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A fatty-acid-binding protein from wheat kernels

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Abstract

A protein of about 7 kDa (W-FABP) has been isolated from mature wheat kernels by H_2O extraction and gel filtration of the extract, followed by two steps of high-performance liquid chromatography. The N-terminal amino acid sequence has been determined up to the 28th residue and found to be identical (except for positions 4 and 5) to that deduced from a barley cDNA (EMBL X15257), which had been improperly classified as a non-specific lipid transfer protein (LTP2). Similarly with LTPs, W-FABP does bind fatty acids, but in contrast, it is not significantly homologous to LTPs, it is not recognized by LTP antibodies, it has a more acidic isoelectric point (pH < 6.8 vs. pH > 9.6), and it does not show antibiotic properties.

Key words: Cereal; Fatty acid binding protein; Plant, Wheat

1. Introduction

Transcripts whose pattern of expression coincides with the accumulation of lipids in developing aleurone cells from barley have been previously investigated [1–4]. A cDNA corresponding to one of these transcripts, B11E (EMBL X15257), has been improperly classified as a non-specific lipid transfer protein, LTP2 [3,4]. Transgenic expression of the coding region of this cDNA in tobacco, under a constitutive promoter, resulted in the premature loss of flower buds [3]. In the course of a systematic purification of low-molecular-mass proteins from wheat kernels, we have isolated a polypeptide of approximatly 7 kDa, designated W-FABP, whose N-terminal amino acid sequence is essentially identical to that deduced for the barley transcript. Although W-FABP does bind fatty acids, as has been reported for LTPs [5,6], it does not share with them their antipathogenic properties and other important features.

2. Materials and methods

2.1. Materials

Kernels from the wheat cv Chinese Spring (*Triticum aestivum*) were ground to a powder on a Culatti mill and used to purify the protein. The bacterial pathogen *Clavibacter michiganensis subsp. sepedonicus*, strain C5 was from the ETSIA collection (Madrid). Wheat protein CM3 was the gift from Dr. R. Sanchez-Monge (Madrid) and thionin and LTP4 were given by Dr. A. Molina (Madrid). Bovine serum albumin and chymotrypsinogen were from Sigma. Radioactive [1-^{14}C]oleic acid was from Amersham.

2.2. Purification procedure

Ground wheat kernels (40 g) were extracted once with 4 volumes of H_2O and the extract was freeze-dried, dissolved in 5 ml of 3 M urea, and loaded into a Sephadex G-100 column (2.5 cm × 80 cm; 25 ml/h). The selected eluate fraction was dialyzed against 5 l of H_2O, using a Spectra/Por 7 (MWCO:2000) membrane (Spectrum Medical Ind. Inc.), and freeze-dried. The fraction was then dissolved in H_2O/0.1% TFA and subjected to RP-HPLC on an ultrapore C3 column (1 × 25 cm; 5μm particle; 30 nm pore), using a 0–50% (180 min) H_2O/acetonitrile gradient, 0.1% TFA, at 0.5 ml/min. The fraction indicated in Fig. 1B was rechromatographed under the same conditions, except that 2-propanol was used instead of acetonitrile. SDS-PAGE in preformed gradient gels (4–20% acrylamide; Bio-Rad) was carried out according to the manufacturers' instructions.

2.3. Fatty acid binding assay

The protein (10 μg) was incubated with [1-^{14}C]oleic acid essentially as described [5,6]. Electrofocusing was carried out on a refrigerated horizontal apparatus from Pharmacia, using pH 3–10 Pharmalyte. Electrode buffers were 1 M NaOH and 0.04 M aspartic acid. Electrofocusing was at 600 V for 45 min and at 2000 V for 11 min. The gel was stained for protein with Coomassie blue R, air-dried, and exposed with X-ray film. Colored isoelectrofocusing standard from Bio-Rad were used.

2.4. Inhibition tests

Proteins were dissolved in water and added to microtiter wells prior to innoculation. Serial dilutions from 0.1 μM to 10 μM were used. Bacteria were added at a final concentration of 10^4 colony forming units/ml at a final volume of 150 μl (100 μl protein + 50 μl nutrient broth, Oxoid), incubated for 24 h at 28°C, and growth recorded in an ELISA plate reader.

3. Results and discussion

Ground wheat kernels were extracted with H_2O and the freeze-dried extract was subjected to gel filtration, as shown in Fig. 1A. Fraction I, eluting with an apparent molecular weight of about 7 kDa, was further fractionated by RP-HPLC (Fig. 1B). Fraction II from this col-

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Abbreviations: LTP, lipid transfer protein; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NAS, normalized alignment score.

