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Comparison of cytokinin-binding proteins from wheat and oat grains

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Cytokinin-binding proteins (CBPs) isolated from mature grains of oat (*Avena sativa* L.) and wheat (*Triticum aestivum* L.) by acid precipitation, ion-exchange and affinity chromatography had similar characteristics, although they differed somewhat in apparent molecular weight of the native protein as determined by gel filtration (109 and 133 kDa, respectively) and subunit size as estimated by SDS-polyacrylamide gel electrophoresis (47 and 55 kDa, respectively). Highly purified oat CBP showed very weak but distinct immunochemical cross-reactivity with anti-wheat CBP IgG, indicating different immunogenic properties of the two CBPs. Nevertheless, both CBPs exhibited very similar binding of different cytokinins and were characterized by high affinity for N^6-benzyladenine (BA)-type and by low affinity for zeatin-type cytokinins to both wheat and oat CBPs and by somewhat higher binding activities of oat CBP compared to wheat CBP (K_ds for BA: 4.6×10^{-7} *M* and 6.8×10^{-7} *M*, respectively). The potential role of CBPs in regulating free BA-type cytokinin levels during cereal grain development and germination is discussed.

Introduction

Cytokinin-binding proteins (CBPs), putative cytokinin receptors, have been detected in different plant organs and organelles (leaves, coleoptiles, hypocotyls, cotyledons, calli, cell suspensions and mitochondria) of several plant species (maize, barley, mung bean, tobacco, cucumber and carrot) (Brinegar 1994, Brault and Maldiney 1999, Brault et al. 1999). The triggering of cytokinin-specific physiological responses was not strictly demonstrated for any of these binding proteins, despite the high specificity of cytokinin binding. Recent identification of a cytokinin response gene (*CRE1*) that encodes a histidine kinase in *Arabidopsis* (Inoue et al. 2001) with sensing and response regulatory functions (Hwang and Sheen 2001) provides strong evidence that CRE1 is a cytokinin receptor which initiates phospho-relay signalling. The function(s) of other cytokinin-binding proteins, if any, remains unknown.

CBP from wheat grains (CBF-1), which was first described by Fox and Erion (1975), is one of the best-characterized CBPs. Native CBF-1 was found to be a homotrimeric protein consisting of three identical subunits of 54 kDa, which bind a single cytokinin molecule (Brinegar and Fox 1985). Using photoaffinity labelling of wheat CBF-1 with [^{14}C] 2-azido-N^6-benzyladenine and subsequent proteolytic digestion, the primary structure of a labelled binding site peptide was determined (Brinegar et al. 1988) and its secondary structure was predicted (Fox 1992). CBF-1 accumulates rapidly in the embryo beginning 2 weeks post-anthesis and accounts for as much as 10% of the soluble embryo protein content at maturation (Brinegar et al. 1985). Characteristics of wheat CBF-1 are closely associated with seed storage proteins, including its fast accumulation during grain filling, localization in tissues surrounding the embryonic axis, and structural similarities with vicilin-type storage proteins (Brinegar et al. 1985, Brinegar and Fox 1987). Some of these properties, namely high concentration in embryos (Brinegar et al. 1985) and relatively low binding

Abbreviations – BA, N^6-benzyladenine; CBF-1, cytokinin-binding protein from wheat; CBP, cytokinin binding protein; iPR, N^6-(2-isopentenyl)adenosine; PPTU, 1-phenyl-3- (thiazolyl)-2-thiourea; thidiazuron, *N*-phenyl-*N*′-1,2,3-thidiazol-5yl urea.

of isoprenoid cytokinins as compared to cytokinins bearing an N^6 aromatic side chain (Keim et al. 1981) suggest that wheat CBF-1 does not function as a cytokinin receptor in the usual sense of that term. The idea that CBF-1 may serve as sequestering protein regulating the access of cytokinins to the embryo during seed maturation and germination (Brinegar et al. 1985) is a much more attractive proposal; however, it cannot be applied to zeatin-type cytokinins which have low affinity for CBF-1. The recent identification of a highly active cytokinin bearing an aromatic side-chain, N^6-(3-hydroxybenzyl)adenosine (Kamínek et al. 1987), as a natural cytokinin in wheat grains (Kamínek et al. 2000) opens the possibility that CBF-1 may be involved in the immobilization of N^6-benzyladenine (BA)-type cytokinins in the course of seed maturation and their release upon germination.

