ORIGINAL ARTICLE

Purification, characterization, and functional groups of an extracellular aflatoxin M$_1$-detoxifizyme from *Bacillus pumilus* E-1-1-1

Xue-Jiao Zhang[1] | Jie Gao[2] | Jun Han[2] | Xiang-Hong Wang[2] | Ya-Xin Sang[2]

[1]College of Science and Technology, Agricultural University of Hebei, Baoding, P. R. China

[2]Faculty of Food Science and Technology, Agricultural University of Hebei, Baoding, P. R. China

Correspondence
Ya-Xin Sang, Faculty of Food Science and Technology, Agricultural University of Hebei, No. 2596, LeKai South Street, Baoding 071000, P. R. China.
Email: sangyaxin@sina.com

Abstract

The experiment was conducted to purify high activity extracellular enzymes, which were produced by a strain that we previously screened was able to degrade aflatoxin effectively, and speculate the functional groups of the enzyme associated with degradation. An extracellular aflatoxin-detoxifizyme (DAFE) from *Bacillus pumilus* E-1-1-1 was purified through a process including ammonium sulfate precipitation, ultrafiltration, Sephadex chromatography, and ion exchange chromatography. The molecular mass of the enzyme assessed by SDS-PAGE was found to be approximately 58 kDa. The optimum reaction temperature and pH for the purified enzyme were 45°C and pH 7, respectively. The enzyme showed temperature stability of up to 60°C. Ba^{2+}, Ca^{2+} Na^+, Mn^{2+}, EDTA, and β-mercaptoethanol showed inhibitory effects on the enzyme activity. Mg^{2+}, Fe^{3+}, Zn^{2+} and K^+ were the activators of enzymes. This enzyme was composed of at least 15 kinds of amino acids. Lysine, tryptophan, and histidine residues were necessary and major functional groups to maintain enzyme activity, disulfide bonds were observed, serine residues had little effect on the enzyme activity, so it was not the necessary group to reflect the enzyme activity, and arginine had no effect on enzyme activity.

KEYWORDS
aflatoxin M$_1$, detoxification, functional groups, purification

1 | INTRODUCTION

Aflatoxins are a class of mycotoxins with similar structure, which are mainly produced by 28 species of *Aspergillus flavus* and *A. parasiticus* (Xiong, Xiong, Zhou, Liu, & Wu, 2018). Aflatoxin B$_1$ (AFB$_1$) can be metabolized into aflatoxin M1 (AFM1), which is highly toxic, carcinogenic, teratogenic, and mutagenic (Duarte et al., 2013 ; Hassan & Kassaify, 2014), when feeding fodder contaminated with AFB1 to mammals such as cattle, sheep, and goats (Hussain & Anwar, 2008; Kaniou-Grigoriadou, Eleftheriadou, Mouratidou, & Katikou, 2005;

Virdis, Corgiolu, Scarano, Pilo, & Santis, 2008). AFM1 is produced by AFB1 in vivo through demethylation, hydroxylation, and epoxidation; the resulting hepatocyte damage can lead to liver cancer (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, World Health Organization, & International Agency for Research on Cancer, 2002; International Agency for Research on Cancer, 1992).

Multiple physical and biological methods to degrade AFB$_1$ have been proposed (Samuel, Sivaramakrishna, & Mehta, 2014; Vijayanandraj et al., 2014; Wang et al., 2011). However, a large proportion of the published studies is related to AFB$_1$. Human exposure

to AFM$_1$ is also a threat. Adults especially children, for whom milk and dairy products are very important food, once consume these contaminated dairy products will face enormous risks (Carraro et al., 2014). Despite this concern, only a handful of current studies on the detoxification of AFM1 contaminated milk can be seen. Various methods including biological, chemical, and physical methods have been proposed for the degradation of AFM$_1$ (Carraro et al., 2014; Deveci, 2007; Elsanhoty, Salam, Ramadan, & Badr, 2014). However, the application of these methods in the food industry has its own limitations due to food safety problems. Studies have shown that microorganisms can be utilized in the elimination of aflatoxin through biodegradation (Elsanhoty et al., 2014). However, the disadvantage to be solved is that microorganisms may use nutrients in food to grow and reproduce, and produce unnecessary metabolites. Therefore, the most practical solution is to apply the biodegradation principle of microorganisms to degradation.

Our previous study examined AFM$_1$, which was degraded by some secretions from *Bacillus pumilus* E-1-1-1, and elucidated the mechanisms of several extracellular enzymes. The objective of this study was to purify the extracellular aflatoxin-detoxifizyme (DAFE) with the highest activity and study the degradation mechanism.

