

The Effect of Lipid Binding and Starch Associated Proteins on Cereal Grain Quality

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Introduction

Numerous studies are performed on the structure and functions of plant lipid binding proteins. This growing interest is mainly justified by the data that have shown the important role of these proteins in both plant physiology (Kader 1996) and technology (Marion et al.1998). In cereal science and technology, most of the research is restricted to two major lipid-binding proteins, lipid transfer proteins (LTPs) and indolines, although it is obvious that many other cereal proteins are capable to bind and interact with lipids. In this review, we report the recent progress that has been realised on the structure and functional properties of LTPs and indolines with both a physiological and a technological outlook and a specific emphasis on the corresponding wheat and barley proteins.

Lipid transfer proteins

Structure and lipid binding properties of nsLTPs

LTPs were initially discovered in the search of proteins involved in the intracellular traffic of lipids in regard to what it was highlighted in yeast and animals (Wirtz 1997). Lipid transfer assays were used to isolate specifically LTPs in plant soluble extracts. These assays consist in following, *in vitro*, the transfer of radiolabelled or fluorescent lipids between artificial and/or natural membranes (Kader 1996).

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1   -IDCGHVDSLVRPCLSYVQG-GPGPSGQCCDGVKNLHNQARSQSDRQSACNCLKGIARGI
2   ALNCGQVDSKMKPCLTYVQG-GPGPSGECCNGVRDLHNQAQSSGDRQTVCNCLKGIARGI
3   -ANCGQVVSYLAPCISYAMGRVSVPGGGCCSGVRGLNAAAATPADRKTTCTCLKQQASGM
4   AISCGQVASAIAPCISYARGQSGPSAGCCSGVRSLNNAARTTADRRAACNCLKNAAGV
5   -AAC--QASQLAVCASAILS-GAKPSGECCGNLR-----AQQGCFQYAKDPTY
6   -AAC--EPAQLAVCASAILG-GTKPSGECCGNLR-----AQQGCLQYVKDPNY

1   HNLNED-NARSIPPKCGVNLPTYISLNIDCSR-
2   HNLNLN-NAASIPSKCNVNPYTIISPDIIDCSRIY
3   GGIKPN-LVAGIPGKCGVNIPYAIISLNIDCSR-
4   SGLNAG-NAASIPSKCGVSIPYTIISTDCSR-
5   GQYIRSPHARDTLTSCGLAVP-----HC----
6   GHYVSSPHARDTLNLCGIPVP-----HC----
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Figure 1. Amino acid sequences of nsLTP1s (1-4) and nsLTP2s (5-6) from wheat (1,3,5), barley (2, 6) and maize (4) seeds.

Plant LTPs, which have been isolated so far, do not share any common structural and functional properties with the corresponding animal and yeast proteins. Especially, plant LTPs are capable to enhance, *in vitro*, the intermembrane transfer and exchange of different polar lipids including phospholipids and glycolipids (Kader 1996) while the corresponding animal proteins display narrow specificities (Wirtz 1997). Therefore, they have been termed non

specific lipid transfer proteins (nsLTP). Ns-LTPs are ubiquitous and are found in almost all plant organs including seeds where they generally account for a significant amount of total soluble proteins (5-10%). Two main multigenic families have been isolated. A family is composed by proteins with a molecular mass around 9kDa and is referred as nsLTP1 while the other, nsLTP2, is composed by proteins with a molecular mass of about 7kDa (Figure 1). Today most of the available data are restricted to nsLTP1s. All nsLTPs are characterised by a conserved cysteine motif in which cysteines are involved in intramolecular disulfide bonds. These basic proteins do not contain tryptophan and in nsLTP1s, phenylalanine is rare. Two conserved tyrosine residues are located towards the N- and the C-terminus of the polypeptide backbone (Figure 1).