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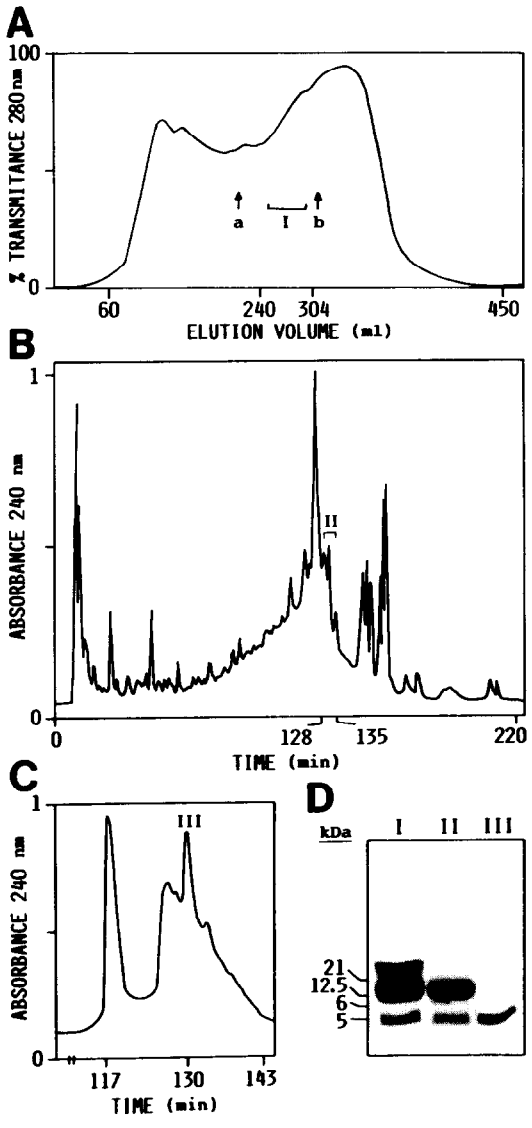

Fig. 1. Purification of W-FABP from wheat kernels. (A) Gel filtration of H_2O extract. Markers of molecular mass were (a) chymotrypsinogen, 25 kDa, and (b) thionin, 5 kDa. (B) RP-HPLC fractionation of fraction I from above, using acetonitrile in the gradient. (C) RP-HPLC fractionation of fraction II from B, using 2-propanol in the gradient. (D) SDS-PAGE of the indicated fractions. Fraction III from C consists of homogeneous W-FABP.

umn, which contained a component that migrated with an apparent molecular mass of 5 kDa in SDS-PAGE (Fig. 1D), was subjected again to RP-HPLC, but using 2-propanol in the gradient, instead of acetonitrile (Fig. 1C). The fraction designated as III contained the apparently homogeneous, fast-moving component, designated W-FABP (Fig. 1D).

The N-terminal amino acid sequence of this component was determined up to the 28th residue and no sequence heterogeneity was observed. Except for residues 4 and 5, this amino acid sequence from wheat was identical to that deduced from a previously reported barley cDNA, B11E, as shown in Fig. 2. The barley sequence has been improperly designated LTP2 (EMBL X69793) in a recent publication [4], but we have designated it as B-FABP for reasons that will be discussed below. The two FABPs have been aligned with LTP1 from barley [7,8], to show that although they have some features in common with LTPs, the probability of homology between B-FABP and LTP1 is extremely low according to the criteria of Doolitle [9]. The normalized alignment score (NAS = 120) for the LTP1/B-FABP comparison is of the same order as those obtained for comparisons between random sequences of the same length, and much below the minimum value of NAS = 300 that would be required for significance [9]. The position of the N-terminus of W-FABP suggests that the N-terminus of the protein deduced from the B11E cDNA (B-FABP) is 6 amino acid residues downstream from where it was originally proposed [1]. The molecular weight of B-FABP protein is 6,989 Da, which is compatible with the elution volume of W-FABP in gel filtration, but higher than the 5 kDa apparent molecular mass estimated for this protein by SDS-PAGE. This is a typical behaviour of lipid- binding proteins: ie, LTP4, whose size is 8.8 kDa, appeared as a 6.5 kDa protein in SDS-PAGE [10].

