining CTAB/*n*-butanol concentration (Fig. 2e). The *n*-hexanol concentration had a significant effect ($p < 0.05$) on the FER. When the content of *n*-hexanol was 10%, a significantly high FER ($p < 0.05$) was reached (73.13%). Without *n*-hexanol in the reverse micelle, the FER was significantly low ($p < 0.05$) and only 29.93%. The reason was that lipophilic alcohols (pentanol, hexanol and octanol) increased the water content in the reverse micelles

(Mathew et al. 2007). The increase in water content resulted in an increase in the size of the reverse micelles, which could further dissolve more protein and extract more protein with higher molecular weight. It was reported that changes in water content can modulate the size, shape and surfactant/cosurfactant composition of CTAB/hexanol reverse micelles (Fuglestad et al. 2019). Adding lipophilic alcohols weakened the repulsive force between micelles, and this condition was more favorable for the protein into the reverse micelles (Krei and Hustedt 1992). However, the high concentration of *n*-hexanol would weaken the electrostatic between the reverse micelles and the protein (Gaikawari et al. 2012a). The high concentrations of organic solvents could make enzyme inactivate. Thus, 10% *n*-hexanol was chosen for the reverse micelles in the subsequent experiments.

Effect of extraction time on forward extraction

We further studied the effect of extraction time on the forward extraction (Fig. 2f). The lipase in the aqueous phase into the organic phase involved three steps. First, the lipase in the aqueous phase diffused into the interface of the two phases. Second, the reverse micelles contained lipase in the interface. Finally, the reverse micelles containing the lipase diffused into the organic phase (Zhang et al. 2002). Therefore, the reverse micelles with the lipase could not reach the saturation state and the extraction rate reduce ($p < 0.05$) when the extraction time was short. When the extraction time was greater than the optimum value, the extraction rate was almost constant ($p > 0.05$). The half-life of the enzyme in the reverse micelles could be several hundred hours (Setti et al. 1995). Therefore, the extraction rate was almost unchanged with prolonged extraction time. Considering the time and cost, 30 min (90.3%) was chosen as the optimum time for the forward extraction in the subsequent experiments.

Backward extraction

The sufficient amount of salt and low pH increased the rate of reverse extraction, because the low pH weakened the attractive interaction between the protein and the cationic surfactant. Moreover, a sufficient amount of salt shielded the interaction between the two molecules. This decreased the size of the reverse micelle and extruded the protein from the reverse micelle. Therefore, the effect of salt strength on BER was studied under the optimum condition of forward extraction. NaCl and KCl allow the protein to be extracted from the reverse micelles during backward extraction. However, NaCl was far from the optimum concentration such that the BER and the PF1 were significantly low ($p < 0.05$) in comparison with KCl when the

salt concentration was 1 M. Considered that high salt concentrations may affect subsequent experiments, we selected KCl as the salt of the aqueous phase during backward extraction.

Figure 3a shows the significantly effect of KCl concentration on BER and PF1 ($p < 0.05$). The maximum BER and PF1 were obtained when KCl was 1.5 M. Under this condition, we performed the recovery of lipase at the backward aqueous phase pH varied from 6.3 to 7.0 (Fig. 3b). The results showed that the optimum pH was 6.5. When $pH < pI$, the polar head of CTAB and the protein molecules positively charged, and both molecules formed an electrostatic repulsion force that facilitated the separation of the protein from the reverse micelle. However, our pre-experiments showed that the lipase inactivated when pH was less than 3.5 (isoelectric point of the lipase). When pH was too high, the attraction between the protein and the surfactant was strong such that the protein was not easily extracted from the reverse micelles. The appropriate pH of 6.5 was selected for back extraction. Chuo et al. (2018) observed that pH of the stripping solution significantly affects the backward extraction of erythromycin, and this effect is higher than that of KCl concentration and sophorolipid.

In this study, the backward extraction time was also investigated. In Fig. 3c, as time was extended, the indicators initially increased ($p < 0.05$) and then became unchanged ($p > 0.05$), because of the saturation of a certain aqueous phase solution to backward extraction of lipase. Considering the time and cost, the back extraction time of 60 min was chosen for further analysis (71.8% and 4.17%).

