UDC 577.112.083

SECRETORY EXPRESSION OF THE GLUCAN ENDO-1,3-BETA-D-GLUCOSIDASE GENE OF *SECALE CEREALE* IN YEAST *PICHIA PASTORIS*

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ABSTRACT

For survival in cold conditions, many organisms have developed unique adaptive mechanisms based on the synthesis of antifreeze proteins, peptides and glycoproteins that prevent ice formation at negative temperatures. These molecules tend to bind ice crystals and lower the freezing point of the solution without the formation of large crystals. Antifreeze proteins (AFP) were found in almost all types of living organisms, including insects, fungus, yeasts, bacteria and plants. The gene of antifreeze protein - *glucan endo-1,3-beta-D-glucosidase* (ScGlu-3) from *Secale cereale* was cloned into shuttle vector pPICZαA. The competent cells of yeast *Pichia pastoris* GS115 were transformed and the producer strain was obtained, which secreted of ScGlu-3 into the culture medium using 3% methanol as the only carbon source. It was found by western blotting that the maximum accumulation of ScGlu-3 in the culture occurs after 48 hours of fermentation on a medium with methanol. Established that rScGlu-3 precipitates at 50-65% of ammonium sulfate.

Key words: antifreeze protein, strain, peptides, *Secale cereale*, gene, shuttle vector, *Pichia pastoris*, ammonium sulfate.

INTRODUCTION

For survival in cold conditions, many organisms have developed unique adaptive mechanisms based on the synthesis of antifreeze proteins, peptides and glycoproteins that prevent ice formation at negative temperatures. These molecules tend to bind ice crystals and lower the freezing point of the solution without the formation of large crystals. This phenomenon is called thermal hysteresis [1]. These proteins were first discovered when studying Antarctic fish in the late 1960s [2]. In the blood of polar fish, glycoproteins have been found in which the carbohydrate fragment is linked to the polypeptide chain [3]. Later, a protein was found in the blood of the winter flounder *Pseudopleuronectes americanus,* which does not contain a carbohydrate fragment, but has antifreeze properties [4]. It was noted that due to antifreeze proteins (AFP), polar fish withstand cooling down to −2°C. In total, five types of AFP are known for fish (types I - IV and antifreeze glycoproteins), which differ depending on their structural characteristics and source [1, 5]. Antifreeze proteins were found in almost all types of living organisms [6], including insects [7-9], fungus [10], yeasts [11-13], bacteria [14-16] and plants [17, 18]. The more studied are the AFP of arthropods, which have a much stronger antifreeze activity than the fish's AFP [19]. At the molecular level, antifreeze proteins are excellent examples of convergent evolution, as ADPs have

completely different protein sequences and structures, but perform the same function. AFP from *Choristoneura fumiferana* [7] and *Tenebrio molitor* [11] have a β-sheet structure, while AFP from a snow flea have a bundle of six tightly packed α-helices of type II polyproline and a hydrophobic ice-binding surface without threonine residues [20, 21].

The ability of AFP to inhibit ice recrystallization and interact with biological membranes makes them interesting molecules for use in various fields. For example, in reproductive biology for cryopreservation of gametes and embryos [22], since the cryoprotective agents used today in high concentrations are cytotoxic [23], while AFP-based cryoprotectants do not have this disadvantage [1, 24]. In the food industry, antifreeze proteins are an important additive to improve the quality of frozen foods [25]. Use of AFPs with the ability to inhibit the growth and recrystallization of ice preserve nutrients [25].

In addition to insects and fish of the circumpolar regions, AFP of plant origin are described in the literature [5, 6]. In contrast to the AFP of fish and insects, a number of AFP of plants have multiple hydrophilic sites for binding to ice [26-28]. For example, wheat APBs have two ice binding sites that are complementary to the prismatic ice surface and are located opposite each other [29]. From the point of view of application in the food industry, antifreeze proteins of plant origin are more preferable than AFP of insects and fish. First of all, due to the low cost of raw materials and the possibility of industrial extraction and the absence of allergenicity, this allows them to be added to food without any risks to the human body. From this point of view, it is interesting to study antifreeze proteins from cereals: wheat, triticale, barley and rye. In particular, glucan endo-1,3-beta-D-glucosidase was found in cereals, which is a protein with a molecular weight of 35.2 kDa and has glucanase activity. And as shown by an analysis of literature sources, this protein has an antifreeze property [30]. It seems promising to obtain AFP of plant origin by the technology of microbial synthesis, since the creation of a microbial producer strain develops possibilities for the industrial production of antifreeze proteins.