2 | MATERIALS AND METHODS

2.1 | DAFE production

2.1.1 | Microbial material

B. pumilus (E-1-1-1, stored at the laboratory) was applied as biological material for the experiment.

2.1.2 | Media

The adopted medium contained 0.8% (w/v) yeast extract, 0.6% (w/v) maltose, and 1% (w/v) NaCl$_2$. Phosphate buffer was used to bring the medium to pH 6.0 prior to sterilization.

2.1.3 | Cultivation

The strain *B. pumilus* E-1-1-1 was cultured in culture medium and incubated for 44 hr at 37°C in a constant temperature incubator shaker. The resulting exocrine secretions of *B. pumilus* were centrifuged at 4.845×10^3 g at 4°C for 20 min to remove contaminants.

2.2 | Enzyme purification

2.2.1 | Extraction of crude enzyme

The cell-free supernatant was purified by ammonium sulfate precipitation. The components obtained at 75% saturation were centrifuged at 4.845×10^3 g at 4°C for 20 min and resuspended in 50 mM phosphate buffer, pH 6.0, to obtain crude enzyme. The crude enzyme was dialyzed against the same buffer overnight.

2.2.2 | Ultrafiltration

Ultrafiltration was performed at room temperature using the Pellicon® mini filter holder (Millipore Corporation, Billerica, MA). Unpurified enzyme was separated by 1, 3, 5, and 10 kDa molecular weight cut-off (MWCO) ultrafiltration membrane. The ultrafiltration membranes used were manufactured by Millipore Corporation, Bedford. Unlike the 1 and 3 kDa membranes processed by recycled cellulose, the 5 and 10 kDa membranes were made from polyether sulfone (Wan, Prudente, & Sathivel, 2012). Prior to enzyme purification, each membrane was assessed by measuring the flux of deionized water. Each membrane was evaluated by measuring the deionized water flux before purifying the enzyme. The portions with each MWCO were concentrated and lyophilized for further use.

2.2.3 | Sephadex chromatography

The enzyme mixture (0.5 ml) was loaded onto a Sephadex G-75 column (1.6 × 300 mm), which was equilibrated and eluted with the deionized water. Then, elution was performed at a flow rate of 1 ml/min, and 2 ml fraction was collected from each tube. All steps of purification were conducted at room temperature and the enzymatic activity of fractions was tested by method described below. The relevant fractions were then collected, concentrated, and lyophilized for further study.

2.2.4 | Ion exchange chromatography

The highly active lyophilized fraction was resuspended in 4 ml of 50 mM Tris–HCl buffer (pH 7.5) and further purified by a DEAE-Sepharose Fast Flow column. A nonlinear gradient of NaCl (0.5 and 1 M) was used for elution, and 4 ml fractions were collected at a flow velocity of 2 ml/min. The fractions with enzymes were grouped according to the elution profile, dialyzed, concentrated, and later lyophilized. The enzyme activity of the collected components was determined by the method as follows.

2.3 | Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to estimate molecular mass by the discontinuous buffer system using a 12% separating and 4% stacking gel. The molecular weight standard used was the molecular mass marker proteins (12–120 kDa). After electrophoresis, the gel was stained with Coomassie brilliant blue (SCRC, China). The method of Bradford (1976) was used to determine the protein concentration, with bovine serum albumin as the standard.

2.4 | Enzyme characterization

2.4.1 | Effect of temperature on AFM1 detoxification properties and enzyme stability of DAFE

The optimum temperature for the purified DAFE activity was measured after culturing in 0.01 mol/L phosphate buffer (pH 6.0) for

12 hr. The temperatures ranged from 20 to 65°C. The thermostability of DAFE was investigated by preincubating the enzyme at temperatures ranging from 45 to 100°C for 10 and 20 min. The residual DAFE activity was measured. The optimum conditions of DAFE were determined by the analysis of relative enzyme activity.

2.4.2 | Effect of pH on the AFM1 detoxification properties of DAFE

In order to ascertain the optimal pH, DAFE was incubated in buffers with different pH values (pH 3.0–8.0 supplied by 50 mM citrate-phosphate buffer, pH 9.0 supplied by 50 mM Tris–HCl buffer) at 37°C for 12 hr, and then, the remaining activities were assayed as described below.

2.4.3 | Activators and inhibitors on the AFM$_1$ detoxification properties of DAFE

The effects of the inhibitors EDTA and β-mercaptoethanol (2-ME) and metal ions on DAFE activity were measured with the addition of 2 mM EDTA, 2-ME or 50 mM of the metal ions Zn^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{3+}, Ba^{2+}, Na^+, and K^+ in the form of their chlorides to the reaction mixtures. The remaining activities were assayed as described below.

