

# Barley Thaumatin-Like Proteins Bind Insoluble (1 → 3)-β-Glucans

*R.I.W. Osmond, M. Hrmova and G.B. Fincher*

Department of Plant Science, University of Adelaide, Waite Campus, Glen Osmond,  
South Australia 5064

## Introduction

Thaumatin-like (TL) proteins are members of the pathogenesis-related group of plant proteins that are expressed constitutively at certain stages of plant development, and in response to pathogen attack. Barley TL proteins have been shown to have antifungal activity *in vitro* (Hejgaard *et al.* 1991) and have a toxic effect against brewers yeast (Cvetkovic *et al.* 1997). More generally, TL protein over-expression in transgenic potatoes delays the onset of disease symptoms caused by *Phytophthora infestans* (Liu *et al.* 1994). It has been proposed that the mechanism of the antifungal activity of TL proteins is through permeabilization of fungal plasma membranes (Roberts and Selitrennikoff, 1990). However, the crystal structures of TL proteins do not have features normally associated with direct pore formation (Batalia *et al.* 1996; Koiwa *et al.* 1999). Furthermore, TL proteins do not affect plant cell membranes, which suggests that TL proteins might recognise specific structures at the fungal cell surface before membrane damage occurs.

The observation that barley TL proteins bind polysaccharide was made by Dr Maria Hrmova (Department of Plant Science, University of Adelaide), who found that TL proteins in a crude protein extract were bound by a NaOH-treated pachyman column when attempting to isolate (1→3)-β-glucanases by affinity chromatography. Linkage analysis of the NaOH-treated pachyman revealed the presence of several different polysaccharides in the pachyman sample. The constituents of the pachyman sample were tested individually for TL protein binding, and the binding agent was shown to be insoluble (1→3)-β-glucan, in agreement with Trudel *et al.* (1998). The aims of this study were to investigate the binding specificity associated with the TL protein/(1→3)-β-glucan interaction.

## Materials and Methods

### *Protein Purification*

Barley (var. Clipper) was surface sterilized and germinated in the dark for 5 days. Protein was extracted at 4°C and fractionally precipitated with ammonium sulphate. The 40-80% saturated ammonium sulphate fraction was redissolved and TL proteins were purified through anion exchange and cation exchange chromatography, followed by gel filtration on a BioGel P-30 column. The resulting protein, which consisted primarily of two TL isoforms, was further fractionated using CM-Sepharose chromatography to yield purified HvTL1 and HvTL2 for use in polysaccharide binding assays.

### *Pachyman Preparation*

Pachyman (Megazyme, Warriewood, NSW, Australia), chitin (Fluka Biochemika, Buchs, Switzerland), avicel cellulose (Fluka) and pustulan (Calbiochem-Novachem Corporation, La Jolla, CA, USA) were heated to 80°C in 1M NaOH for 30 min. Insoluble material was recovered by centrifugation, thoroughly washed with H<sub>2</sub>O and resuspended in 50 mM sodium acetate buffer (pH 5.0) for use in binding studies. Pachyman is an essentially linear (1→3)-β-glucan of DP ~ 250, but contains some internal (1→6)-β-linkages and branching (Saito *et al.* 1968). Debranched pachyman was generated through periodate oxidation, borohydride reduction and mild acid hydrolysis, according to the method of Chihara *et al.* (1970). Curdlan, a linear (1→3)-β-glucan, was obtained from Sigma Chemical Company (St. Louis, MO, USA).

### *TL-Polysaccharide Binding Studies*

Various amounts of protein were incubated with a fixed amount of polysaccharide (10 mg/ml in 50 mM sodium acetate buffer, pH 5.0) to give a final assay volume of 0.5 ml. The protein-polysaccharide mixtures were incubated at 25°C for 1 hour on an orbital rotor, and centrifuged at 1,000 x g for 3 min to remove insoluble material. The supernatant was recovered and analyzed for residual protein using ELISA (Evans and Hejgaard, 1999) and Coomassie Brilliant Blue (Bradford, 1976).

## **Results and Discussion**

Barley TL proteins, designated HvTL1 and HvTL2, were isolated from germinated barley grains in the ratio of approximately 1:1.5. NH<sub>2</sub>-Terminal sequencing of the first 60 amino acid residues of HvTL1 and HvTL2 revealed that they were almost identical to PR-R and PR-S respectively, isolated previously by Hejgaard *et al.* (1991). Previous studies have shown that PR-R and PR-S are antifungal, with PR-S three times more effective (Hejgaard *et al.* 1991). Furthermore, Cvetkovic *et al.* (1997) found that barley TL proteins have inhibitory activity towards brewers yeast, again with PR-S more potent.