The aim of this work was to obtain recombinant glucan endo-1,3-beta-D-glucosidase (ScGlu-3) of *Secale cereale* by secretory expression of the target gene in the yeast *Pichia pastoris.*

Materials and methods

Vectors, strains, enzymes and chemicals. Vector pPICZαA (Invitrogen, USA) was used to construct the expression cassette. The gene of *glucan endo-1,3-beta-D-glucosidase* (921 bp) was used from NCB lab collection. Polymerases Pfu (Thermo Fisher, USA) and Taq (NCB, Kazakhstan) and restriction enzyme PmeI (NEB, USA) were employed for the amplification and cloning of the target gene. Escherichia coli strain DH5α was acquired from Thermo Fisher Scientific. *P. pastoris* GS115 cells were purchased from Invitrogen (USA). The chemical reagents used in this study were of molecular biology or pure analytical grade and purchased from Sigma-Aldrich (St. Louis, USA) and AppliChem (Darmstadt, Germany). The vectors and enzyme were stored at -20°C, the strains were stored at -80°C and chemicals were stored accordingly manufactured recommendations.

Cloning of the gene and vector construction. The *ScGlu-3* gene was amplified from pET-28c(+)/ScGlu-3 with PCR primers Glu3-Rfw (5’-GTATCTCTCGAGAAAA GAGAGGCTGAAGCTATCGGCGTCTGTACGGCGT-3’) and Glu3-Rrv (5’-GGAAC AGTCATGTCTAAGGCTACAAACTCAGAACTGGATGGCGTAGGCCG-3’). PCRs (50 μL final volume) contained 5 μL of 10X Pfu Buffer (Thermo Fisher), 5 μL of

dNTPs (a 2 mM stock solution), 1 μL of each primer (a 10 μM stock solution), 100 ng of a DNA template, 1 μL of Pfu polymerase (1250 U/mL), and 34 μL of nuclease-free water. The amplification parameters were following: initial denaturation at 98°C for 3 min; then 30 cycles of 98°C for 30 sec, 55°C for 1 min, and 68°C for 2 min; and final extension at 68°C for 10 min. The PCR-product was purified by phenol/chloroform extraction and was cloned into the pPICZαA vector by restriction free methodology [31]. PCRs (50 μL final volume) contained 5 μL of 10X Pfu Buffer (Thermo Fisher), 5 μL of dNTPs (a 2 mM stock solution), 1.5 μL of DMSO (a 100% stock solutiion) 100 ng of a PCR product from the first step, 30 ng of pPICZαA plasmid, 2 μL of Pfu polymerase (1250 U/mL), and up to 50 μL of nuclease-free water. Amplification parameters were following: initial denaturation at 98°C for 1 min; then 18 cycles of 98°C for 30 sec, 60°C for 1 min, and 68°C for 5.5 min; and final extension at 68°C for 11 min. The PCR-product was treated by DpnI for 1 h, and E.coli DH5α cells were transformed by resulting treated linear vector. Obtained clones were selected on zeocin (25 μg/mL) agar plates and the shuttle plasmid pPICZαA/ScGlu-3 was purified from cells by the plasmid Midiprep kit (Invitrogen, USA). The encoded ScGlu-3 protein carries an N-terminal α-factor signal peptide for secretion in yeast culture.

Sequencing. Sequencing of target genes was carried out on an ABI 3730xl Genetic Analyzer (Applied Biosystems) using BigDye Terminator v3.1 (Applied Biosystems).