The three dimensional structure of the wheat, barley, maize and rice seed nsLTP1s have been determined by NMR or X-ray crystallography (Gincel et al. 1994; Shin et al. 1995; Heinemann et al. 1996; Lee et al. 1998). This nsLTP fold is characterised by a four helix bundle surrounded in part by a C-terminus arm formed by turns. The most interesting feature of this fold is the presence of a large internal cavity. The surface of this cavity is covered by the side chains of hydrophobic residues provided by the amphipathic helices and by the C-terminal arm. The size of this cavity is variable from one isoform to another when comparing the structure of different nsLTP1s (Lee et al. 1998). In fact rather than a cavity, we have a tunnel following the long axis of the protein. This tunnel is partly closed on one side by the highly conserved tyrosine that is located in the C-terminus. Loops 1 and 3 which are composed of different hydrophilic and hydrophobic amino acids close in part the tunnel on the other side (Charvolin et al. 1999). On lipid binding, the tunnel displays a high plasticity and its volume can increase to bind one or two monoacylated lipids (Shin et al. 1995; Lerche et al. 1997; Charvolin et al. 1999) or a diacylated lipid (Sodano et al. 1997). The complex is stabilised by hydrogen bonds between the tyrosine of the C-terminus and the phosphatidic or carboxylate group of the lipid. Moreover, the anionic groups of the bound lipids are in close contact with an arginine residue that is highly conserved in nsLTP1s. When two lysophosphatidylcholines are bound to the wheat nsLTP1 (Charvolin et al. 1999), the second lipid adopt a dissimilar orientation. Its polar head protrudes outside the protein between helix H1 and H3 and its fatty backbone is mainly in interaction with the fatty backbone of the first lipid. This latter orientation was observed for barley nsLTP1 complexed with a molecule of palmitic coenzyme A and palmitic acid (Lersche et al. 1997; Lerche and Poulsen 1998). This is rather surprising since every structure of nsLTP1 complexed with only one lipid had shown an orientation with the polar head protruding between loop 2 and the C-terminal region, that is, at the opposite side (Shin et al. 1995 ; Sodano et al. 1997).

NsLTPs : plant defence and/or formation of surface hydrophobic layers?

The first demonstration that nsLTP1s are not involved in the intracellular lipid transport came from the discovery that nsLTP1 are synthesised as pre-proteins containing a signal peptide of 20-25 amino acids (Tchang et al. 1988). Finally the routing of nsLTPs through the secretory pathway was definitely confirmed when it was shown that nsLTP1 could be located on the cell walls (Thoma et al. 1993) or secreted in the medium of embryogenic cell cultures (Sterk et al. 1991). A second hypothesis suggested that nsLTP1s are involved in the formation of cutin layers (Sterk et al. 1991) by transporting the hydrophobic monomers (fatty acids, fatty alcohols and hydroxy-fatty acids) which compose these polymeric and hydrophobic layers in most aerial organs (Kollatukuddy 1981). This role is strengthened by the fact that nsLTP1 genes are mainly expressed in epidermal tissues of plants and can be isolated from surface waxes (Kader 1996). Furthermore a relationship has been highlighted between an increase of the thickness of cuticle wax layer and over-expression of an nsLTP1 gene in leaf epidermis of

