

Antimicrobial Activity of Malting Barley Grain Thaumatin-Like Protein Isoforms, S and R

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ABSTRACT

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Two basic proteins were isolated to homogeneity from malting barley (*Hordeum vulgare* L.) grain. Proteins were identified as members of a Thaumatin-Like Protein (TLP) family, by Western blot. Isoforms, assigned as TLP-S and TLP-R, have slightly different mobility at about 22 and 27 kDa in nonreducing and reducing conditions, and pI values of 9.5 and 9.4, respectively. The antifungal potency of malting barley grain TLPs isoforms was examined on *Micrococcus lysodeikticus*, *Saccharomyces cerevisiae*, *Candida albicans* and plant pathogen *Fusarium sporotrichioides* growth *in vitro*. It was found that that IC₅₀ value for TLP-S was two fold higher. Antibacterial and antifungal activities of both isoforms were completely abolished by divalent (Ca²⁺, Mn²⁺, Mg²⁺) and monovalent (K⁺) cations, at concentrations approximating physiological ionic strength and higher. Glucanase activity was not observed; neither TLP-S nor TLP-R digested glucan. On the basis of these results, the importance of TLP for barley grain protection against fungal diseases has been discussed together with the mechanism of antimicrobial action.

Key words: Antimicrobial activity, barley (*Hordeum vulgare* L.) grain, growth inhibition, sugar binding, thaumatin-like protein.

INTRODUCTION

Plant proteins, induced in a pathological or related situation, named pathogenesis-related (PR) proteins, have been classified into 17 families based on amino acid sequence, serological relationship and enzymatic or biological activity²¹. Although PR proteins were considered as inducible proteins elicited by pathogen attack and other stressors, they are present constitutively in different plant organs including grains¹¹.

Multiple isoforms of Thaumatin-Like Protein (TLP), which belong to the PR-5 family, have been found in the

crop species barley (*Hordeum vulgare* L.)^{15,28}, rice (*Oryza sativa*)²⁷ and wheat (*Avena sativa*)²⁰. They are named after their amino-acid sequence and structural similarities to the sweet tasting protein thaumatin from the fruits of the West African rain forest shrub *Thaumatococcus daniellii* Benth.

Two isoforms, named TLP-S and TLP-R, in accordance with the nomenclature used for tobacco proteins, were isolated from high-lysine mutant barley grain¹⁵. They are basic with a pI around 9, MW around 22 kDa, with mainly a β structure, with high β turn content and a small helix, stabilized by eight disulphide bridges. Isoforms are highly homologous, with 60% amino acids at identical positions¹⁵.

In our previous paper⁸, we reported that barley grain TLP-S and TLP-R are glycosylated. The content of neutral sugars is different, 2.8% and 5% for TLP-R and TLP-S, respectively. Moreover, TLP-S possesses Concanavalin A (a lectin that binds to mannose and glucose) binding ability⁸. A difference in sugar binding among TLPs isoforms from germinated barley grain has been noticed and only one of two isoforms, designated HvPR5c that resembles TLP-R, binds β -1,3-glucan, the type commonly found in fungal walls²⁵.

Mutant barley grain TLPs inhibit growth of *Trichoderma viride* and *Candida albicans*¹⁵. Mixtures of TLP-R and TLP-S from common barley grain, in their natural ratio, inhibit the metabolic activities of *Saccharomyces cerevisiae*, causing cell constituent leakage and they can also exert a lethal effect on certain yeasts^{8,10}.

TLPs exert an antifungal effect through membrane permeabilization^{1,2} or a pore forming mechanism⁹. Occurrence of positive charges on the TLPs surface enables them to interact with the surface of the yeast plasma membrane, while hydrophobic interactions are responsible for the increase in permeability⁹.

Later studies indicated that TLPs react with the cell wall rather than with the membrane. Some TLPs bind to β -1,3-glucan^{25,34} and exhibit glucanase (laminarinase) activity, but possessing glucanase activity does not necessarily mean antifungal activity²². Osmotin, a PR-5 family member, binds the cell wall phosphomannoprotein¹⁷. In contrast to most PR proteins, osmotin antifungal activity is not abolished but rather is potentiated by physiological concentrations of some cations³⁰ such as Ca²⁺ and Mn²⁺.

