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THIONINS OF WHEAT *Triticum kiharae* Dorof. et Migush. ARE NOVEL POTENT INHIBITORS OF *Candida albicans* (C.P. Robin) Berkhout

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Abstract

Plants serve as a source of biologically active compounds, the most important of which are antimicrobial peptides (AMPs). AMPs represent an integral part of the defense arsenal of all living beings. Members of the thionin family found only in plants are effective inhibitors of plant pathogens, including bacteria and fungi, which opens up prospects for their practical application as biopesticides to protect plants from diseases. However, the effect of thionins on animal and human pathogens has not been sufficiently studied. Yeast-like fungi of the genus *Candida* are opportunistic pathogenic microorganisms that occur in 70 % of people without causing disease (M. Dadar et al., 2018). However, in immune-compromised individuals, they can cause a number of serious diseases, the frequency of which has increased significantly in the last two decades. Antimycotics traditionally used to treat *Candida* infections are not always effective and safe for humans. In this regard, the world is constantly searching for new natural antifungal agents. The aim of this work was to isolate thionins from the kernels of the highly pathogen-resistant wheat species *Triticum kiharae* Dorof. et Migush., determine their primary structure, and assay antifungal activity against *Candida albicans*. For the first time from the wheat *T. kiharae* using chromatography on chitin and reversed-phase high-performance liquid chromatography (HPLC), 2 thionins Tk-AMP-BP and Tk-AMP-AP1 were isolated, and their amino acid sequences were determined by automated Edman degradation. The primary structure of Tk-AMP-BP was confirmed by transcriptome high-throughput sequencing (NGS) of wheat seedlings. The study of antimicrobial activity of Tk-AMP-BP showed that it has potent fungicidal effect on *C. albicans* cells at very low concentrations (MIC = 0.78 μg/ml). The biological activity of the wheat thionin against *C. albicans* was higher than that of thionins from other plant species. The results obtained in this work allow us to consider the wheat thionin as a promising molecule for the development on its basis of next-generation drugs to treat *C. albicans* infections.

Keywords: plant immunity, antimicrobial peptides, *Triticum kiharae* Dorof. et Migush., wheat species, thionins, mycoses, *Candida albicans*

Although the plants have no adaptive immunity system as compared to superior vertebrates they possess congenital immunity enabling in finding pathogens and inhibit growth thereof in plant tissues [1]. Range of "chemical means" of protection including secondary metabolites and proteic substances like antimicrobial proteins (AMPr) and peptides (AMP) are used to inhibit plant pathogens [2-4]. Antimicrobial peptides are components of congenital immunity both in animals and plants. They are short (less than 100 amino-acid residues) positively charged amphiphilic polypeptides differed in primary and spatial structures and the so-called cysteine motif, i.e. arrangement of cysteine residues inside the peptide molecule [5-9]. There are several AMP families marked out under the struc-

tural similarity thereof: thionins, defensins, nonspecific lipid-transfer proteins, hevein- and knottin-like peptides, harpinins, as well as macrocyclic peptides (cyclotides) [5-7]. AMPs may be practically applied in agriculture as biopesticides to protect plants against diseases and in medicine when making new drug groups. As compared with usual antibiotics, the AMPs have some advantages: immediate impact, wide spectrum of antimicrobial activity, activity against antibiotic-resistant pathogen isolates, no persistent form of pathogens, if applied together with antibiotics they enhance the effect thereof, they possess activities useful for humans (e.g. inhibit sepsis).