The ability of the FABP to bind fatty acids was investigated by a previously described electrofocusing technique [6], using [1-^{14}C]oleic acid. A wheat protein (CM3), which has hydrophobic domains and is soluble in chloroform/methanol mixtures [11], was used as a negative control, and bovine serum albumin (BSA) as a positive one. As shown in Fig. 3, W-FABP did bind oleic acid in a similar manner as LTP4 and its isoelectric point was more acidic than that of LTP4 (< 6.8 versus >9.6). A highly charged motif in the central part of the sequence and a hydrophobic stretch downstream from it have been proposed as the phospholipid-binding sites of LTPs [12]. These sequences, which are conserved in LTPs, are not obviously present in FABPs. Antibodies raised against a mixture of barley LTPs [14], which recognized LTPs

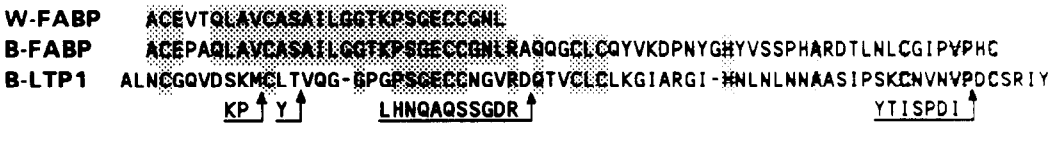

Fig. 2. N-terminal amino acid sequence of W-FABP aligned with those corresponding to B-FABP, deduced from B11E cDNA [1], and LTP1 from barley [7,8]. Identical residues are shadowed, gaps (−) and insertions (↑) are indicated. A highly similar LTP1 sequence from wheat has been reported [17].

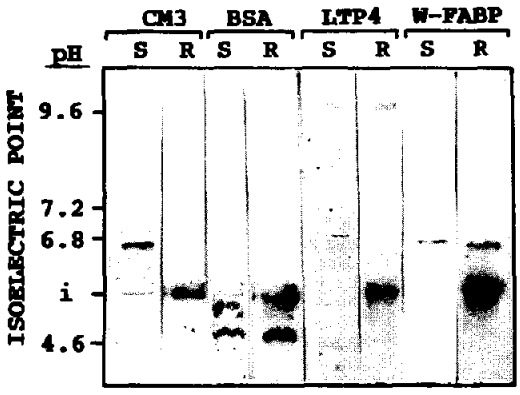

Fig. 3. Binding of radioactive oleic acid to W-FABP. Bovine serum albumin (BSA) and barley LTP4 [10] were used as positive controls, wheat CM3 [11] as a negative one. The gel was stained with Coomassie blue (S) and then exposed to X-ray film (R). The insertion point is indicated (i).

from other species, including spinach and Arabidopsis, do not recognize W-FABP (not shown).

We have recently reported that all LTPs tested are potent inhibitors of plant pathogens [10,13]. W-FABP was not active against the test microorganism at up to 10 μM concentration, tenfold higher than those giving complete inhibition with LTPs or with thionins [10,13], which were used as positive controls in the experiment (not shown).

Based on the above dissimilarities of W-FABP with respect to the LTPs, both in their amino acid sequences and in their in vitro properties, it would be advisable that the amino acid sequence deduced from the barley B11E cDNA should not be identified as a member of the LTP family, as has been proposed (EMBL X69793 and [4]). The designation LTP2 has been recently given to a protein from barley leaves which is significantly homologous to LTP1 [10,14].

Barley B11E mRNA was first detected at 10 days after pollination (DAP), reached a maximum at about 25 DAP, and then declined rapidly to an undetectable level in mature aleurone tissue [1,2], whereas W-FABP is present in and can be readily purified from the mature kernel, which suggests that it is stable well beyond the moment in which its gene is switched off. This persistence points more to a structural role in association with lipids than to a catalytic function in lipid metabolism. Further research will be needed to elucidate the function(s) of both FABPs and LTPs, as their in vitro properties, such as binding of fatty acids, coenzyme-A esters, and phospholipids, or lipid transfer between artificial liposomes and mitochondria [5,6,15,16], cannot be extrapolated into the in vivo situation. The subcellular location of LTPs seems to be incompatible with some of the metabolic roles proposed for them (see [14]). The subcellular location(s) of FABPs need to be investigated. From the above results it can be concluded that W-FABP and B-FABP represent a distinct protein family with respect to the LTPs, although both families might be involved in lipid metabolism. A definite designation of this family should wait until more is known about their roles in vivo.

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