Expression of Zearalenone-Degrading Enzyme in Bacillus subtilis and Its Effect on Sow Reproductive Performance (Postprint)

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Abstract

The study aimed to establish an expression system for the zearalenone (ZEN) degrading enzyme gene ZEN-jjm in *Bacillus subtilis* and to determine the biodegradation activity of its product and its effects on sow reproductive performance. The ZEN-jjm gene was cloned, subjected to double digestion with EcoRI and NotI, and ligated into the pHT01 expression vector to construct a recombinant plasmid. *Bacillus subtilis* was transformed, and the expression level of ZEN-jjm protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The ZEN-degrading activity of the expressed ZEN-jjm protein was detected by high-performance liquid chromatography (HPLC). The effect of ZEN degradation by the enzyme on sow reproductive performance was determined through evaluation of reproductive parameters in sows. Double digestion and sequencing results confirmed successful insertion of ZEN-jjm into pHT01. SDS-PAGE analysis revealed that a recombinant *Bacillus subtilis* strain efficiently expressing the target protein was obtained, with a molecular weight of approximately 29 kDa. HPLC results demonstrated that the expressed ZEN-jjm protein could effectively degrade ZEN, and could significantly alleviate the toxic effects of ZEN on reproductive sows ($P<0.05$). In summary: 1) This study successfully constructed an expression vector for ZEN-jjm in *Bacillus subtilis* and achieved its expression in this host. 2) The expressed ZEN-jjm protein possessed biological activity for ZEN degradation. 3) Dietary supplementation with ZEN-jjm protein could significantly reduce the detrimental effects of ZEN on sow reproductive performance.

Full Text

Expression of Zearalenone Degrading Enzyme in *Bacillus subtilis* and Its Effects on Reproductive Performance of Sows

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Abstract

This study aimed to establish an expression system for the zearalenone (ZEN) degrading enzyme gene *ZEN-jjm* in *Bacillus subtilis* and to determine the biodegradation activity of its expression product and its effects on sow reproductive performance. The *ZEN-jjm* gene was cloned and ligated into the pHT01 expression vector after double digestion with EcoR and Not to construct the recombinant plasmid, which was then transformed into *Bacillus subtilis*. Protein expression levels were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the degrading activity of the expressed ZEN-jjm protein was detected by high-performance liquid chromatography (HPLC). The impact of ZEN degradation on sow reproductive performance was evaluated through feeding trials. Double enzyme digestion and sequencing results confirmed successful insertion of *ZEN-jjm* into pHT01. SDS-PAGE analysis identified a recombinant *Bacillus subtilis* strain with high-level expression of the target protein at approximately 29 ku. HPLC results demonstrated that the expressed ZEN-jjm protein could effectively degrade ZEN. Furthermore, the expressed enzyme significantly alleviated the toxic effects of ZEN on breeding sows ($P<0.05$). In conclusion: (1) this study successfully constructed a *ZEN-jjm* expression vector and achieved expression in *Bacillus subtilis*; (2) the expressed ZEN-jjm protein possesses biological activity for ZEN degradation; and (3) dietary supplementation with ZEN-jjm protein can significantly reduce the detrimental effects of ZEN on sow reproductive performance.

Keywords: zearalenone; *ZEN-jjm* gene; *Bacillus subtilis*; sows

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Zearalenone (ZEN) poses a major food safety hazard and significantly impacts animal husbandry, particularly causing veterinary obstetric diseases. Enzyme preparations play a crucial role in degrading mycotoxins due to their specificity and high efficiency, enabling complete decomposition of ZEN without leaving

toxic residues. Previous studies have isolated lactonohydrolases from fungi such as *Pichia pastoris* that effectively degrade ZEN (gene designated as *zhd101*). The *zhd101* gene encodes a pantothenic acid lactonohydrolase in *Clonostachys rosea*. While Bakutis et al. and Kakeya et al. expressed *zhd101* in *Escherichia coli* and fission yeast respectively, Cheng et al. cloned a similar ZEN degrading enzyme gene (*ZEN-jjm*) from *Clonostachys rosea* and expressed it in prokaryotic systems. However, several aspects require further investigation: first, research on *ZEN-jjm* expression in *Bacillus subtilis* remains limited; second, no animal trials have evaluated the effects of ZEN degrading enzymes on sow reproductive performance. Therefore, this study reconstructed the *ZEN-jjm* expression system in *Bacillus subtilis* to determine whether the expressed enzyme possesses biological activity for ZEN degradation and can mitigate reproductive toxicity in sows.