2.5 | Amino acid composition analysis of DAFE

The high-performance liquid chromatography (HPLC) was used to analyze amino acid composition. The freeze-dried DAFE sample was loaded into glass ampoules. The ampoules with 6 M HCl (without phenol) were incubated at 110°C for 24 hr (Yu et al., 2014). The digested ampoule was filtered through a 0.45 μm membrane filter when cooled to room temperature. The sample was preliminary purified by centrifugal to remove the residues and then enriched by a vacuum rotary evaporator at 50°C, and finally redissolved in 0.1 mol/L HCl. Fifty microliters of sample solution was incubated with 200 μl buffer and 100 μl derivative in a centrifuge tube. The centrifuge tube was incubated in a 90°C water bath for 90 min. After the centrifuge tube was cooled down to room temperature, 10% acetic acid was added to the mixture to a volume of 1 ml. The sample was filtered through a 0.45 μm membrane and loaded into the HPLC (Waters) to estimate the amino acids according to a standard program. The detection limit for amino acids is 0.24–2.37 μg/ml.

2.6 | Functional group analysis of DAFE

DAFE was selectively modified by N-bromosuccinimide (NBS), phenylmethylsulfonyl fluoride (PMSF), bromoacetic acid (BrAc), succinic anhydride (SUAN), acetylacetone (BD), 2-ME, and carbodiimide (EDC) (Chen, Zhang, Zhou, & Zhuang, 2000; Granjeiro, Ferreira, Cavagis, Granjeiro, & Hiroshi, 2003; Liu, Xia, & Liu, 2008; Liu, Wang, Wang, & Zhang, 2008). Nine hundred microliters of DAFE solution was mixed with 1 ml of different concentrations of modification reagents in the corresponding buffer at 37°C for 30 min. Then, the reaction system was incubated with 100 μl AFM$_1$ at 37°C for 12 hr. The remaining activity was assayed under the blank group. The DAFE activity is expressed as the percent relative activity and that without modification of enzyme activity was 100%.

2.7 | Extraction of residual AFM$_1$

The reaction system was added to 5 ml methanol-water (70:30, v/v) and 5 ml chloroform with ultrasonic extraction for 10 min in separatory funnel. This step is repeated three times for full extraction. The underlying solution was collected, evaporated, and finally dissolved in 1 ml acetonitrile to load to HCLP (Gu, Sun, Cui, Wang, & Sang, 2018).

2.8 | HPLC analysis of residual AFM$_1$

AFM$_1$ levels were determined by a Waters HPLC system (Waters), which is equipped with a Waters 2475 Series auto sampler and fluorescence detector. Ten microlitre of the prepared sample was loaded into HPLC. The excitation wavelength was 360 nm and the emission wavelength was 410 nm. An Agilent silica gel C18 column (Agilent Associates, 250 × 4.6 mm, 10 μm) was used for detection. The mobile phase was isocratic acetonitrile: water (25:75, v/v), with a flow rate of 1 ml/min. Continuous step concentration of AFM1 acetonitrile solution was prepared for drawing the standard curve(Gu et al., 2018).

2.9 | Analysis of residual AFM$_1$

$$Y = (1 - X_1/X_2) \times 100\%$$

where X_1 is the contents of AFM$_1$ in the treated group, X_2 is the contents of AFM$_1$ in the negative control, and Y is the detoxification ratio.