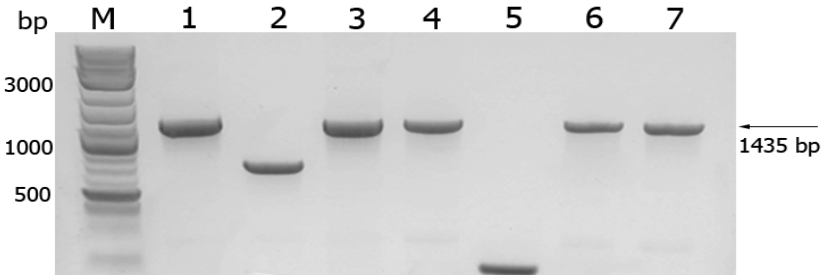
Transformation of *P. pastoris* cells and producer strain preparation. The pPICZαA/ScGlu-3 vector was linearized with endonuclease PmeI in Cut Smart Buffer (NEB, USA). The linearized vector was purified by phenol/chloroform extraction followed by ethanol precipitation. *P. pastoris* GS115 cells were electroporated with the linearized vector as follows. Fresh competent *P. pastoris* cells were prepared and transformed according to the EasyselectTM Pichia Expression Kit manual (Invitrogen). To 80 μL of the cells, 3 μg of purified linearized plasmid DNA was added, and then the suspension was pulsed in a 0.2 cm electroporation cuvette at 2 kV for 4.8 ms with electoporator (MicroPulserTM, Bio-Rad, USA). Clones of *P. pastoris* GS115/pPICZαA/ScGlu-3 were selected on an agar plate with zeocin (200 μg/mL). After that, the clones were screened for the presence of the insert by PCR with primers AOX1fw (5'- GACTGGTTCCAATTGACAAGC-3') and AOX1rv (5'- GCAAATGGCATTCTG ACATCC-3'). The clones positive for ScGlu-3 insert were analyzed for ScGlu-3 expression by a SDS-PAGE and western blotting. Clone with the highest ScGlu-3 expression was preserved with glycerol at -80°C and served as a producer strain.

Protein production in shake flasks and ammonium sulfate precipitation. The *Pichia pastoris* GS115/pPICZαA/ScGlu-3 strain cells were inoculated into a 50 mL flask containing 5 mL of YPD with zeocin (200 μg/mL) and were cultured overnight at 30°C and 250 rpm in a shaking incubator (KS 4000 i control, IKA, Germany). The overnight culture was inoculated into 50 mL of YPD in a 500 mL shake flask and was incubated at 30°C and 250 rpm for overnight. The overnight culture was inoculated into fresh 500 mL of YP in a 5 L shake flask and was incubated in a shaking incubator (Climo-Shaker ISF1-X, Kuhner, Switzerlend) at 28°C and 250 rpm for 120 h with daily addition of methanol (finally 3%). The cells were collected by centrifugation at 3500×g, for 15 min, at 4°C (Avanti J-26SXP with rotor JA-10, Beckman Coulter, USA) and discarded. The supernatant was subjected to ammonium sulfate fractionation. The 20%, 35%, 50% and 65% of (NH4)2SO4 was used for protein precipitation. The supernatant was incubated with appropriate ammonium sulfate for 1h at 4°C and centrifuged at 18000 × g. The supernatant and pellet were analyzed by SDS-PAGE.

SDS-PAGE and western blotting. Electrophoretic separation of proteins was performed by SDS-PAGE in a 12% polyacrylamide gel according to the Laemmli method [32] in a miniProtean-IV cell (Bio-Rad Laboratories Inc). Electrophoresis conditions were as follows: 120 V for 90 min. The gel was stained in 2% Coomassie Brilliant Blue R250 and destained with 7.5% acetic acid in 25% ethanol. For western blotting, we applied polyclonal antibodies raised in rabbits against *ScGlu-3*. Western blotting was performed according to the standard protocol [33]. Briefly, protein samples were separated by SDS-PAGE in a 12% (w/v) gel and transferred to a polyvinylidene difluoride (PVDF) membrane. To verify the protein transfer, the membrane was stained with the Ponceau S dye [0.1% (w/v) of Ponceau S in 5% (v/v) acetic acid]. After membrane blocking with 0.5% (w/v) bovine casein in Tween 20/Tris-buffered saline (TBST: 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% [w/v] Tween 20), the ScGlu-3 protein was detected with the rabbit polyclonal antibody against calf prochymosin (1:5000) as a primary antibody and a goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Sigma-Aldrich Chimie S.a.r.l., Lyon, France) (1:10,000) as a secondary antibody. The bands were detected by the Enhanced Chemi-Luminescence Detection Kit (Applichem GmbH, Darmstadt, Germany), and an X-ray film was then exposed to the membrane (AgfaPhoto GmbH, Germany).