Materials and Methods

Plasmids and Strains The plasmid containing the target gene was constructed and preserved by Beijing Aoke Dingsheng Biotechnology Co., Ltd. The *Bacillus subtilis* expression vector, *Escherichia coli* DH5α, and *Bacillus subtilis* strains were also maintained by Beijing Aoke Dingsheng Biotechnology Co., Ltd.

Reagents PCR amplification kits, low molecular weight protein markers, DNA markers, RNase inhibitors, diethyl pyrocarbonate, distilled water, buffers, competent cell preparation kits, plasmid extraction kits, gel recovery kits, peptone, DNA gel recovery kits, restriction enzymes (EcoR and Not), and Pfu DNA polymerase were purchased from Beijing Aoke Dingsheng Biotechnology Co., Ltd.

Instruments PCR amplifier (Eppendorf, Germany), electroporator (Bio-Rad, USA), high-speed centrifuge (Eppendorf, Germany), high-performance liquid chromatograph (Agilent, Germany), and gel imaging analyzer (Bio-Rad, USA) were used in this study.

Experimental Animals and Materials Experimental sows were provided by Guangzhou Conghua Breeding Pig Farm. ZEN standards were purchased from Sigma-Aldrich.

Primer Design and Restriction Site Design Based on the known full-length sequence of the *ZEN-jjm* gene, full-length amplification primers were designed using Oligo 6 software. The upstream primer was 5' -ATGCGCACTCGCAGCACAAT-3' with an EcoR site, and the downstream primer was 5' -TCAAAGATGCTTCTGCGTAGTTTCC-3' with a Not site. Primers were synthesized by Beijing Aoke Dingsheng Biotechnology Co., Ltd.

PCR Amplification of *ZEN-jjm* Gene Using 2×Pfu PCR MasterMix from Beijing Tiangen Biotech Co., Ltd., PCR was performed with reverse-transcribed cDNA from mRNA as template. The target gene was approximately 750 bp. The 25 µL reaction system contained 1 µL cDNA, 1 µL each of upstream and downstream primers (10 µmol/L), 12.5 µL 2×Pfu PCR MasterMix, and ddH_2O to 25 µL. The PCR program consisted of initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min.

PCR Product Purification After amplification, 5 µL of the product was analyzed by 1.5% agarose gel electrophoresis. The target band was excised and purified using a gel recovery kit.

Construction of Recombinant Expression Vector pHT01-ZEN-jjm The purified PCR product and pHT01 vector were double-digested with EcoR and Not to facilitate insertion of the foreign gene. The digested fragments were ligated using T4 ligase at 22°C for 3 h. The ligation product was transformed into *E. coli* DH5α competent cells, which were then plated on LB agar containing 100 mg/mL ampicillin for selection of recombinants. Positive clones were identified by PCR and EcoR /Not double digestion.

DNA Sequencing and Analysis Clones confirmed by PCR and enzyme digestion were sent to Beijing Aoke Dingsheng Biotechnology Co., Ltd. for DNA sequencing. Sequence alignment confirmed correct insertion, and the verified plasmid was used for subsequent protein expression.

Preparation of *Bacillus subtilis* Competent Cells Competent cells were prepared using growth medium following the method described in reference [10].

Transformation of *Bacillus subtilis* The recombinant plasmid pHT01-ZEN-jjm was transformed into *Bacillus subtilis* competent cells. Two clones were selected for induced expression of the target protein.