3 | RESULTS AND DISCUSSION

3.1 | Purification of enzyme

The crude enzyme was precipitated using ammonium sulfate precipitation of the supernatant obtained after fermentation of B. pumilus. Maximum enzyme recovery was obtained at 75% saturated ammonium sulfate. The molecular weight of the active enzyme was preliminarily measured by ultrafiltration. Unpurified enzyme was filtered with 1, 3, 5, and 10 kDa MWCO membranes (Figure 1). Five components had varying degrees of degradation of AFM$_1$, but the degradation level was low. However, the total degradation rate of the five components reached 94.3%, close to the degradation rate by microbes. These findings suggested that the ability of the strain to degrade AFM1 may be due to the synergistic action of multiple enzymes. The crude enzyme was further purified by Sephadex chromatography (Figure 2) and ion exchange chromatography (Figure 3).
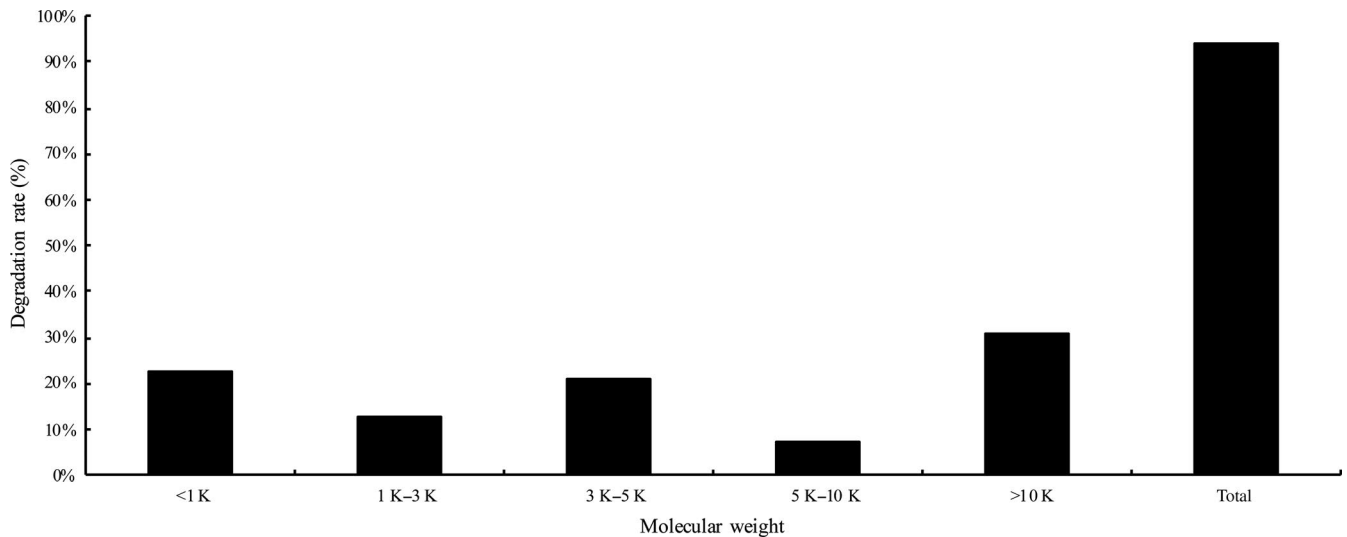
FIGURE 1 The AFM_1 degradation rate of proteins with different molecular weights. Unpurified enzyme was filtered with 1, 3, 5, and 10 kDa molecular weight cut-off membranes. The highest enzyme activity was 30.7% at above 10 K
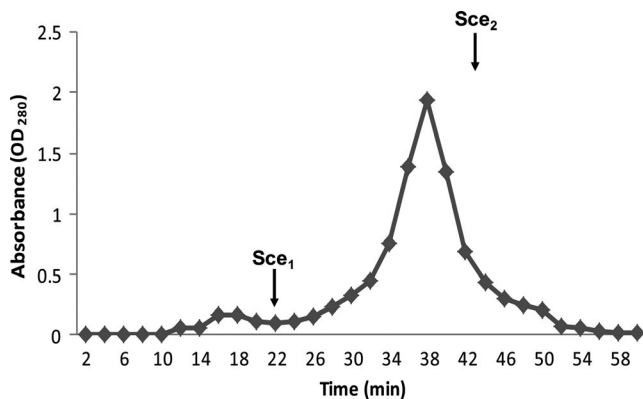

FIGURE 2 After equilibration of the column with deionized water, enzymes were eluted with deionized water at a flow rate of 1 ml/min. Sce_1 degraded AFM_1, but the Sce_2 wave crest showed no degradation of AFM_1

Details of the purification steps are summarized in Table 1. Various enzymes produced by different microorganisms can be purified by ammonium sulfate precipitation and column chromatography (Brzezinska, Jankiewicz, & Walczak, 2013; Chanwicha, Katekaew, Aimi, & Boonlue, 2015; Karthik, Binod, & Pandey, 2015; Liu et al., 2001; Tripathi, Singh, Bharti, & Thakur, 2014). The DAFE was purified 2.37-fold with a specific activity of 7.65×10^{-2} U/mg and a recovery rate of 0.014%. DAFE degradation of AFM_1 may be accomplished by synergy with other enzymes.