Different CBPs, which may possess a similar function to wheat CBF-1, were detected in embryos of several cereal species. Interestingly, their binding activities towards BA do not always correlate with their immunochemical cross-reactivity with anti-wheat embryo CBF-1 specific antibodies as indicated by experiments with crude embryo protein extracts (Brinegar and Fox 1987). We present here a comparison of the biochemical properties, cytokinin-binding activity and immunological cross-reactivity of highly purified wheat CBF-1 and oat CBP. Their potential function in the control of free cytokinin levels during development and germination of wheat and oat grains with respect to the recent identification of a BA-type cytokinin in developing wheat grains is discussed.

Materials and methods

Plant material and chemicals

Matured grains of field-grown oat (*Avena sativa* L. cv. Tiger) and wheat (*Triticum aestivum* L. cv. Munk) were used in this study. Unless otherwise stated all reagents were from Sigma, St. Louis, USA. [^{3}H] BA (0.9 TBq mmol^{-1}) was synthesized by Dr J. Hanuš, Institute of Experimental Botany, Prague, Czech Republic, and anti-wheat embryo CBF-1 polyclonal rabbit antibodies were obtained from Dr C. Brinegar, San Jose State University, San Jose, CA, USA.

Isolation of cytokinin-binding proteins

CBPs were isolated using a modified method of Brinegar et al. (1985). Briefly, 100 g of cold grains were ground to a powder using a cooled blade homogenizer and extracted with 500 ml of cold (−5°C) acetone under gentle stirring. All further operations proceeded at 4°C and all centrifugations were performed at 20 000 *g* for 20 min. Defatted material was dried on filter paper and extracted under stirring for 30 min with 200 ml of ice-cold 50 m*M* Tris-HCl buffer (pH 8.5) containing 100 m*M* KCl, 1 m*M* dithiothreitol and 0.025 mg ml^{-1} soybean trypsin inhibitor. After centrifugation CBPs were precipitated from the supernatant by lowering its pH to 5.0 with 50% (v/v) acetic acid. After gentle stirring for 30 min the precipitate was separated by centrifugation, re-suspended in 15 ml of 500 m*M* KCl in buffer A (25 m*M* TES, 5 m*M* 2-mercaptoethanol and 0.1 m*M* Na_2EDTA, adjusted with Tris base to pH 8.5) and extracted under stirring for 30 min. Following centrifugation the salt concentration of the supernatant was lowered by the addition of four volumes of buffer A (pH 7.5) and the supernatant was applied onto cellulose phosphate (Whatman P-11) column (2.4 × 15 cm) equilibrated with buffer A containing 50 m*M* KCl (pH 7.5) at a flow rate of 48 ml h^{-1}. After removal of unbound material with 150 m*M* KCl in buffer A (pH 7.5) the column was eluted with 500 m*M* KCl in the same buffer. The protein-containing fraction was pooled and dialysed overnight against 2 l 25 *M* Tris-acetate buffer (pH 7.5) containing 50 m*M* K-acetate, 2.5 m*M* $CaCl_2$ and 1 m*M* dithiothreitol. After centrifugation the supernatant was applied onto a BA affinity column (1.2 × 6 cm, BA-Sepharose 4A) equilibrated with the dialysis buffer. The column was washed with the same buffer supplemented with 2 *M* KCl and CBP was eluted with the same buffer containing 2 *M* KCl and 1 *M* urea. Protein-containing fractions were pooled and dialysed at 4°C against 0.2 *M* NH_4HCO_3, pH 8.0. Following dialysis, solids were removed by centrifugation and aliquots of concentrated dialysate were frozen in liquid nitrogen and stored at −70°C.