RESULTS AND DISCUSSION

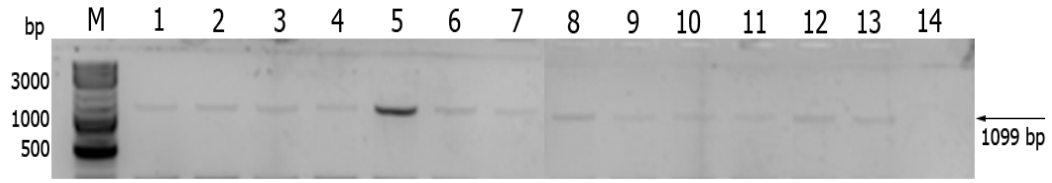
Restriction free cloning is very effective method for cloning target gene into vector [31]. Using this method, by two-step PCR, the pPICZαA/ScGlu-3 construction was obtained. Figure 1 shows the results of PCR screening of *Escherichia coli* DH5α clones transformed with the pPICZαA/ScGlu-3 vector. As can be seen from the Figure1, 5 out of 7 clones carry an insert corresponding to the length of the *ScGlu-3* gene with an additional secretory domain (255 bp) and AOX1 priming cite (182 bp). The total length of AOX1 region with insert is 1435 bp. Plasmids were isolated from clones #1,3,4,6,7 and sequenced on the AOX1 region.



M-DNA Ladder; 1-7 - clones

Fig. 1. Results of PCR screening of DH5α-pPICZαA/ScGlu-3 clones on AOX1 region

Thus, using the method of restriction-free cloning, the pPICZαA / ScGlu-3 construct was obtained, in which the glucan *endo-1,3-beta-D-glucosidase* gene was inserted under the control of the methanol-induced AOX1 (alcohol oxidase 1) promoter. By hydrolyzing the shuttle vector pPICZαA/ScGlu-3 with the PmeI restriction enzyme, the transformation cassette was obtained. The competent cells of yeast *Pichia pastoris* strain GS115 were electroporated with this cassette. The transformation was high efficiency and results to insertion of *glucan endo-1,3-beta-D-glucosidase* gene into chromosomal DNA of yeast. Figure 2 shows the results of PCR screening of 14 yeast clones grown on YPD agar with zeocin.

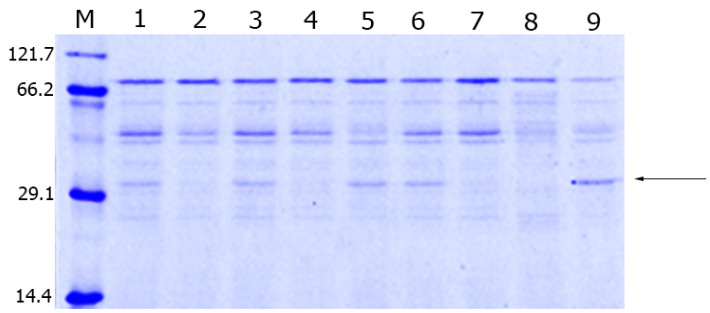


M-DNA Ladder; 1-14 - clones

Fig. 2. Results of PCR screening for genomic DNA of clones GS115-pPICZαA/ ScGlu-3 with Glu3-RFfw and AOX1rv primers

As shown on Figure 2, clones ##1-13 contain ScGlu-3 insert in the chromosomal DNA. Clone #5 has maximum signal, it can be explain with multiple insertion cassette with Glu3-RF inside AOX region of yeast. *Pichia pastoris* (also known as *Komagataella phaffi*) is methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. The first step in methanol metabolism is the oxidation of methanol with alcohol oxidase (AOX1 or AOX2) in the presence of molecular oxygen to formaldehyde, along with which hydrogen peroxide is formed. Expression of the AOX1 gene is regulated and induced by methanol to high levels in cells grown with methanol. AOX1 promoter is used to regulate expression of the gene of interest encoding the desired heterologous protein [34, 35]. *Pichia pastoris* can secrete the heterological protein to supernatant within cultivation. Secretion requires the presence of a signal sequence on the expressed protein. The secretion signal sequence from *Saccharomyces cerevisiae* factor prepro peptide has been used with the most success [36].

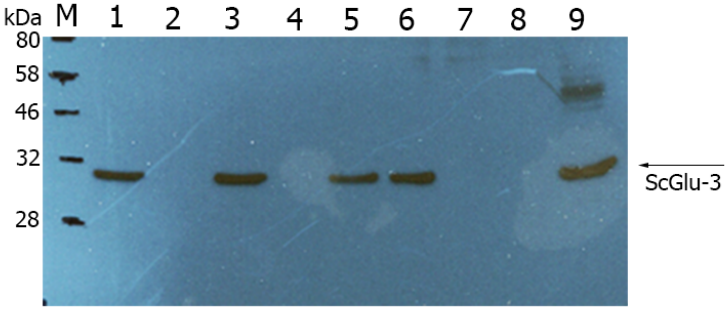
Culturing of 9 clones in YPD medium followed by replacement with YP medium supplemented with 3% methanol as sole carbon source for 120 hours provided protein secretion into the culture medium (Figure 3). SDS-PAGE shows the presence of a protein with a mass according to the calculated mass of ScGlu-3.