Alcohol molecules have been used to improve the protein backward extraction because short-chain alcohols reduce not only the electrostatic interaction between protein and surfactant but also W_0 (Hong and Kuboi 1999). ANOVA results showed that the ethanol ranged from 5 to 10% significantly affected the BER ($p < 0.05$), whereas the ethanol ranged from 10 to 15% did not influenced the BER ($p > 0.05$). Considering the cost, 10% ethanol selected. The time for stripping also slightly increased. Ethanol is believed to change the fluidity of micelle membranes and affect the structure of micelles, resulting in changes in the extraction time. Figure 3d1, d2 shows that the BER and PF1 were $85.05\% \pm 2.9\%$ and 4.76 ± 0.092 , respectively, when the amount of ethanol was 10% and the stripping time was 1.5 h.

SDS-PAGE

This study showed the feasibility of the new and simple method of combined acetone precipitation and reverse micelle extraction to purify the sn-1,3 extracellular lipase

from *A. niger* GZUF36. This could purify the target enzyme to almost a pure electrophoretic grade. Figure 4 shows that the molecular weight of the enzyme was approximately 42.7 kDa.

Analysis of sequencing and positional selectivity of the enzyme

The amino acid sequence of the enzyme was obtained through LC–MS/MS analysis. The results were as follows: MFSGRFGVLLTAHAALSAAAPTPLDVRSVSTLDE LQLFSQWSAAAYCSNNIDSDSNVTCTADACPSVEE ASTKMLLEFDLTNNFGGTAGFLAADNTNKRLVVAFRGSSTIKNWIADLDFILQDNDLCTGCKVHTGFWKA WEAAADNLTSKIKSAMSTYSYGYTYFTGHS LGGAL ATLGATVLRNDGYSVELYTYGCPRVGNALAEHITS QGSGANFRVTHLNDIVPRLPPMDFGFSQPSPEYWITS GTGASVTASDIELIEGINSTAGNAGEATVDVLAHLW YFFAISECLL.

According to NCBI, the amino acid sequence of the purified enzyme was similar to that of ABG73613.1 triacylglycerol lipase A from *A. niger*. TLC showed that the purified enzyme displayed the sn-1,3 positional selectivity when it hydrolyzed triolein, which was similar to PPL's selectivity (Fig. 5). Furthermore, 65% triolein contained low amount of 1,2-diolein and 1,3-diolein (Fig. 51). After 65% triolein and 99% triolein were hydrolyzed, the extracellular lipase displayed high sn-1,3 selectivity to triglyceride. This new enzyme was our target lipase. In line with the 297-residue sequence, the sn-1,3 lipase molecular weight was 31.72 kDa, and SDS-PAGE showed that the molecular weight of the enzyme was approximately

Fig. 4 SDS-PAGE of the enzyme

Fig. 5 TLC analysis: (1) hydrolyzates from 65% triolein hydrolyzed by GZUF36 lipase, (2) 65% triolein; (3) and (4) hydrolyzates from 99% triolein hydrolyzed by GZUF36 lipase, (5) and (6) hydrolyzates from 99% triolein hydrolyzed by PPL, (7) 99% triolein

42.7 kDa. The molecular weight difference indicated that this lipase was likely to be glycosylated.

Conclusion

Herein, we presented the report on the preliminary purification of a sn-1,3 extracellular lipase from *A. niger* GZUF36. The results demonstrated that almost an electrophoretically pure lipase could be obtained using the new and simple method of combination of reverse micelle extraction and the lipase showed some unique extraction characteristics. The FER was as high as $90.3\% \pm 3.2\%$ under optimized extraction conditions. Adding ethanol increased the BER by 14%. Under this condition, the extraction rate was $85.05\% \pm 2.9\%$. This study established a preliminary foundation for the application of lipase in the synthesis of structural lipids such as 1,3-DAG and for subsequent studies on the structure, property, and rational design of the sn-1,3 lipase. In the future, studies will focus on the mechanism of the reverse micelle extraction of proteins and their thermodynamic and kinetic processes. With the development of safe surfactants and simple product separation techniques, the industrial application of this technology will be facilitated.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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