The purpose of this work was to isolate and purify both isoforms from domestic cultivars of malting barley grain

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to homogeneity, characterize them and examine their individual influence on *S. cerevisiae*, *C. albicans*, *Fusarium sporotrichioides* and *Micrococcus lysodeikticus* growth. Inhibition of tested species, and most importantly the phytopathogenic *Fusarium*, known to provoke the cereal disease *Fusarium* head blight, points to TLPs role in malting barley grain protection. This work is the first evidence of TLPs antibacterial activity. We have examined the effect of cation presence on protein antimicrobial activity in order to elucidate if the mechanism of TLP-R and TLP-S action is metal mediated in a manner similar to that with osmotins³⁰. We were interested whether both or only one isoform possesses glucanase activity, in order to elucidate differences in isolated isoform antimicrobial activity, as well as whether TLPs glucanase activity may be important for beer quality, i.e., for improvement in lautering and beer clarity.

On the basis of the results obtained, the importance of TLP-R and TLP-S for malting barley grain protection and the mechanism of their action on fungal and bacterial growth will be discussed.

MATERIALS AND METHODS

Biological materials

The grains of five domestic cultivars of malting barley (*H. vulgare*), NS 294, NS 293, NS 448, NS 456 and NS 519, were obtained from the Institute of Field and Vegetable Crops, Novi Sad, Serbia. Strains of brewers yeast *Saccharomyces cerevisiae* (*S. uvarum* var. *carlsbergensis*), *Candida albicans* ATCC 24433, *Fusarium sporotrichioides* (collection number: B.M.-2/C-3-7) and *Micrococcus lysodeikticus* ATCC 4698 were used. *F. sporotrichioides* was obtained from the Maize Research Institute "Zemun polje", Serbia.

Estimation of TLPs presence in malting barley grain cultivars using dot-blot

Ground samples of five malting barley cultivars of mature grain (0.1 g) were mixed with 1 mL of extraction buffer⁵ (25 mM sodium phosphate, 50 mM NaCl and 5 mM EDTA, pH 7) and agitated for 1 h. The extracted proteins (the supernatant collected after centrifugation (20 min at 15,000 × g)) were loaded directly onto a nitrocellulose membrane using the dot-blot manifold. Blocked membrane (5% skim milk/TBS buffer for 1 h) was soaked with the antibody (sormatin raised using rabbits twice injected with purified sorghum protein at a concentration 1:100 (10 µL of antibody in 100 mL of buffer)) and after 12 h of incubation rinsed with TBS buffer and incubated with goat anti-rabbit IgG antibody³ (concentration 1:2500 (4 µL of antibody in 100 mL of buffer)) for 2 h. After incubation, the membranes were rinsed with buffer and proteins visualized using BCIP/NBT (5-Bromo-4-Chloro-3'-Indolylphosphate p-Toluidine Salt/Nitro-Blue Tetrazolium Chloride) as developing solution (developing time 3 min). Membranes were air-dried and scanned. Scanned images were analyzed using Image J program (<http://rsb.info.nih.gov/ij/download.html>). Protein isolated from sorghum was used as a standard.

Isolation of TLP-S and TLP-R from malting barley grain

Malting barley grain (cultivar NS 294) was ground to a fine flour in a rotating mill equipped with a 1 mm screen. The flour was extracted for 20 h with 50 mM citrate buffer, pH 5.5, at 6°C. After extraction, the slurry was passed through cheesecloth and centrifuged (15 min at 10,000 × g). The crude extract was fractionated by ammonium sulphate precipitation. The pellet obtained by 40–80% (w/v) saturation was dissolved in a minimal volume of starting buffer, dialyzed extensively (20 h) against 50 mM Tris-HCl buffer, pH 7.8, and applied to a 2.6 × 20 cm column of DEAE-Sephacel (Pharmacia LKB, Uppsala, Sweden), equilibrated with 50 mM Tris-HCl buffer, pH 7.8. The unbound (basic) proteins were eluted with the same buffer, while acid proteins were eluted after 0.5 M NaCl addition. Flow rate was 20 mL/h, fraction volume 5 mL. The basic protein fraction, concentrated by ultrafiltration, was applied to a 2 × 100 cm column of Sephadex G-75-50 (Sigma Chemical Co, St. Louis, USA) equilibrated with Tris-HCl buffer, pH 7.8. Flow rate was 16 mL/h, fraction volume 4 mL. Fraction II, concentrated by ultrafiltration and dissolved in minimal volume of 50 mM citrate-phosphate buffer, pH 5.8., was applied to a 1.4 × 15 cm column of CM-Sepharose CL-6B (Sigma Chemical Co, St. Louis, USA) equilibrated with 50 mM citrate-phosphate buffer, pH 5.8. Proteins were eluted with the linear gradient of NaCl (0–0.2 M). Flow rate was 9 mL/h, fraction volume 3 mL. The protein elution was monitored spectrophotometrically by measuring absorbance at 280 nm.