M- protein marker; 1-9 - clones

Fig. 3. SDS-PAGE of GS115-pPICZαA/ScGlu-3 clones supernatants after 120 h cultivation

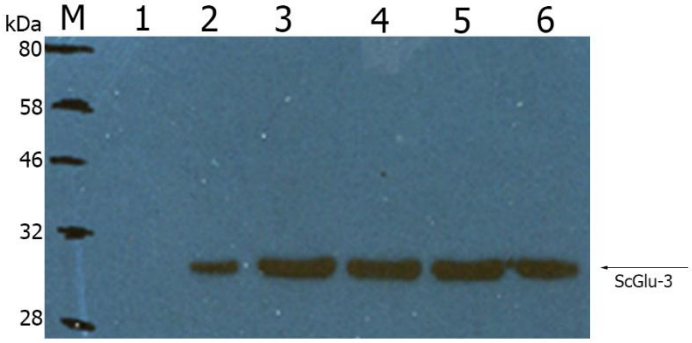
Figure 3 shows that clones 1,3,5,6,9 produce a recombinant protein ScGlu-3. The belonging of this protein to the target protein ScGlu-3 was confirmed by the results of Western blotting using anti-rScGlu-3 polyclonal antibodies, previously obtained by immunization with purified rabbit ScGlu-3 protein (Fig. 4).



M- protein marker; 1-9 - clones

Fig. 4. Western blotting of GS115-pPICZαA/ScGlu-3 clones supernatants after 120 h cultivation

As follows from Figure 4, clones #1, 3, 5, 6 and 9 cultured on YP medium, after induction with 3% methanol, produce the recombinant protein rScGlu-3 with approximately the same expression level, and clone 9 was chosen as the producer strain for the recombinant protein rScGlu-3. An experiment to determine the dynamics of secretory expression of the rScGlu-3 protein by the GS115-pPICZαA/ScGlu-3 strain confirmed the accumulation of rScGlu-3 in the supernatant (Fig.5).

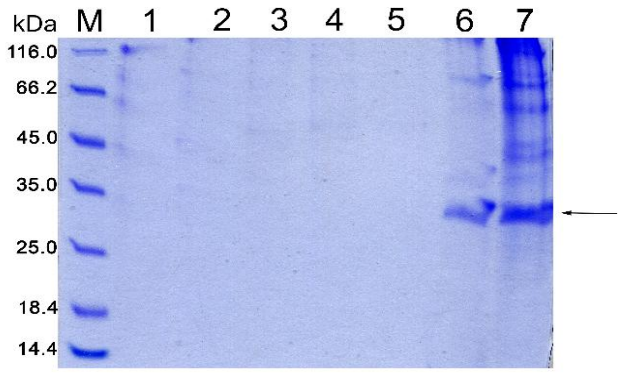


M-protein marker; 1- 0 h; 2- 24 h; 3- 48 h; 4- 72 h; 5- 96 h; 6- 120 h

Fig. 5. Dynamics of accumulation of recombinant protein ScGlu-3 during cultivation of clone GS115-pPICZαA/ScGlu-3 depending on time

As can be seen from the results of Western blotting (Fig. 5), already after 24 hours of induction with methanol, the recombinant protein ScGlu-3 is present in the supernatant and after 48 hours it reaches a maximum. Further cultivation of the strain on a medium with methanol has practically no effect on the production of the ScGlu-3 protein.

Ammonium sulfate precipitation is often the first step in the purification of untagged recombinant proteins from yeast culture [37]. This approach allows to significantly concentrate the recombinant protein and get rid of some impurity proteins, while not disrupting the structure and properties of the target proteins [38, 39]. It was experimentally found that recombinant ScGlu-3 is precipitated only when the concentration of ammonium sulfate reaches 50-65% (Fig. 6).



M-protein marker, 1- 0% (supernatant); 2- 20% (supernatant); 3- 35% (supernatant); 4- 50% (supernatant); 5- 65% (supernatant); 6 - 50% (pellet); 7 - 65% (pellet)

Fig. 6. Ammonium sulfate precipitation of rScGlu-3 from yeast culture

At a lower concentration of ammonium sulfate, precipitation of the recombinant protein is not observed. This information will be useful for purification of rScGlu-3.