3.2 | Gel electrophoresis

Enzyme activity was determined, and the enzymes were then concentrated and subjected to SDS-PAGE. The molecular mass of the target sample was 58 kDa on SDS-PAGE (Figure 4a,b).
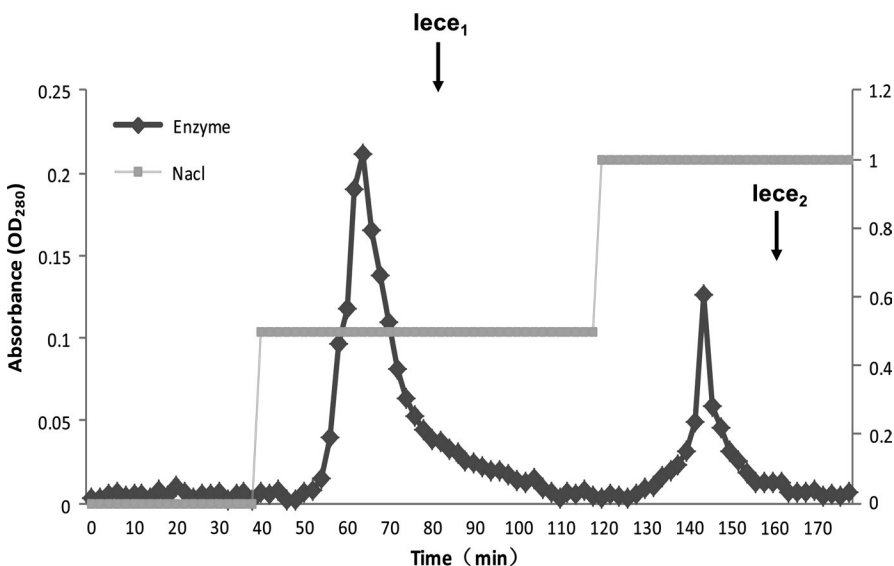

FIGURE 3 Ion exchange chromatography on DEAE-Sepharose Fast Flow: enzyme and NaCl concentration (mol/L). After equilibration of the column with 50 mM Tris–HCl buffer (pH 7.5), enzymes were eluted with a gradient of sodium chloride at a flow rate of 2 ml/min. The $lece_2$ showed degradation of AFM_1, but the $lece_1$ wave crest did not degrade AFM_1

TABLE 1 Purification of *Bacillus pumilus* extracellular aflatoxin-detoxifizyme

Purification step	Volume (ml)	Total proteins (mg/ml × 10^{-2})	Total activity (U/ml)	Specific activity (U/ml)	Purification (fold)	Yield % (total activity)
Culture filtrate	1,000	5,746.28	185.19	3.22	1	100
$(NH_4)_2SO_4$ precipitation (75%)	100	1,342.61	79.96	5.96	1.85	43.18
Ultrafiltration	20	343.42	10.09	2.91	0.90	5.45
Sephadex G–75 chromatography	20	21.86	1.16	5.29	1.64	0.62
DEAE-Sepharose Fast Flow	20	0.34	0.03	7.65	2.37	0.014
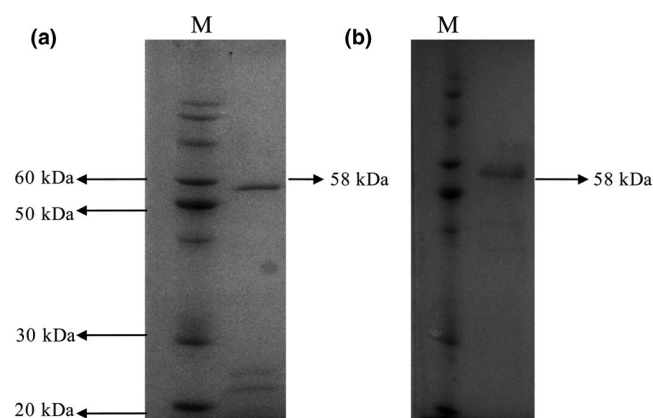
FIGURE 4 SDS-PAGE analysis at various stages of purification of extracellular aflatoxin-detoxifizyme (DAFE). (a) Sephadex chromatography G-75 purification of DAFE, (b) DEAE-Sepharose Fast Flow purification of DAFE. M, low-molecular weight protein marker

3.3 | Enzyme characterization

To determine the effect of temperature on enzymatic activity, we incubated DAFE solution at a temperature range from 20°C to 65°C in 0.01 mol/L phosphate buffer (pH 6.0). The effect of temperature is shown in Figure 5a. The optimum reaction temperature of the enzyme was 45°C. When the temperature rises to more than 55°C, the enzyme activity was <40% of the highest enzyme activity. Within the scope of 35–50°C, the enzyme activity was maintained at more than 40% of the highest enzyme activity.

The effect of pH on the activity of DAFE was analyzed under different buffer conditions. As shown in Figure 5b, the optimal pH was 7.0. The enzyme activity was less than half of the highest enzyme activity at pH < 5.0 or pH > 9.0, and under conditions close to neutral (pH 6.0–8.0), enzyme activity was maintained at more than 80% of the highest enzyme activity. The enzyme activity had a wide range at pH values of 5.0–8.0 and could reach more than 40% of the highest enzyme activity; the highest enzyme activity was observed at pH 7.0.

To determine the thermal stability of DAFE, we incubated DAFE samples at different temperatures varying from 45 to 100°C for a period of 10 and 20 min. The thermal stability study indicated that more than 80% of its original enzyme activity was preserved in a temperature range of 45–50°C (Figure 5c), while at 60°C, the enzyme retained 60% of the highest enzyme activity.