Purity test, molecular weight and pI determination

Analytical SDS-PAGE was performed on a polyacrylamide gel (4% (w/v) stacking gel and 10% (w/v) resolving gel), both in reducing and non-reducing conditions. Molecular weight markers were: lactalbumin (14 kDa), trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa) and human sera albumin (67 kDa).

Isoelectric focusing was performed on a LKB 2117 Multiphore system (LKB, Bromma, Sweden) in ultrathin polyacrylamide layers formed on the "Gel-Bond" using Ampholine (LKB, Uppsala, Sweden) pH 3.5–10 as carrier ampholytes.

After SDS-PAGE and IEF, proteins were stained with Coomassie Brilliant Blue G-250 and R-250 dye, respectively³⁶.

Protein analysis methods

The protein concentration was estimated using the Bradford assay, with BSA as standard⁴. Analysis of free sulfhydryl groups in the unfolded malting barley grain TLP-S and TLP-R, before and after reduction, was performed colorimetrically under the conditions described by Ellman⁷.

Immunodetection by Western blot

The protein samples were subjected on SDS-PAGE (4% (w/v) stacking gel and 12% (w/v) resolving gel) with reducing conditions, and then electrotransferred onto a

nitrocellulose membrane, as described by Towbin³³. Immunodetection was essentially performed according to the method of Harlow and Lane¹⁴. In brief, a monoclonal antibody BG148¹³ was used as the primary antibody, and alkaline phosphatase anti-mouse IgG (Sigma Chemical Co, St. Louis, USA) was used as the secondary antibody.

In vitro antimicrobial activity assay

Antifungal activity of malting barley grain TLP-R and TLP-S was measured by microspectrophotometry. Synthetic growth medium with a low ionic strength (SMF⁻) consisted of K₂HPO₄ 2.5 mM, MgSO₄ 50 μM, CaCl₂ 50 μM, FeSO₄ 5 μM, CoCl₂ 0.1 μM, CuSO₄ 0.1 μM, Na₂MoO₄ 2 μM, H₃BO₃ 0.5 μM, KJ 0.1 μM, ZnSO₄ 0.5 μM, MnSO₄ 0.1 μM, glucose (10 g/L), asparagine (1 mg/L), methionine (20 mg/L), myo-inositol (2 mg/L), biotin (0.2 mg/L), thiamine-HCl (1 mg/L) and pyridoxine-HCl (0.2 mg/L). The cell number of *S. cerevisiae*, *C. albicans*, *F. sporotrichioides* and *M. lysodeikticus* was 10⁵ CFU/mL. After incubation for 24 h the absorbance at 595 nm was determined with an enzyme-linked immunosorbent assay reader³². The antimicrobial activity was measured in the same medium supplemented with several divalent cations added separately (SMF⁺), at different concentrations (1, 2.5, 5 and 10 mM of CaCl₂, MgCl₂, MnCl₂) or with monovalent potassium cation (10, 50 and 100 mM KCl).

Laminarin and barley (1,3;1,4)-β-glucan hydrolysis assay

One microlitre of each purified protein adjusted to 1 μM, was mixed with 5 μL of substrate (barley (1,3;1,4)-β-glucan or laminarin) (2.5% (w/v)) in 10 μM ammonium acetate, pH 6.0, and 1 mM DTT. Following 12 h of incubation at 20°C, samples were assayed for the release of reducing sugars according to the Somogyi-Nelson method²⁴. Absorbance was measured at 660 nm.