barley (Hollenbach et al. 1997). However, some nsLTPs (Krause et al. 1994; Song et al. 1998) are synthesized in cutin-free organs, e.g. roots. However, the integuments of underground organs are composed of another hydrophobic layer, suberin which is a complex co-polymer of phenolic and hydroxy-fatty acid compounds (Kollatukuddy 1981). In this regard, it is noteworthy that root nsLTPs belong to the nsLTP2 type (Krause et al. 1994; Song et al. 1998) while the genes encoding nsLTP1 are not or weakly expressed in this organ (Kader, 1996). In wheat and barley seeds where both cutin and suberin layers are synthesised both nsLTP1 and nsLTP2 are found (Désormeaux et al. 1992 ; Kalla et al. 1994). Therefore, these results could suggest that nsLTP1 is involved in the transport of hydrophobic cutin monomers while nsLTP2 is involved in the transport of hydrophobic suberin monomers. Finally it has been suggested that nsLTPs play a major role in the defence of plants, mainly because some nsLTPs were efficient to inhibit growth of bacteria and fungi *in vitro* (Garcia-Olmedo et al. 1995). However some nsLTPs such as the wheat aleurone nsLTP1 is devoided of antimicrobial activity although it is capable to synergically enhance the antimicrobial properties of wheat thionins (Dubreil et al. 1998a). In fact a role in the defence of plants is compatible with a role in the formation of external hydrophobic layers since it has been observed that hydrophobic layers can be re-synthesised on fungal attacks (Kollatukuddy, 1981). The antimicrobial activity of nsLTPs could be a secondary activity to the primary biological function in the formation of hydrophobic layers.

Barley ns-LTP1 and formation of beer foams

Concerning beer it has been shown that the barley aleurone nsLTP1 concentrates in beer foam and contributes to foam formation while protein Z confers foam stability. In fact barley seed nsLTP1 display poor foaming properties (Sørensen et al. 1993). It is only after denaturation occurring on wort boiling that the protein becomes a foam promoting agent (Bech et al. 1995). Recently we have shown that different nsLTP1s entities are formed during the malting and the brewing processes. These forms include unfolded nsLTP1s, glycosylated (Maillard reactions) and proteolytic fragments of nsLTP1s (Jégoux et al. 1999). These modifications should improve the availability of hydrophobic residues and amphipathic domains for adsorption at air-water interfaces. However it is noteworthy that a significant part of the barley nsLTP1 is not denaturated and could prevent beer foams from destabilisation by lipids.

Indolines

Structure and lipid binding properties of indolines

Indolines have been extracted for the first time from wheat kernels using Triton X114 phase partitioning (Blochet et al. 1991), a procedure generally used to isolate transmembrane proteins (Bordier 1981). As nsLTPs, indolines are basic and cysteine-rich proteins. Wheat seed indolines are composed of two main isoforms, puroindoline-a (PIN-a) and puroindoline-b (PIN-b). This name was given in regard to the unique tryptophan-rich domain (Trp-Arg-Trp-Trp-Lys-Trp-Trp-Lys) of the major isoform (puros, a greek word for wheat and indoline, for the indole ring of tryptophan)(Blochet et al. 1993). This domain is truncated in PIN-b (Trp-Pro-Thr-Trp-Trp-Lys) (Gautier et al. 1994) (Figure 2), a minor isoform of most wheat cultivars (Igrejas et al., 1999). Indolines have been found in oat, barley and rye seeds (Tanchak et al. 1998). Some primary and secondary structure similarities have been found between puroindolines and nsLTP1, except in the region containing the tryptophan-rich domain (Le Bihan et al, 1996)(Figure 2). Interestingly, the two cysteines that enclosed the tryptophan-rich domain form a disulphide bond while the eight other cysteine residues display nsLTP-like pairing (Marion et al. 1998). These similarities suggest that nsLTPs and puroindolines display similar folds. Other plant proteins that display similar cysteine pairing

have an nsLTP fold such as the hydrophobic protein of soybean (Baud et al. 1993) and the 2S seed storage proteins (Rico et al. 1996). These proteins do not possess an hydrophobic tunnel.