Thionins are short (~ 5 kDa) cysteine-rich peptides first found in wheat flour [10]. Later they were found in a wide range of mono- and dicotyledonous plants. Over 100 thionin sequences from 15 plant species are known nowadays [11]. Thionins are divided into two main groups by number of cysteine residues, 6 and 8 ones, forming disulphide bonds [5]. There are 5 classes of thionins by structural features thereof [12]. For more than 60 years thionins are known to inhibit growth of pathogenic bacteria and fungi in vitro [13]. Inhibition of pathogenic bacteria growth was first demonstrated by R. Fernandez de Caleya et al. [14], and they made an assumption on the protection role of these proteins in plants. According to further analysis, thionins proved to inhibit the growth of both gram-positive and gram-negative bacteria and a number of phytopathogenic fungi and Oomycetes as well, with IC_{50} usually being within 1 to 15 *r*g/ml [11, 15, 16]. Therewith biological thionin activity with regard to pathogens and opportunistic pathogens in humans is poorly studied.

Candida albicans is a yeast-like fungus found in normal flora of 70% of humans (oral cavity, esophagus, intestinal tract, genital tracts and skin) provoking no diseases [17]. Immune compromised humans (HIV infected, oncology patients and after organ transplantation) may be seriously affected by this fungus, including blennosis and general lesion [18-20]. Infections induced by *Candida* spp. include full spectrum of such serious diseases as invasive candidiasis (*Candida* fungi in blood), chronic disseminated candidiasis, endocarditis, cerebral fever and entophthalmia [19].

Five classes of antimycotic agents are applied at invasive mycosis: azoles (imidazole and triazole derivatives), polyenes (polyene antibiotics), allylamines, echinocandins and fluoropyrimidines [21, 22]. However, treatment of infections with the above mentioned drugs is limited by spectrum of activity thereof, resistance of pathogens thereto, and fungistatic but not fungicidal activity thereof [23]. Besides, many antimycotic agents are toxic to mammalian cells. Therefore, natural antibiotics (AMP, like thionins) may be considered as the promising molecules against mycosis both in humans and animals.

Thionins of synthetic hexaploid wheat *Triticum kiharae* Dorof. et Migush were analyzed herein. They possess high resistance to phytopathogens due to large variety of AMPs found in seeds of this plant among which there are two thionins called Tk-AMP-BP and Tk-AMP-AP [24]. Short N-terminal amino acid sequences thereof were found by us earlier, and these AMPs were demonstrated to efficiently inhibit phytopathogenic fungi [24, our not published data] and exhibit antimutagenic activity protecting human cells against toxic effect of cadmium ions [25]. At the same time, full primary structure of these AMPs, and ability thereof to inhibit growth and development of pathogens in humans were not studied yet.

Primary structure of two thionins of *T. kiharae* wheat was first discovered herein, while the sequence of one of the, the Tk-AMP-BP, was confirmed by NGS (next-generation sequencing) method, and this thionin was demonstrated to have fungicidal effect on *C. albicans* cells at very low concentrations.

The aim of this paper was to isolate thionins from seeds of *T. kiharae* wheat, to find full amino acid sequences thereof, and analyze antifungal activity against *C. albicans*.

Techniques. Thionins were isolated from grinder-milled seeds of *T. kiharae* (10 g). The flour was being extracted by 50 ml of acid mixture (1 M HCl and 5% HCOOH, Khimmed, Russia) for 1 hour with constant stirring thereof. Protein-peptide fraction was pelleted (10,000 g, 15 min) from supernatant by five volumes of chilled acetone (high purity, Khimmed, Russia) during night at 4 °C. The residue was air-dried, dissolved in 5 ml of 50 mM ammonium-bicarbonate buffer (pH 7.8), and centrifuged (1,000 g, 10 min). The supernatant was mixed for 1 hour with 1 g of chitin (Sigma, USA) pre-washed with two volumes of 0.1% trifluoroacetic acid (TFA) (puriss. p.a., Fluka, Switzerland), two volumes of MQ water and balanced by three volumes of 50 mM NH_4HCO_3 (chemically pure, Reakhim, Russia). After immobilization of protein-peptide fraction on chitin, the latter was washed three times with 20 ml of 50 mM NH_4HCO_3 to remove not-bound components, then the chitin-bonded protein-peptide fraction was eluted with 30 ml of 0.1% trifluoroacetic acid (TFA) and separated by reverse phase highly performance liquid chromatography method (RP-HPLC) in Reprosil C_{18} column (4×250 mm, Dr. Maisch GmbH, Germany) in gradient of acetonitrile concentrations (UHPLC Supergradient, PanReac Quimica SLU, Spain): 10-50% solution B (80% acetonitrile in 0.1% TFA) in solution A (0.1% TFA) within 60 min (elution rate of 1 ml/min, detection at λ = 214 nm). The fractions were mass-spectrometricall analyzed (Bruker Daltonik GmbH, Germany).