Induced Expression in Recombinant *Bacillus subtilis* Transformed *Bacillus subtilis* were inoculated into fermentation medium and cultured at 37°C with shaking at 200 r/min until logarithmic growth phase. Expression was induced with 1 mmol/L isopropyl β-D-1-thiogalactopyranoside for 6 h. Cells were harvested by centrifugation, and the supernatant was mixed with loading buffer and boiled for denaturation. Ten microliters were loaded for SDS-PAGE analysis.

Detection of ZEN Degrading Enzyme Activity The supernatant from induced recombinant *Bacillus subtilis* culture was used for ZEN degradation assays. ZEN solution (standard provided by Beijing Aoke Dingsheng Biotechnology Co., Ltd., final concentration 1 g/mL) was added to the supernatant,

with non-expressed culture supernatant as negative control. Degradation was performed at 30% proportion for 9 h, followed by HPLC detection of residual ZEN.

Evaluation of ZEN Degrading Enzyme Effects on Sow Reproductive Performance A single-factor experimental design was employed using 60 gestating sows (90 days post-coitum) weighing approximately 200 kg. Sows were divided into three groups of 20 each based on similar parity and body weight, with one sow per crate. The experimental groups are shown in Table 1 . Diets were formulated according to NRC (2012) standards for gestating sows. The basal diet was a powdered corn-soybean-fish meal complete feed with nutrient levels of 13.00 MJ/kg digestible energy, 15.63% crude protein, 0.67% lysine, 0.87% calcium, 0.67% total phosphorus, 0.42% available phosphorus, and 0.40% salt. HPLC analysis detected 267 μg/kg ZEN in the basal diet, meeting national feed hygiene standards. Experimental groups were fed basal diet supplemented with either 1.5 mg/kg ZEN or 1.5 mg/kg ZEN + 100 mg/kg ZEN degrading enzyme. Sows were fed at 07:00 and 17:00 daily with 2.5 kg/d feed, had ad libitum access to water, and received routine health management according to farm protocols. Feed intake was zero on the day of farrowing. Reproductive performance parameters including total litter size, live births, stillbirths, mummies, and individual piglet birth weights were recorded within 12 h of farrowing.

Statistical Analysis Data were analyzed by one-way ANOVA using SPSS software. Duncan' s multiple range test was used for post-hoc comparisons. Results are expressed as "mean ± standard error." $P<0.05$ was considered statistically significant, and $P<0.01$ was considered highly significant.

Results

PCR Amplification of *ZEN-jjm* Gene PCR amplification of the *ZEN-jjm* gene fragment yielded a product of approximately 800 bp [Figure 1: see original paper], consistent with the target gene size in NCBI database.

Construction and Identification of pHT01-ZEN-jjm Recombinant Vector As shown in [Figure 2: see original paper], double digestion with EcoR and Not of three selected clones produced bands corresponding to the target gene (approximately 800 bp) in two clones. Sequencing analysis confirmed successful construction of the pHT01-ZEN-jjm expression vector.

Screening of ZEN-jjm Protein Expression SDS-PAGE analysis [Figure 3: see original paper] showed that two recombinant *Bacillus subtilis* strains expressed proteins with molecular mass approximately 29 ku, matching the expected size.

Detection of ZEN Degrading Activity HPLC analysis results are presented in Table 2 . After incubating ZEN-contaminated corn with the expressed *Bacillus subtilis* culture supernatant for 30 min, the degradation rate approached 70%. Following 90 min of enzymatic hydrolysis, ZEN residues were nearly undetectable in the contaminated corn, with degradation rates exceeding 92%. These results demonstrate that the expressed ZEN degrading enzyme effectively degrades ZEN with low production cost and safe application.

Effects of ZEN Degrading Enzyme on Sow Reproductive Performance As shown in Table 3 , sows fed diets containing 1.5 mg/kg ZEN exhibited significantly higher numbers of stillbirths and weak piglets compared to the control group ($P<0.05$), while total litter size and piglet birth weight tended to decrease ($P>0.05$). Compared with experimental group 1, supplementation with 1.5 mg/kg ZEN + 100 mg/kg ZEN degrading enzyme significantly reduced ZEN-induced stillbirths and weak piglets ($P<0.05$).