The effects of metal ions with different concentrations on enzyme activity are described in Table 2. Mn^{2+}, EDTA, and 2-ME inhibited the DAFE activity, while the presence of Ba^{2+}, Ca^{2+}, and Na^+ at a concentration of 50 mM triggered a slight inhibitory effect on the DAFE activity. Mg^{2+} and Fe^{3+} were activators, and the presence of Zn^{2+} and K^+ at a concentration of 50 mM stimulated the DAFE slightly.
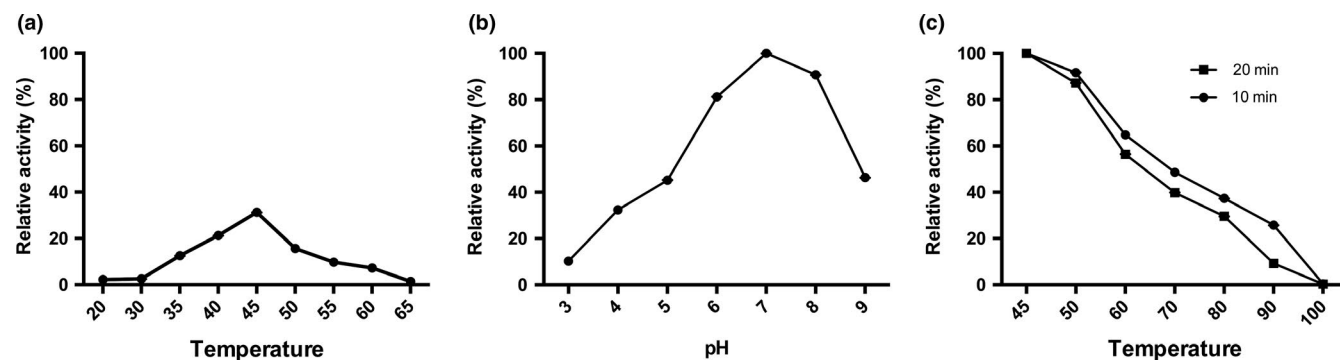

FIGURE 5 Enzyme characterization of extracellular aflatoxin-detoxifizyme (DAFE). (a) Effect of temperature on DAFE activity, (b) effect of pH on DAFE activity, (c) thermal stability of DAFE.

TABLE 2 The effects of metallic ions and β-mercaptoethanol (2-ME) on extracellular aflatoxin-detoxifizyme

Metallic ion	Relative activity (%)
Control	100.00
Fe^{3+}	124.72
Mn^{2+}	38.57
Ba^{2+}	85.69
Ca^{2+}	81.40
Zn^{2+}	113.62
Mg^{2+}	149.18
K^+	115.60
Na+	88.76
2-ME	27.97
EDTA	20.1

3.4 | Amino acid composition analysis

Amino acids were determined using HPLC. DAFE compared with 19 standard amino acids is shown in Figure 6a,b. Table 3 shows the amino acid content of DAFE. Fifteen amino acids were detected in DAFE, including seven essential amino acids (EAAs) in addition to methionine. The total amino acid (TAA) content in DAFE was 35.36%. The percentage ratios of EAAs in DAFE were 0.17% of the TAA. The major amino acids of DAFE were glutamic acid and proline, which accounted for 15.21% and 40.81% of the TAA, respectively.

3.5 | Functional group analysis

In order to clarify which amino acid residues play a role in the catalytic process of DAFE, we preincubated the enzyme in the

TABLE 3 Amino acid profiles of the extracellular aflatoxin-detoxifizyme by dry weight (g/100 g)

Amino acids	Content (g/100 g)
Aspartic acid (Asp)	1.18
Glutamic acid (Glu)	5.38
Histidine (His)	2.93
Serine (Ser)	3.52
Arginine (Arg)	0.61
Glycine (Gly)	2.93
Threonine (Thr)	0.96
Proline (Pro)	14.43
Alanine (Ala)	1.05
Valine (Val)	0.06
Isoleucine (Ile)	0.59
Leucine (Leu)	0.42
Tryptophan (Trp)	1.28
Phenylalanine (Phe)	0.52
Lysine (Lys)	2.17
Total amino acid (TAA)	35.36
Essential amino acid (EAA)	5.99
Nonessential amino acid (NEAA)	29.37
EAA/NEAA	0.20
EAA/TAA	0.17