To isolate individual components the thionin-containing fraction was re-chromatographed using Luna C_{18} column (4.6×150 mm, Phenomenex, Inc., USA) in gradient of acetonitrile concentrations (20-50 % solution B in solution A) for 30 min (elution rate of 0.75 ml/min, detection at λ = 214 nm).

Disulphide bonds were restored and alkylated as indicated in [24]. For this purpose 10 μg of the dried peptide was dissolved in 40 μl solution containing 6 M guanidine hydrochloride and 2 mM EDTA (BioUltra, Sigma-Aldrich, USA) in 0.5 M Tris-HCl buffer (pH 8.5). Then 2 μl of 1.4 M dithiothreitol water solution (BioUltra, Sigma-Aldrich, USA) was added. The obtained reaction mixture was vortexed and incubated for 4 hours at 40 °C, then 2 μl of 4-vinylpyridine (M_w ~ 60,000, Sigma-Aldrich, USA) was added. The mixture was incubated for 20 min at room temperature in the dark, then diluted by 100 rl of 0.1% TFA and injected onto the Luna C_{18} column (4.6×150 mm, Phenomenex, Inc., USA). The reaction products were separated in gradient of acetonitrile concentrations (0-50% in 0.1% of TFA) for 30 min.

RNA was isolated from wheat seedlings using Plant RNA Isolation Aid (Ambion, Inc., USA). Quality of RNA preparation was checked by Agilent 2100 Bioanalyzer (Agilent, USA). Ribosomal RNA fraction, unbound ribonucleotides and any residues of genomic DNA was removed from total RNA with the application of ready-to-use chemical sets (Illumina, Inc., USA) according to the manufacturer's protocol.

cDNA libraries were constructed as indicated in [26]. The cDNA libraries were sequenced (Genome Analyzer IIx, Illumina, Inc., USA). Transcriptomes were assembled by Trinity application (version 2.1.0) [27] with the digital normalization and maximum 50× coverage).

The elaborated algorithm described in [26] was applied for searching thionin precursor transcripts.

Mass-spectra were fixed by MALDI-TOF-MS (Ultraflex II TOF/TOF, Bruker Daltonik GmbH, Germany) either in linear mode or positive ion mode

using reflectron. 2.5-Dihydroxybenzoic acid (Ultra Pure, Sigma-Aldrich, USA) in concentration of 10 mg/ml in 50% (volume/volume) acetonitrile containing 0.1% TFA (volume/volume) was a matrix. Standard set of peptides and proteins in the molecular weight range of 700-66,000 Da (Sigma-Aldrich, USA) was applied for calibration.

For sequencing, reduced and alkylated thionins were vaporized in Savant SpeedVac Concentrator (Thermo Fisher Scientific, USA) till 50 μl volume. Amino acids were sequenced by Edman degradation (Procise 492 Sequencer, Applied Biosystems, Inc., USA) according to the manufacturer's protocol. Homologous sequences were found in the databases GenBank (https://www.ncbi.nlm.nih.gov/genbank/)/EMBL-Bank (http://www.ebi.ac.uk/embl/).