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1  -----IDCGHVDSLVRPCLSYVQG-----GPGPSQ-----GCCDGVK
2  -----ALNCGQVDSKMKPCLTYYVQG-----GPGPSG-----ECCNGVR
3  EVGGGGGSQQCPQERPKLSSCKDYVMERCFTMKDFPVTWP-TKWWKGGCEHEVREKCCQQLS
4  DVAGGGGAQQCPVET-KLNSCRNYLLDRCS'TMKDFPVTWRWWKWWKGGCQ-ELLGECSSRLG

1  NLHNQARSQSDR---QSA1CN2CLKGIARGIHNLNEDNARSIPPK3CGVNL4PYTISLNID5CSRV-----
2  DLHNQAQSSGDR---QTV1CN2CLKGIARGIHNLN--NAASIPSK3CNVN4VPYTISP5DDID6CSRIY
3  QIAPQ1CR2CDSIRRVIQGRLGGFLGIWRGEVFKQLQRAQSLPSK3CN4MG-----AD5CK6FPS--GYW
4  QMP1PQ2CR3CNI4IQGSIQGD5LG6IFGFQRDRASKVIQEAKNLPPR7CN8Q9GPP-----CN10IPGTIGYYW

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Figure 2 Alignment of the amino acid sequences of PIN-a (4), PIN-b (3), wheat (1) and barley (2) nsLTP1s.

About 4-5 binding sites are found on puroindolines in accordance with their capacity to insert more or less deeply in lipid aggregates such as micelles or bilayers liposomes (Wilde et al. 1993 ; Dubreil et al. 1997). These basic proteins interact more with anionic phospholipids than with neutral polar lipids such as phosphatidylcholine or galactosylglycerides (Dubreil et al. 1997). Furthermore an important difference of the interaction with neutral lipids is observed between PIN-a and PIN-b which is probably due to the loss of tryptophan residues in the corresponding domain (Dubreil et al. 1997). However it is interesting to note that PIN-b interacts more strongly with anionic lipids than PIN-a (Dubreil et al. 1997). Since PIN-b displays a higher positive net charge than PIN-a, these results suggest that the electrostatic contribution is important for the interaction of puroindolines with polar lipids.

Surface properties of indolines

nsLTPs and puroindolines are surface active proteins and are capable to adsorb spontaneously at air-water interfaces as most low molecular mass surfactants (Subirade et al. 1995; Koijman et al. 1998). However only puroindoline films are capable to form very stable foams. Above all, puroindoline foams are highly resistant to destabilisation by lipids (Wilde et al. 1993; Husband et al. 1995; Clark et al. 1994 ; Dubreil et al. 1997) and in the presence of some polar lipids, the foam stability is improved (Wilde et al 1993 ; Dubreil et al 1997). Such foaming properties are unique since polar lipids and surfactants compete with proteins for the air-water interfaces (Dickinson 1992). The synergistic enhancement of foam stability is well related to the affinity of puroindolines with lipids since this effect is less pronounced for PIN-b which interacts less strongly with most polar lipids (Husband et al. 1995; Dubreil et al. 1997).

These high surface properties are also expressed in complex system such as beer and bread. In beer, it has been shown that slight amounts of puroindolines can restore foam destabilised by different neutral and polar lipids (Clark et al. 1994). In bread, puroindolines lead to crumb with an homogeneous structure composed of fine gas cells. These effects were observed by adding relatively low amount of puroindolines (0.05 to 0.2%) to flours obtained from puroindoline-free cultivars (Dubreil et al. 1998b). The fine gas cells of bread crumb are quite similar to the fine bubbles of puroindoline foams. Preliminary experiments suggest that puroindolines act on the foaming of dough liquor by preventing foam destabilisation by oil globules and also by their capacity to synergistically increase the surface properties of wheat polar lipids (Dubreil 1997).

Puroindolines and kernel hardness : lipid or starch binding proteins ?