RESULTS

Purification and characterization of the malting barley grain TLP-S and TLP-R

The presence of TLPs in five different cultivars of domestic malting barley grain, grown under the same environmental conditions, was tested immunochemically, us-

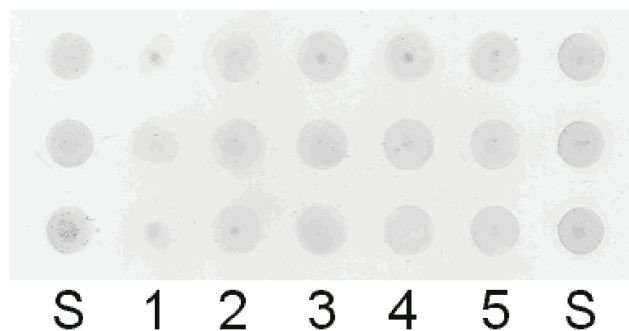


Fig. 1. TLPs presence in five domestic cultivars of malting barley grain, tested by dot-blot method (analysis performed in triplicate). S – standard; 1 – NS 519; 2 – NS 293; 3 – NS 294; 4 – NS 448; 5 – NS 556.

ing the dot-blot method (Fig. 1) and quantified. Barley varied in amount of TLPs. Cultivar NS 294 (Lane 3), having the highest, was chosen for protein isolation. However, NS 294 may not necessarily possess the greatest amount of TLPs if the tested cultivars were grown under other environmental conditions.

The TLPs contained in the raw extract of malting barley grain were concentrated by fractional precipitation at 40–80% (w/v) saturation. The fraction enriched with TLP was depleted of acidic proteins by means of anion-exchange chromatography (Fig. 2A). Basic (unbound) proteins concentrated in the leading peak (fraction I) were separated by gel-permeation chromatography (Fig. 2B). Finally, cation-exchange chromatography resulted in the separation of two TLP isoforms, S and R (Fig. 2C). TLP-S appeared at 90 mM NaCl while TLP-R at 130 mM NaCl. The amount of malting barley grain TLP-R and TLP-S, that can be purified by this isolation procedure, approximates 25 and 17 mg/100 g grain, respectively. Assuming a malting barley grain specific weight (1.25 g/mL) and the

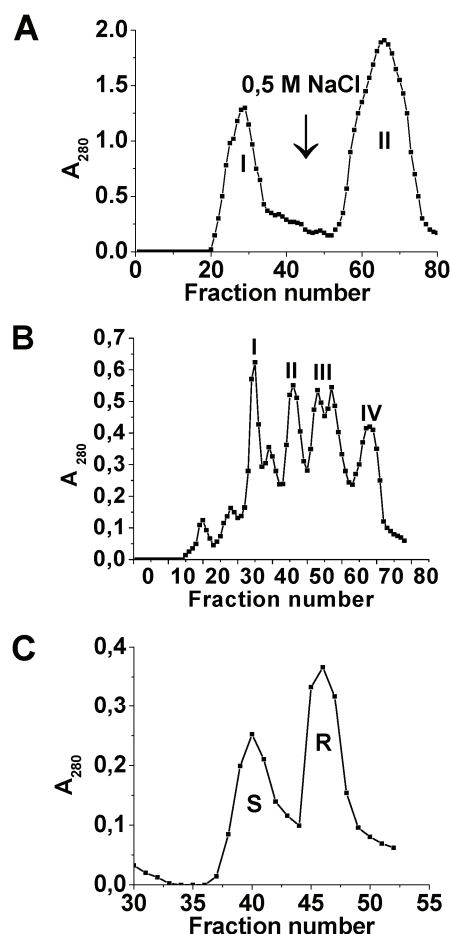


Fig. 2. (A) Isolation of the basic proteins from the fraction obtained by ammonium sulphate precipitation (40–80% (w/v) saturation) of barley grain extract, using anion-exchange chromatography on a DEAE-Sephacel column (fraction I – basic proteins); (B) Gel-permeation chromatography of the basic protein fraction on a Sephadex G-75 column (fraction II – mixture of TLPs, S and R); (C) Separation of TLP isoforms, S and R, using cation-exchange chromatography on a CM-Sepharose column.

amount of TLP-R and TLP-S in barley grain, their *in vivo* level approximates 312.5 and 212.5 µg/mL, respectively.