CONCLUSION

Thus, by cloning the *glucan endo-1,3-beta-D-glucosidase* gene in the chromosomal DNA of the methylotrophic yeast *Pichia pastoris*, the recombinant strain was obtained. The strain GS115- pPICZαA/ScGlu-3 when cultured on a medium with 3% methanol as sole carbon source secretes the protein with a mass of 28 kDa. Secretory protein is glucan endo-1,3-beta-D-glucosidase, that was confirmed by western blotting with specific antibodies. Established that rScGlu-3 precipitates at 50-65% of ammonium sulfate. rScGlu-3 will be tested for antifreeze properties.

Acknowledgements

The work was supported by the Ministry of Education and Science of the Republic of Kazakhstan, Science-Technical Program OR11465422 «Creation of a biobank of rare and endangered species of flora and fauna of Kazakhstan for the conservation of biodiversity» for 2021-2022.

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PICHIA PASTORIS АШЫТҚЫСЫНДАҒЫ *SECALE CEREALE* ГЛЮКАН ЭНДО-1,3-БЕТА-D-ГЛЮКОЗИДАЗА ГЕНІНІҢ СЕКРЕТОРЛЫҚ ЭКСПРЕССИЯСЫ

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ТҮЙІН

Суық жағдайда өмір сүру үшін көптеген ағзалар теріс температуралар кезінде мұздың пайда болуына жол бермейтін антифриз ақуыздарының, пептидтердің және гликопротеиндердің синтезіне негізделген ерекше бейімделу механизмдеріне ие болды. Бұл молекулалар мұз кристалдарымен байланысады және үлкен кристалдар түзбей, ерітіндінің қату температурасын төмендетеді. Антифриз ақуыздары (AFB) тірі ағзалардың барлық түрлерінде кездеседі, соның ішінде: жәндіктер, саңырауқұлақтар, ашытқылар, бактериялар және өсімдіктер.

Антифриз ақуызының гені - глюкан эндо-1,3-бета- D -глюкозидаза (ScGlu-3) қара бидайдан (*Secale cereale*) pPICZαA векторының құрамында клондалды. Ашытқы *Pichia pastoris* штаммының GS115 компетентті жасушалары осы вектормен түрлендірілді және ScGlu-3-ті қоректік ортаға бөлетін штамм, 3% метанолды көміртектің жалғыз көзі ретінде қолдана отырып алынды. Вестерн-блоттинг әдісімен культурада ScGlu-3 максималды жинақталуы метанол ортасында 48 сағаттық ашытудан кейін пайда болатындығы анықталды. rScGlu-3 аммоний сульфатының 50-65% тұндырылатыны анықталды.

Негізгі сөздер: антифриз ақуыздары, штамм, пептид, *Secale cereale*, ген, тасымалдау векторы, *Pichia pastoris*, аммоний сульфаты.

СЕКРЕТОРНАЯ ЭКСПРЕССИЯ ГЕНА ГЛЮКАН ЭНДО-1,3-БЕТА-D-ГЛЮКОЗИДАЗЫ *SECALE CEREALE* В ДРОЖЖАХ *PICHIA PASTORIS*

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АБСТРАКТ

Чтобы выжить в холодных условиях, многие организмы разработали уникальные адаптивные механизмы, основанные на синтезе белков антифриза, пептидов и гликопротеинов, которые предотвращают образование льда при отрицательных температурах. Эти молекулы, как правило, связываются с кристаллами льда и снижают температуру замерзания раствора без образования крупных кристаллов. Антифризные белки (АФБ) обнаружены почти во всех типах живых организмов, включая: насекомые, грибы, дрожжи, бактерии и растения.

Ген антифризного белка - глюкан эндо-1,3-бета-D-глюкозидаза (ScGlu-3) из ржи посевной (*Secale cereale*) был клонирован в составе челночного вектора pPICZαA. Компетентные клетки дрожжей *Pichia pastoris* штамма GS115 были трансформированы данным вектором и путем селекции был получен штамм, секретирующий ScGlu-3 в культуральную среду, используя 3% метанол в качестве единственного источника углерода. Методом вестерн-блоттинга было установлено, что максимальное накопление ScGlu-3 в культуре происходит после 48 часов ферментации на среде с метанолом. Установлено, что rScGlu-3 осаждается на 50-65% сульфата аммония.

Ключевые слова: антифризный белок, штамм, пептид, *Secale cereale,* ген, челночный вектор, *Pichia pastoris*, сульфат аммония.