Antifungal activity was studied with 3-day culture of *C. albicans* grown on Sabouraud agar. Fungal cells were suspended in Sabouraud nutrient broth (1×10^6 cells/ml). Peptide sample was dissolved in nutrient broth to 200 μg/ml concentration and series of ten two-fold dilutions was prepared. A 50 μl aliquot of fungal suspension and 50 μl of peptide solution were added to wells of 96-well polystyrene plate for biological tests (BioCell, USA). Nutrient broth was a negative control, while 50 μl of fungal suspension and 50 μl of nutrient broth was the positive control. The plate was incubated for 72 hours at 28 °C. Then the control sample (without peptide) was diluted with Sabouraud medium (1:1000) and 100 μl was plated onto Sabouraud agar in Petri dishes. The dished were incubated for 48 hours at 28 °C. Fungus growth in control was taken as 100%. Test samples with various peptide concentrations were analyzed in the same way. Peptide minimum inhibiting concentration (MIC) fully (100 %) inhibiting the fungus growth was defined. The experiment was arranged in 3 replications.

Results. Wheat thionins were extracted in several steps: first, protein-peptide fraction was extracted by acid mixture [24], second, AMPs were immobilized on chitin [28], third, AMPs were separated by RP-HPLC method thereby obtaining thionin fraction; at the final step individual thionins were purified by re-chromatography. Chromatographic separation profile of wheat AMPs in the reversed phase column Reprosil C_{18} is presented in Figure 1.

According to mass-spectrometry analysis, the retention time for thionin mixture is 46.35 min. Components 4918 Da, 4801 Da and 4921 Da were found in this fraction, with the first two prevailing (see Fig.1). Re-chromatography of thionin-containing fraction in Luna C_{18} column (data not presented) resulted in purification of two peptides of 4918 Da and 4801 Da.

Amino acid sequences of the reduced and alkylated thionins were found by Edman automatic sequencing method (Fig. 2). Amino acid sequences of *T. aestivum* hexaploid wheat thionins and diploid species *T. urartu* and *T. monococcum* which are supposed donors of genome A of wheat polyploid species, as well as barley and rye thionins deposited in GenBank, are also indicated for comparison. Polypeptide chain length of both isolated purothionins is 45 amino acid residues. The *T. kiharae* 4918 Da thionin corresponded in weight and retention time to thionin Tk-AMP-BP characterized previously by us for N-terminal amino acid sequence [24]. Full amino acid sequencing of this peptide confirmed identity thereof to Tk-AMP-BP thionin. The second peptide with molecular weight of 4801 Da proved to be the new one and was not described previously. Identification of amino acid sequence thereof and comparison with the identified cereals thionins has indicated that it is also a thionin but referring to α-thionin family as opposed to Tk-AMP-BP. This thionin was marked by us as Tk-AMP-AP1. Both *T. kiharae* thionins just like other cereals thionins contain 8 cysteine residues (see Fig. 2). Alkylation of non-reduced peptides by vinylpyridine resulted in no changes in molecular weight thereof, and hence the molecules

thereof contain no free sulfhydryl groups, and all SH-groups form disulfide bonds typical to plant AMPs.