Homogeneity of the purified TLPs was tested by means of SDS-PAGE and IEF. Both fractions obtained exhibited a single band both with SDS-PAGE (Fig. 3A) and IEF (result not shown). The two isoforms had slightly different mobilities (about 22 and 27 kDa under nonreducing and reducing conditions). TLPs have anomalous migration in SDS-PAGE probably due to the high number of disulphide bridges. An isoelectric point of 9.5 and 9.4, for S and R respectively, was estimated by IEF. As expected, both isoforms indicated the presence of eight disulphide bridges. Isolated protein identity was confirmed using an immuno-blotting assay (Fig. 3B).

Effect of the malting barley grain TLP-S and TLP-R on fungal and bacterial growth

To assess the antimicrobial activity of malting barley grain TLP-R and TLP-S, their influence on *S. cerevisiae*, *C. albicans*, *F. sporotrichioides* and Gram-positive bacteria *M. lysodeikticus* growth was tested using a micro-

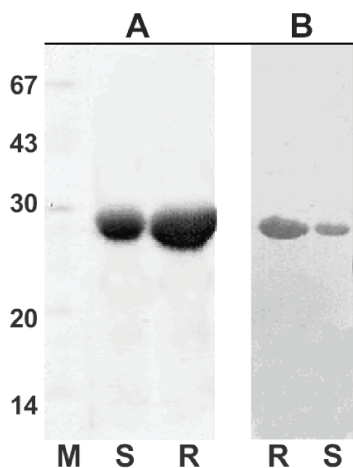


Fig. 3. The purity test and molecular weight determination of malting barley grain TLP-S and TLP-R, performed on SDS-PAGE (M – molecular weight standards) (A); Immunodetection of TLP-S and TLP-R by Western blot analysis (B).

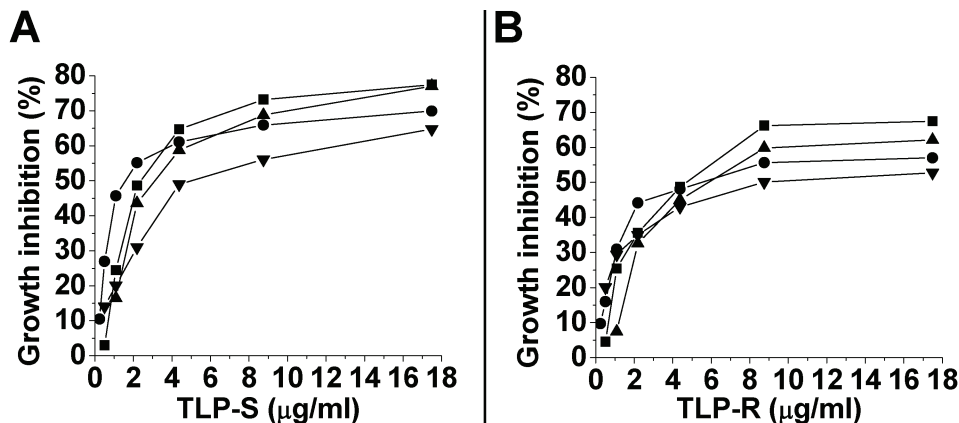


Fig. 4. *In vitro* antimicrobial activity of malting barley grain TLP-S (A) and TLP-R (B). Dose-response curves determined after 24 h incubation of *S. cerevisiae* (▲), *F. sporotrichioides* (■), *C. albicans* (●), and *M. lysodeikticus* (▼) in synthetic low ionic strength medium.