In contrast with nsLTPs, puroindolines are synthesised as preproteins containing in their

N-terminus, a signal peptide followed by a sequence of 8-10 amino acids (Gautier et al 1994). Furthermore at the C-terminus an aromatic tripeptide (Tyr-Tyr-Trp) is cleaved in the mature protein. This post-translational processing could correspond to a specific cell routing and function. Puroindolines are found both in the aleurone layer and the starchy endosperm. The results available today indicate that PIN-a is located in the starchy endosperm while PIN-b is located either only in aleurone cells or both aleurone cells and starchy endosperm (Dubreil et al., 1998a; Digeon et al. 1999). These proteins are not found in leaves and roots suggesting that puroindolines are seed specific proteins (Gautier et al 1994). In aleurone cells, puroindolines are localised in the aleurone grain. In the mature starchy endosperm puroindolines are recovered in the protein matrix and at the interface between starch granules and starchy endosperm (Dubreil et al., 1998a). This interface contains membrane remnants (Al-Saleh et al. 1986). Puroindoline synthesis begins early during grain maturation as storage proteins in agreement with a probable localisation in protein bodies (Jolly et al., 1993 ; Dubreil et al. 1998a). Therefore, puroindolines follow the secretory pathway and are probably stored in protein bodies.

By SDS-PAGE, Greenwell and Schofield (1986) reported for the first time a relationship between the presence of a 15kDa protein on the surface of water-washed starch and softness. This protein has been named friabilin or grain softness protein (Jolly et al., 1993). Grain hardness/softness is an important parameter for the end uses of wheat seeds and results mainly in the expression of a gene, the hardness (*Ha*) gene, located on the short arm of chromosome 5D (Malven et al. 1973). Puroindolines are the main components of friabilin/GSP proteins (Jolly et al. 1993; Morris et al. 1994). However, puroindolines are present in most hard and soft wheat although there is a tendency for lower amounts in hard wheat kernels (Dubreil et al. 1998b ; Igrejas et al. 1999). These results suggest that friabilin/puroindoline do not determine endosperm texture but are only markers. The presence of puroindolines on the surface of starch could be lipid mediated (Greenblatt et al 1994) in agreement with the strong binding properties of these proteins with wheat polar lipids (Dubreil et al. 1997) and the localisation studies (Dubreil et al. 1998a).

It is noteworthy that PIN-a free cultivars are always hard or very hard wheats (Dubreil et al. 1998; Giroux and Morris 1998). PIN-a gene is located on the short arm of chromosome 5D at proximity of the *Ha* gene (Sourdille et al. 1996). An interesting allelic variation has been observed in PIN-b gene that leads to a glycine to serine change (Gly46Ser). This allelic variation was apparently highly associated to hardness (Giroux and Morris 1998). If such a mutation is probably without major consequence on the structure and functionality of PIN-b, it could be at least considered as a genetic marker. Although this PIN-b mutation is not systematically observed in hard wheat, the other hard wheat are PIN-a null variants (Giroux and Morris 1998). Such a conclusion was not confirmed for some Australian hard wheats where both the "soft PIN-b allele" without the null PIN-a allelic variation were highlighted (Turnbull et al. 1999). Recently on a sample of 40 French wheat cultivars grown in four different locations it has been shown that the PIN-b content was more related to grain hardness than the PIN-a content. Furthermore this study demonstrated that heritability of hardness and PIN-b content are similar (Igrejas et al. 1999). These contradictory results between wheat samples suggest that puroindolines are not the product of the *Ha* gene but are probably closely related to it. Finally although puroindolines are not starch binding proteins and are not directly involved in endosperm texture, they are obviously the only markers available for further investigations of the *Ha* gene, an important determinant of wheat end uses.

Conclusion

Today many exciting data are available on the major lipid binding proteins of cereal seeds, lipid transfer proteins. NsLTPs could play a major role in the formation of cutin and suberin layers that protect cereal grains from water diffusion and fungal attacks. They are also important protein for the formation and stability of beer foams. Indolines are highly surface active proteins that has been shown to change the texture of bread crumb. Indolines are also proteins which could interact with starch through their surface lipids and is today the only marker for the texture of grain. Therefore these proteins have promising perspectives to improve grain quality and their end uses. With these proteins and especially with indolines we have for the first time an alternative to the manipulation of the genes of storage proteins to improve the breadmaking and brewing qualities of wheat and barley.

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