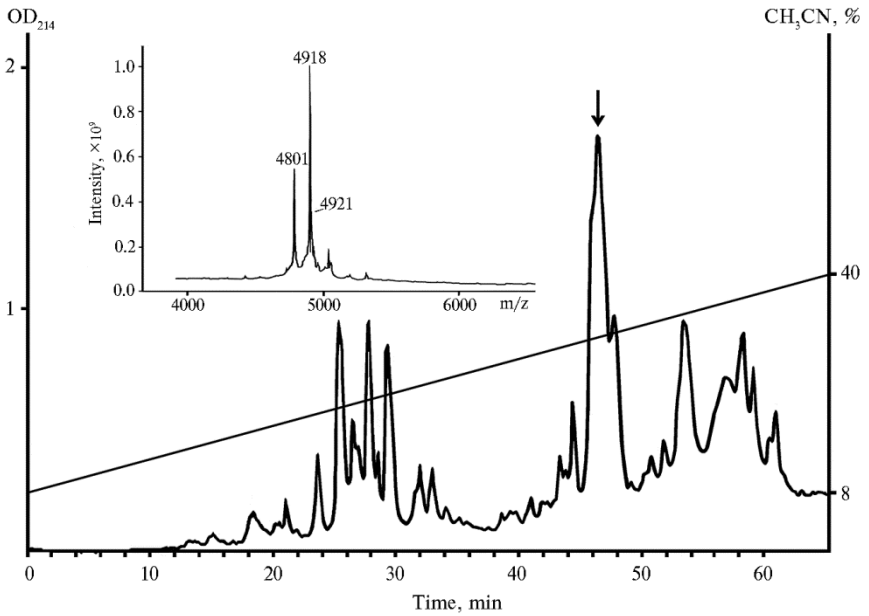

Fig. 1. Separation of antimicrobial peptides isolated from wheat (*Triticum kiharae*) seed by RP-HPLC method (highly performance liquid chromatography) in Reprosil C_{18} column (4×250 mm, Dr. Maisch GmbH, Germany) in gradient of acetonitrile concentrations. Thionin-containing fraction is marked by arrow. Mass-spectrum of this fraction is given in the insert (MALDI-TOF-MS, Bruker Daltonik GmbH, Germany). For description of chromatography conditions and mass-spectrometry, see *Techniques* section.

Species	Amino acid sequence	Peptide
Triticum kiharae	KSCCKSTLGRNCYNLCRARGAQKLCANVCRCKLTSGLSCPKDFPK	TK-AMP-BP
T. kiharae	KSCCRSTLGRNCYNLCRARGAQKLCAGVCRCKIASGLSCPKGFPK	TK-AMP-AP1
T. aestivum (CAA65312.1)	KSCCKSTLGRNCYNLCRARGAQKLCANVCRCKLTSGLSCPKDFPK	β-purothionin
T. aestivum (CAA65313.1)	KSCCRSTLGRNCYNLCRARGAQKLCAGVCRCKISSGLSCPKGFPK	α_B-purothionin
T. urartu (ACL11920.1)	KSCCKSTLGRNCYNLCRARGAQKLCANVCRCKLTSGLSCPKDFPK	β-purothionin
T. urartu (ACL11926.1)	KSCCKSTLGRDCYNLCRARGAQKLCANVCRCKLTSGLSCPKDFPK	β-purothionin
T. monococcum (ACL11901.1)	KSCCKSILGRNCYNLCRARGAQKLCANVCRCKLTSGLSCPKDFPK	β-purothionin
T. monococcum (ACL11917.1)	KSCCKSTLGRNCYNLCRARGAQKLCANVCRCKLTSGLSCPKDFPK	β-purothionin
Secale cereale (CAA65316.1)	KSCCKSTLGRNCYNLCRTRGAQKLCANFCRCKLISSTSCPKEFPK	purothionin
Hordeum vulgare (AAA32966.1)	KSCCRSTLGRNCYNLCRVRGAQKLCAGVCRCKLTSSGKCPTGFPK	α-hordothionin
H. vulgare (1206255A)	KSCCRSTLGRNCYNLCRVRGAQKLCANACRCKLTSGLKCPSSFPK	β-hordothionin

Fig. 2. Amino acid sequences of *Triticum kiharae* thionins and thionins of other cereals. Multiple alignments are made by CLUSTAL W2 program. Sequence numbers in GenBank are given in brackets. Cysteine residues are marked by white letters on the black background, divergent amino acid residues are marked by grey color.

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Tk-amp-bp   MGSKGLKGVMVCLLILGLVLEQVQVEGKSCCKSTLGRNCYNLCRARGAQKLCANVCRCKLTSGLSCPKDFPK
Tk-amp-ap2  MGSKGLKGVMVCLLILGLVLEQVQVEGKSCCRTTLGRNCYNLCRSRGAQKLCSTVCRCKLTSGLSCPKGFPK

Tk-amp-bp   LVLESNSDEPDTMEYCNLGCRSSLCDYMVNAAADDEEMKLYVEQCGDACVNFCNADAGLTSLDA
Tk-amp-ap2  LALESNSDEPDTIEYCNLGCRSSVCDYMVNAAADDEEMKLYVENCGDACVNFCNGDAGLTSLDA
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Fig. 3. Amino acid sequences of thionin precursors of *T. kiharae* wheat. The signal peptide is underlined, the mature peptide is italicized. Cysteine residues in mature peptide are marked by white letters on the black background, divergent amino acid residues are marked by grey color.