assay method³². In each case, the antimicrobial activity was tested in a defined synthetic medium with a low ionic strength. IC₅₀ values for inhibition after 24 h of incubation were determined from dose-response curves (percentage of growth inhibition versus protein concentration). Both isoforms displayed very strong antimicrobial activity on *S. cerevisiae*, *F. sporotrichioides*, *C. albicans* and *M. lysodeikticus* (Fig. 4). As seen from Fig. 4, TLP-S possessed a more potent inhibitory effect on the fungi, as well as the bacteria, in comparison to TLP-R. In contrast to curves obtained for TLP-R, each tending to saturation, curves obtained for TLP-S in the case of *S. cerevisiae* and *M. lysodeikticus* were still growing at the highest concentration (17.5 µg/mL). As seen from Table I, the IC₅₀ value for TLP-S was about two fold higher than for TLP-R. Also, the percentage of growth inhibition at the final concentration (17.5 g/mL) was significantly higher for TLP-S than for TLP-R.

Effect of cations on malting barley grain TLPs antimicrobial activity

The sensitivity of the antimicrobial activity of TLPs to the presence of various cations in the growth medium was tested. The medium was supplemented separately with different concentrations of divalent (1, 2.5, 5 and 10 mM of CaCl₂, MgCl₂, MnCl₂) and monovalent (10, 50 and 100 mM KCl) cations. The protein concentration range was the same as in the previous experiment. No antimicrobial activity was observed regardless of protein and/or cation concentration (Table I).

Malting barley grain TLPs glucanase activity

Glucanase activity, tested using the laminarin hydrolysis assay, was not observed; neither TLP-S nor TLP-R digested laminarin. Using the Somogyi-Nelson method²⁴, the release of reducing sugars upon protein incubation with laminarin as substrate was not detected. The possibility that malting barley grain TLPs digest barley glucan was tested by the same procedure, using barley (1,3;1,4)-β-glucan as substrate, in order to reveal if TLPs could contribute to lautering and beer clarity. Negative results for both isoforms were obtained.

Table I. Antimicrobial activity of malting barley grain TLP-S and TLP-R in low (SMF-) and high (SMF+) ionic strength synthetic medium; growth inhibition (GI) at final protein concentration and IC₅₀ value.

	TLP-S			TLP-R		
	GI (%) 17.5 µg/mL	IC ₅₀ (µg/mL)		GI (%) 17.5 µg/mL	IC ₅₀ (µg/mL)	
		SMF-	SMF+		SMF-	SMF+
<i>C. albicans</i>	69.9	1.6	—	57.0	5.5	—
<i>F. sporotrichioides</i>	77.4	2.3	—	67.5	4.8	—
<i>S. cerevisiae</i>	77.1	3.1	—	62.1	6.0	—
<i>M. lysodeikticus</i>	64.8	4.9	—	52.7	9.0	—

The IC₅₀ values of TLP-R and TLP-S were determined in a synthetic growth medium of low ionic strength (SMF-) and the same medium supplemented with 1, 2.5, 5 and 10 mM CaCl₂, MgCl₂, MnCl₂ respectively and 10, 50, 100 mM KCl respectively (SMF+).

DISCUSSION

Malting barley grain is a rich source of TLPs. Using conventional methods for protein isolation these can readily be purified in large quantities. Malting barley grain TLP-R and TLP-S were isolated to homogeneity, using ammonium sulfate precipitation followed by anion-exchange, gel-permeation and cation exchange chromatography.

The isolated proteins resembled the physico-chemical features of the mutant barley isoforms TLP-R and TLP-S¹⁵. They appeared at a similar position with salt gradient cation-exchange chromatography, protein TLP-R being prevalent in the mixture; they were basic (pI above 9), shared similar mobility in SDS-PAGE under non-reducing conditions and had the same number of disulphide bridges. Finally, the identity of the isolated proteins was confirmed using an immuno-blot assay.

Both isoforms strongly inhibited the growth of test microorganisms, most importantly the phytopathogenic *Fusarium* and thus might be involved in protection of grain during dormancy and germination. Their *in vivo* level exceeded the IC₅₀ values for the microorganisms tested. Since TLPs are preferably localised in the aleurone cell layer of barley grain endosperm, their concentration *in situ* must be much higher. Moreover, their effectiveness may be amplified due to synergistic interactions with other PR proteins³⁵. Also, there is a possibility that other molecules or ions around the protein could potentiate their antimicrobial activity²⁹. Finding that cereals with higher amounts of TLPs are more resistant supports the idea about the effectiveness of the TLPs³.