The NaOH-treated pachyman was analyzed for linkage types, and it was revealed that the major species present was (1→3)-glucosyl residues. However, there was also significant amounts of (1→4)-glucosyl, (1→6)-glucosyl, (1→3,1→6)-glucosyl and (1→4)-*N*-acetyl glucosaminyl residues present in the sample. To determine which of these components was responsible for barley TL protein binding, they were individually assayed and the results are shown in Table 1.

**Table 1.** + indicates binding observed, - indicates no binding observed.

	<b>Pachyman</b>	<b>Pustulan</b>	<b>Chitin</b>	<b>Cellulose</b>
<b>HvTL1</b>	+	-	-	-
<b>HvTL2</b>	+	-	-	-

The data reveals that TL protein binding was significant only for pachyman, in agreement with Trudel *et al.* (1998). This was further investigated using debranched pachyman, and the linear (1→3)-β-glucan curdlan (Table 2). The data shows that there is no binding to either untreated pachyman or curdlan, which may reflect an inability of TL proteins to gain access

polysaccharide chains when in the compacted state. However, when treated, binding for both HvTL1 and HvTL2 was greatest on debranched pachyman and curdlan, indicating specificity for (1→3)-β-linkages. Furthermore, HvTL1 bound in far greater amounts than HvTL2. From

**Table 2.** Binding of HvTL1 and HvTL2 on various (1→3)-β-glucans.

	Untreated Pachyman	Debranched Pachyman	Debranched Pachyman, (NaOH-treated)	NaOH-treated Pachyman	Untreated Curdlan	Boiled Curdlan
<b>HvTL1</b>	No binding	High binding	High binding	Medium binding	No binding	High binding
<b>HvTL2</b>	No binding	Low binding	Low binding	Low binding	No binding	Low binding

the data presented in Table 2, it is apparent that HvTL1 has a higher affinity for pachyman, and also binds in greater amounts than HvTL2. In contrast, HvTL2 has more potent antifungal activity than HvTL1 on the fungal isolates and yeasts tested (Hejgaard *et al.* 1991; Cvetkovic *et al.* 1997), which may indicate that binding of insoluble (1→3)-β-glucan is not involved directly with the antifungal activity of HvTL2. HvTL1 may be involved with the plant cell wall during plant stress, as the deposition of the (1→3)-β-glucan callose is associated with plant stress. Alternatively, HvTL1 may act in concert with other plant defence compounds to exert its antifungal effect.

The importance of (1→3)-β-glucan binding in the antifungal activity of HvTL1 and HvTL2 may be further investigated with competition assays using soluble and insoluble (1→3)-β-glucans, which may provide an insight of the importance of (1→3)-β-glucan binding to antifungal activity.

## References

- Batalia, M.A., Monzingo, A.F., Ernst, S., Roberts, W.K. and Robertus, J.D. (1996) *Nature Struct. Biol.* **3**: 19 - 23
- Bradford, M. (1976) *Anal. Biochem.* **72**: 248 - 254
- Chihara, G., Hamuro, J., Maeda, Y., Arai, Y. and Fukuoka, F. (1970) *Nature* **225**: 943 - 944
- Cvetkovic, A., Blagojevic, S., Hranisavljevic, J. and Vucelic, D. (1997) *J. Inst. Brew.* **103**: 183 - 186
- Evans, E. and Hejgaard, J. (1999) *J. Inst. Brew.* **105**: 159 - 169
- Hejgaard, J., Jacobsen, S. and Svendsen, J. (1991) *FEBS Lett.* **291**: 127 - 131
- Koiwa, H., Kato, H., Nakatsu, T., Oda, J., Yamada, Y. and Sato, F. (1999) *J. Mol. Biol.* **286**: 1137 - 1145
- Liu, D., Ragothama, K.G., Hasegawa, P.M. and Bressan, R.A. (1994) *Proc. Natl. Acad. Sci. USA* **91**: 1888 - 1892
- Roberts, W.K. and Selitrennikoff, C.P. (1990) *J. Gen. Microbiol.* **136**: 1771 - 1778
- Saito, H., Misaki, A. and Harada, T. (1968) *Agric. Biol. Chem.* **32**: 1261 - 1269
- Trudel, J., Grenier, J., Potvin, C. and Asselin, A. (1998) *Plant Physiol.* **118**: 1431 - 1438