Sephacryl S-300 chromatography

Native oat CBP and wheat CBF-1 were loaded in 10% (v/v) glycerol onto a Sephacryl S-300 column (1.6 × 90 cm) equilibrated with 0.2 *M* NH_4HCO_3 buffer (pH 8.0) and eluted with the same buffer at a flow rate of 8 ml h^{-1}. Fractions of 1.5 ml were collected and assayed for cytokinin-binding activity by equilibrium dialysis.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE of purified wheat CBF-1 and oat CBP was carried out on 7.5% gels according to Laemmli (1970) and proteins were visualized by Coomassie Brilliant Blue R-250 staining.

Ouchterlony double immunodiffusion

The assay was performed in 5-cm Petri dishes containing 8 ml of 1% (w/v) agarose buffered with 0.2 *M* NaCl in 0.1 *M* Tricine-HCl (pH 8.2). Affinity purified rabbit polyclonal anti-wheat CBF-1 antibody (15 µg) was applied into the central well and purified oat CBP and wheat CBF-1 (0.8, 4.0 and 20 µg) were introduced into peripheral wells (all in 20 µl of 0.2 *M* NaCl in 0.1 *M* Tricine-HCl, pH 8.0). After a 3-day incubation at room temperature, non-precipitated proteins were removed by washing with the above buffer and precipitation zones

were visualized by staining with Coomassie Brilliant Blue R-250.

Cytokinin-binding assay

Ligand binding to oat CBP and wheat CBF-1 was determined by equilibrium dialysis assay arranged according to Brinegar et al. (1985) with some modifications. As compared to other binding assays it allows the simultaneous assay of multiple samples, and together with the ultrafiltration assay, gives the most reliable results (Kamínek and Fox 1992). Aliquots of 0.3 ml of CBPs in 0.2 *M* NH_4HCO_3, pH 8.0, containing 4 m*M* 2-mercaptoethanol inside of dialysis bags (Spectrapor, 10 mm diameter, molecular weight cut-off 12 000–14 000) were dialysed against 500 ml of the same buffer supplemented with 1 n*M* [^{3}H] BA (1 TBq mmol^{-1}) and unlabelled competitor for 18 h at 4°C. Radioactivity in 0.2 ml aliquots of the dialysates was measured by scintillation counting and corrected for background counts in 0.2 ml dialysis buffer. All assays were run in triplicate. Estimation of dissociation constants (K_d) is based on the assumption that the concentration of unlabelled competitor required to yield 50% competition of the radiolabelled ligand binding is equal to the K_d of the binding protein for the ligand. A prerequisite for the validity of the assay is that the concentration of the labelled ligand is much less than the K_d of binding protein for the ligand and that both the competitor and the ligand interact with the same site (Cheng and Prusoff 1973, Keim et al. 1981).

Results

Chromatography of native oat CBP and wheat CBF-1 preparations on a Sephacryl 300 column yielded single peaks of protein which closely correlated with the peaks of [^{3}H] BAP binding and corresponded to apparent molecular weights of 109 and 133 kDa, respectively (Fig. 1). When analysed by SDS-PAGE, each of the two CBPs yielded a single polypeptide with an apparent molecular weight of 47 kDa and 55 kDa, respectively, indicating their presumed trimeric structure consisting of three identical subunits. While wheat CBF-1 52 kDa subunit yielded a single sharp band after SDS-PAGE, the oat CBP band was accompanied by a number of closely adjacent bands of very low densities, implying microheterogenity of the oat CBP (Fig. 2).