We have reported previously that malting barley TLPs isoforms are glycosylated with different sugar content, TLP-S being two fold higher and with the ability to bind concanavalin A⁸. It is worthy to note that the number of newly recognized glycoproteins among the TLPs family is growing^{13,16,18,19,23,26,37}.

We have demonstrated TLP-S has higher antifungal and antibacterial activity than TLP-R, regarding the IC₅₀ value. Previously, we have revealed that TLP-S has a more potent inhibitory effect on *S. cerevisiae* fermentation in comparison to TLP-R. The difference in the antifungal activity between TLPs isoforms from barley mutant grain on *T. viride* and *C. albicans* has been reported by Hejgaard¹⁵. Also, protein Pr22-3 (similar to TLP-S), isolated from barley leaves infested with *Rhynchosporium secalis*, was more active in the spore bioassay than Pr22-2

(similar to TLP-R); the difference was explained by the finding that Pr22-3 digests laminarin while Pr22-2 does not³⁸. Both TLPs isoforms from malting barley grain do not digest laminarin, i.e., a difference in glucanase activity between the isoforms was not observed. This could be considered as additional evidence that TLPs with glucanase activity do not necessarily possess antifungal activity and *vice versa*.

The observed difference in antifungal activity between the malting barley grain isoforms may be related to the difference in the sugar amount and the sugar binding ability. Our results, taken together with demonstrations that some TLPs bind to glucan^{25,34} and phosphomannan¹⁷ components of the cell wall, suggest that sugar binding is their common feature and potentially important for activity.

Complete abolishment of TLP-S and TLP-R antimicrobial activity in the presence of cations supports the assumption that TLPs could not act on a microbial cell without previous attachment to the cell surface. The presence of cations could disturb protein binding to the cell wall components, due to their interaction with the cell surface, or with proteins themselves. The antimicrobial activity of most PR proteins has been reported to be drastically reduced in the presence of physiological concentrations of inorganic cations and thus such activity *in vivo* may be questioned. A concentration of 1 mM Ca²⁺ drastically reduces the antifungal activity of hevein- or knottin-type peptides, defensins and non-specific lipid-transfer protein (ns-LTP)¹². Antifungal activity of radish ns-LTP is abolished by 5 mM and 3-fold reduced by 1 mM Ca²⁺³¹. An exception to the rule, besides onion grain ns-LTP⁶ is osmotin, a PR-5 family member. Cytotoxicity of osmotin against *S. cerevisiae* was abolished by K⁺, but was not suppressed by 100 mM Ca²⁺. Actually, Ca²⁺ in concentrations from 0.2 to 2.7 mM facilitated its activity, as well as binding to the cell surface³⁰.

Although malting barley grain TLP-S and TLP-R, and osmotin belong to the PR-5 family, the mechanism of their action appears to be different, i.e. either TLP-R or TLP-S binding is not metal mediated. Osmotin is a calcium binding protein, having epidermal growth factor-like calcium-binding domains³⁰. Searching NCBI Entrez Protein Data Bank for primary structure similarity using BLAST revealed that TLP-R and TLP-S do not possess calcium-like domains. Also, the difference in the mechanism between osmotin and TLP action could indicate the different target molecules on the cell surface.

CONCLUSIONS

Two basic isoforms of TLP, assigned as S and R, have been isolated to homogeneity from malting barley grain and characterized. The effect of pure proteins on the growth of microorganism has been studied. It was found that TLP-R and TLP-S inhibited the growth of *M. lyso-deikticus*, *C. albicans*, *S. cerevisiae* and the plant pathogen *F. sporotrichioides*, known to provoke the cereal disease *Fusarium* head blight, causing problems in the beer industry. Inhibition of the phytopathogenic *Fusarium* points to TLPs role in malting barley grain protection. *In situ* concentration of both isoforms is much higher than what is required for *Fusarium* growth inhibition. This work is the first report of TLPs antibacterial activity. TLP-S has more potent antimicrobial activity than TLP-R. The presence of the tested cations completely abolished the activity of both isoforms. Both isoforms do not possess glucanase activity.

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