Amino acid sequence of Tk-AMP-BP purothionins of *T. kiharae* wheat was confirmed under transcriptome analysis of seedlings by NGS method. Previously made algorithm was used for searching precursor transcripts in the dataset of sequencing [26]. As a result 15 transcripts encoding precursors of thionin-like peptides consisting of signal peptide, mature peptide and S-terminal prodomain were found in wheat. Sequence of the mature peptide in one of precursors fully

coincided with Tk-AMP-BP purothionin isolated from kernel (Fig. 3). In addition to this transcript another Tk-amp-ap2 transcript encoding close homolog of Tk-AMP-AP1 purothionin was found.

Comparison between sequences of the obtained purothionins and that of thionins of other cereals varieties has shown that Tk-AMP-BP sequence of *T. kiharae* coincides with β-purothionin sequence of *T. aestivum*, as well as with one of two identified β-purothionin sequences of *T. urartu* and *T. monococcum* (see Fig. 2). Hexaploid wheat *T. aestivum* is known to have three thionins marked as β, α_B and α_D purothionins. They are encoded by *pur A1*, *pur B1* and *pur D1* genes located on long arms of chromosome 1A, chromosome 1B and chromosome 1D, respectively [29]. As β-purothionin gene is bound to genome A, the similarity found between sequences of these thionins for genome A carriers (*T. kiharae* (AAGGDD), *T. aestivum* (AABBDD), *T. urartu* (A^uA^u) and *T. monococcum* (A^mA^m) is not surprising. Sequences of β-purothionins are also highly conserved: sequences of Tk-AMP-AP1 of *T. kiharae* and α-purothionins of *T. aestivum* differ by substitution in position 34 (Ser→Ala).

As Tk-AMP-BP thionin of *T. kiharae* was obtained in quantity enough for biological test, it was used for analysis of inhibiting activity against *C. albicans*. According to the study made this thionin under low concentration thereof (MIC = 0.78 μg/ml) is proved to fully inhibit growth of *C. albicans*, i.e. possessing high fungicidal activity against this pathogen. It should be mentioned that thionin activity of other plant species was lower. The thionin of *Arabidopsis* inhibited *C. albicans* growth by 80% at 2.5 μg/ml concentration only [18], while thionin-like protein of *Capsicum annuum* was less active (IC_{50} = 10 μg/ml) [30]. Mechanism underlying effects of wheat thionin on *C. albicans* cells is not known. Biological activity of thionin is considered to be due to direct interaction with cell-target membranes, and three models of such interaction were proposed [11]. Study of purothionin affection on *Rhizoctonia solani* demonstrated that cytolysis of this fungus was caused by steep increase of membrane permeability [31], which was likely due to pore formation [32]. The similar mechanism may be also supposed in *C. albicans* but this assumption is to be experimentally studied.

Thus, two purothionins, the Tk-AMP-BP and Tk-AMP-AP1, were isolated by us for the first time from wheat (*Triticum kiharae*) kernel, with full amino acid sequence thereof being found. Sequence of Tk-AMP-BP purothionin was confirmed under transcriptome analysis of seedlings by NGS method. Tk-AMP-BP purothionin is found to have high fungicidal impact on *Candida albicans* cells acting at very low concentrations (MIC = 0.78 μg/ml) and being superior to thionins of other plant species in activity. High fungitoxicity of wheat thionins against *C. albicans* offers great opportunities in applying this antimicrobial peptide for making new drug products thereunder against candidiases.

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