corresponding buffer at 37°C for 30 min with different amino acid-modifying reagents and then incubated the samples with 100 μl AFM_1 at 37°C for 72 hr. The effects of different amino acid-modifying reagents on enzyme activity are shown in Figure 7. The importance of disulfide bonds, lysine residues, and serine residue in the activity of DAFE was investigated by the reactions with
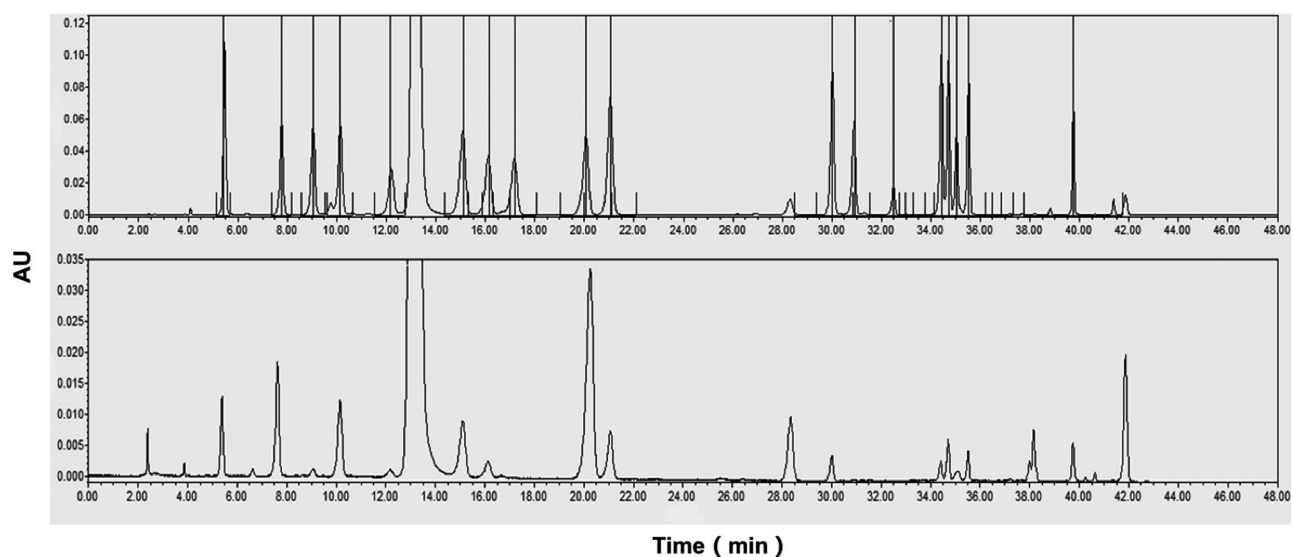

FIGURE 6 Amino acid composition analysis of extracellular aflatoxin-detoxifizyme (DAFE). (a) high-performance liquid chromatography (HPLC) analysis of standard amino acids, (b) HPLC analysis of purified DAFE

FIGURE 7 The effects of different amino acid-modifying reagents on enzyme activity. (a) Effect of β-mercaptoethanol (2-ME) on extracellular aflatoxin-detoxifizyme (DAFE) activity (b) Effect of succinic anhydride (SUAN) on DAFE activity (c) Effect of acetylacetone (BD) on DAFE activity (d) effect of phenylmethylsulfonyl fluoride (PMSF) on DAFE activity (e) effect of N-bromosuccinimide (NBS) on DAFE activity (f) effect of bromoacetic acid (BrAc) on DAFE activity
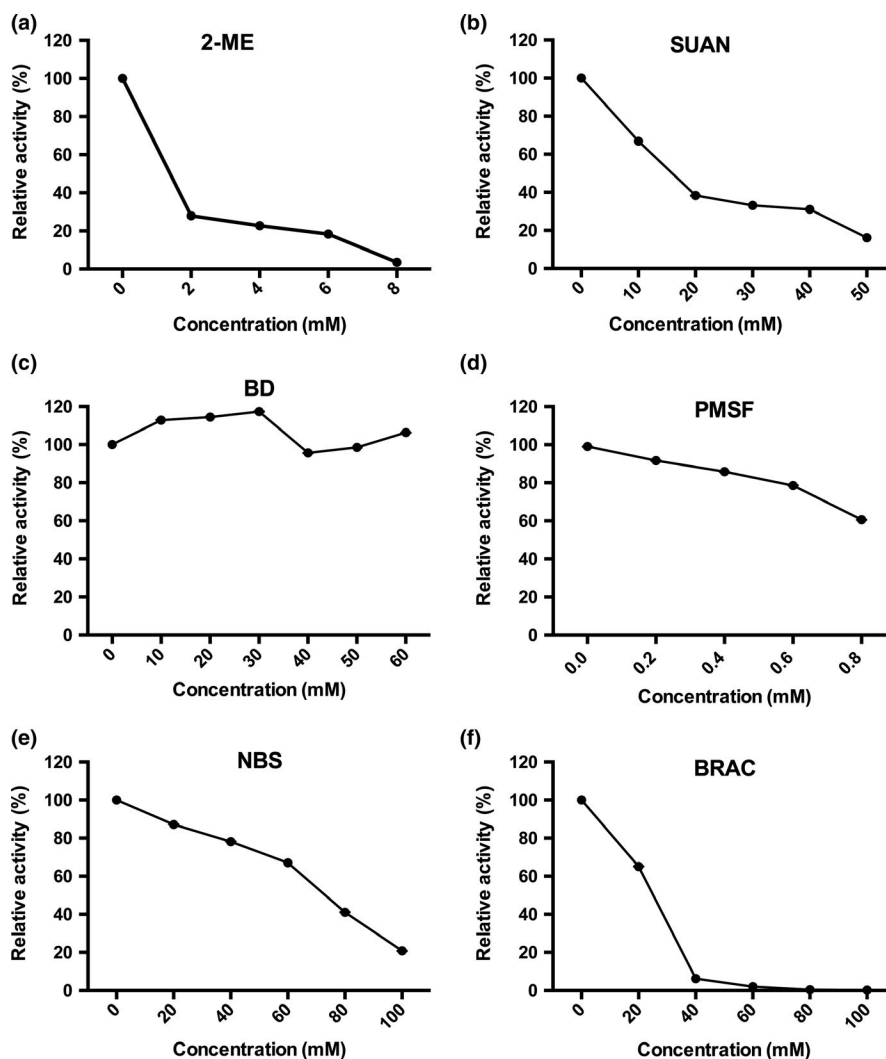
2-ME, SUAN, and PMSF, which were freshly prepared in 50 mM Tris–HCl, pH 8.2. Enzyme activity rapidly decreased with the increase in concentration of 2-ME, and the enzyme activity at 8 mM of 2-ME was almost zero (Figure 7a). This finding showed that disulfide bonds probably played an important role in maintaining enzyme degradation capacity. The lysine residues are probably the major contributor to enzyme activity since it was inhibited (60%) at 20 mM of SUAN (Figure 7b). The enzyme activity decreased slowly with the increase in concentration of PMSF (Figure 7d). These findings indicated that serine residues were involve in the process of enzyme expression, but were not the key point.