Discussion

ZEN represents a significant food safety concern and substantially impacts livestock production, particularly causing veterinary obstetric diseases. Minerals such as montmorillonite and aluminosilicates, along with microorganisms including *Bacillus*, lactic acid bacteria, and yeasts, play important roles in adsorbing and degrading ZEN. Bakutis et al. reported that yeast cell wall extracts, primarily composed of oligoglucan products, effectively adsorb and remove ZEN. Kakeya et al. found that *Clonostachys rosea* produces two types of conidiophores —penicillate and verticillate—and relies on secreted extracellular degrading enzymes to reduce ZEN toxicity in reproductive systems. ZEN affects mitochondrial metabolism, plasma membrane permeability, and cell cycle progression in prostate cancer cells. At concentrations of 100 and 0.3 nm, ZEN decreases mitochondrial oxidative activity, induces lactate dehydrogenase release, triggers apoptosis, and increases G0/G1 phase cell numbers. As a 2,4-dihydroxybenzoic acid lactone compound, ZEN toxicity can be eliminated by destroying its ester ring, converting it to non-estrogenic products.

Takahashi-Ando et al. demonstrated that the ZEN lactonohydrolase *zhd101* expressed in *E. coli* exhibits strong ZEN detoxification activity at pH 10.5 in a 37–45°C water bath, representing an irreversible inactivation reaction. The lactonohydrolase *zhd101* is effective against five ZEN analogs, though degradation efficiency varies among them. Molnar et al. identified a novel yeast strain, *Trichosporon mycotoxinivorans*, capable of degrading ZEN to carbon dioxide and other non-toxic metabolites, though field validation is required for its application as a feed detoxifier in livestock and aquaculture. Mokoena et al. reported that lactic acid bacteria can significantly degrade 68–75% of ZEN in cereals such as corn, soybean meal, barley, and bran during 96 h of liquid fermentation. In this study, we expressed ZEN degrading protease using the pHT01 vector in *Bacillus subtilis*, which offers advantages including minimal interference from

host proteins, diverse growth environments, rich nutrient utilization, abundant endogenous enzyme systems, high target protein expression levels, and maintained biological activity, making ZEN degradation safer, more effective, and cost-efficient.

Research indicates that ZEN poisoning significantly affects swine health, causing functional changes in reproductive organs. Minervini et al. fed piglets diets containing 9 mg/kg ZEN from 32 days post-weaning for 30 days, resulting in abnormal reproductive systems characterized by impaired oocyte maturation and chromosomal abnormalities. Jadamus et al. fed gestating sows diets containing 180 µg/kg ZEN across three reproductive cycles, observing increased return-to-estrus rates and abortion rates; the first cycle showed abnormal early estrus. Jan Obremski et al. administered 0.2 and 0.4 mg/kg BW ZEN to gestating sows for one week, finding follicular atresia, ovarian granulosa cell apoptosis, and uterine/oviduct cell proliferation in ZEN-treated groups. Our results similarly demonstrate that even low dietary ZEN levels (1.5 mg/kg) significantly reduce healthy live fetuses and increase weak piglet incidence, consistent with low-dose ZEN and its metabolites disrupting endocrine systems and affecting estrous cycles, ovulation, and embryo implantation. However, supplementation with ZEN degrading enzyme in experimental group 2 did not show adverse effects on total litter size, stillbirths, weak piglets, or birth weight, indicating that ZEN degrading enzyme effectively mitigates multiple toxic effects of ZEN on sow production and reproductive performance.

ZEN reduces reproductive performance in gestating sows, with low-dose contamination (1.5 mg/kg) significantly decreasing live fetuses and increasing weak piglet incidence. In this study, genetically engineered *Bacillus subtilis* expressing the *zhd101* gene effectively removed high concentrations of ZEN (1.5 mg/kg). Evaluation of total litter size, stillbirths, weak piglets, and birth weight demonstrates that the constructed ZEN degrading enzyme effectively alleviates ZEN toxicity on sow reproductive performance during gestation.

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Note: Figure translations are in progress. See original paper for figures.