The enzyme activity was <90% of the highest enzyme activity at 40 mM of BrAc in 0.1 M citric acid-phosphate buffer, pH 5.8 (Figure 7f). The results showed that histidine played an important role in the activity of enzyme expression. Under acid condition, NBS can selectively oxidize the tryptophan side chain indole (Patchornik, Lawson, & Witkop, 1958; Ramachandran & Witkop, 1959). As shown in the experimental results, tryptophan plays a role in maintaining the activity of the enzyme and may be an essential group in the enzyme structure (Figure 7e).

Under alkaline conditions, BD can react with the guanidine of arginine and change the structure of the side chain groups, which affect the enzyme activity. DAFE was modified by BD in 50 mM Tris–HCl, pH 7.8 (Figure 7c). The BD had no effect on enzyme activity with increasing concentrations. These findings indicate that arginine residues do not participate in the structure of the enzyme activity center.

4 | CONCLUSIONS

A 58 kDa extracellular aflatoxin-detoxifizyme from *B. pumilus* E-1-1-1 was purified. The enzyme had remarkable activity in the neutral pH range. The optimum reaction temperature for the enzyme was 45°C. The enzyme in a temperature range of 45–50°C was relatively stable. Ba^{2+}, Ca^{2+} Na^+, Mn^{2+}, EDTA and 2-ME showed inhibitory effects on the DAFE activity. The enzyme was stimulated by Mg^{2+}, Fe^{3+}, Zn^{2+}, and K^+. DAFE is composed of at least 15 kinds of amino acids. Lysine, tryptophan, and histidine residues were closely related to the maintenance of enzyme activity, disulfide bond played an important role in maintaining the active structure of enzyme, serine residues were not necessary to maintain the level of enzyme activity, arginine had no effect on

the enzyme activity. Overall, the DAFE from the *B. pumilus* can be usefully exploited for the degradation of AFM$_1$. In the following work, we plan to use mass spectrometry to identify the purified enzyme, and with this information, the protein may be purified to homogeneity after heterologous expression. Then we can use it in more accurate experiments to the possible reaction mechanism or the reaction product that would reinforce the findings from B. pumilus E-1-1-1.

ACKNOWLEDGMENTS

CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

XZ conceived and designed the experiments, conducted the data analyses and wrote the manuscript. JG polished experiment plan, put forward constructive analysis to the manuscript. X-HW and JH improved experimental operation, contributed to data analysis, and proofread the final manuscript. Y-XS contributed enormously to analysis and manuscript preparation.

ETHICS STATEMENT

None required.

DATA ACCESSIBILITY

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

ORCID

Xue-Jiao Zhang https://orcid.org/0000-0002-9255-6676

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