Immunochemical cross-reactivity of highly purified native oat CBP and wheat CBF-1 against anti-wheat CBF-1 IgG was tested using Ouchterlony imunodiffusion. As expected, strong imunoprecipitation zones were generated following immunodiffusion of wheat CBF-1 against anti-CBF-1 antibody. However, only very weak but distinct precipitation lines were found with oat CBP diffused against the same wheat IgG (Fig. 3), indicating considerably different imunogenic properties of the two CBPs.

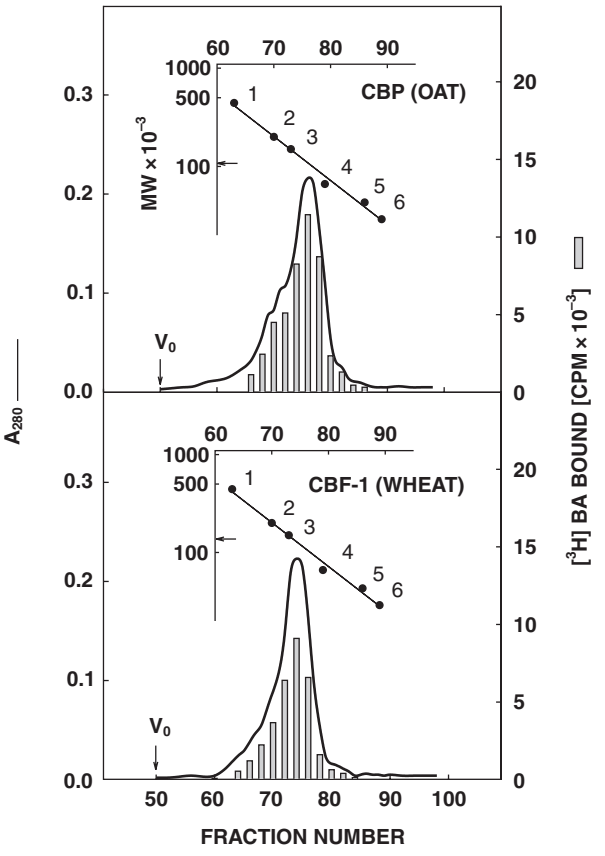

Fig. 1. Molecular weight estimations of native oat CBP and wheat CBF-1 by gel filtration on Sephacryl S-300. Molecular weight markers: apoferritin, horse spleen (1); I-amylase, sweet potato (2); alcohol dehydrogenase, yeast (3); bovine serum albumin (4); ovalbumin (5); and carbonic anhydrase, bovine erythrocytes (6).

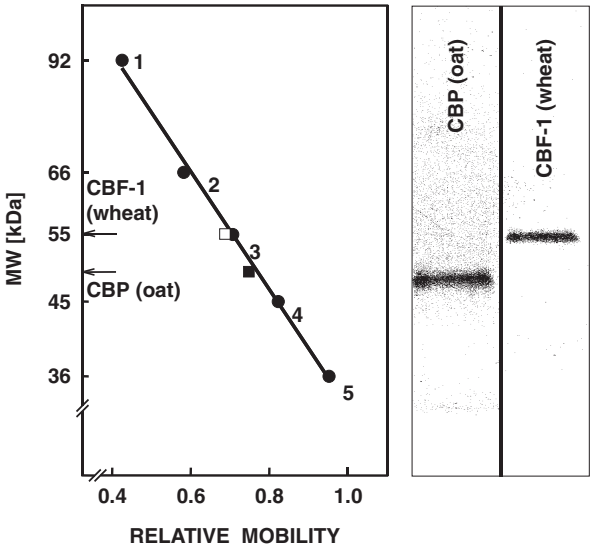

Fig. 2. Molecular weight estimations of oat CBP and wheat CBF-1 subunits by SDS-polyacrylamide gel electrophoresis. Molecular weight markers: phosphorylase b, rabbit muscle (1); albumin, bovine serum (2); glutamate dehydrogenase, bovine liver (3); ovalbumin, chicken egg (4); and lactate dehydrogenase (5). The stained electrophoretogram is shown in the right panel.

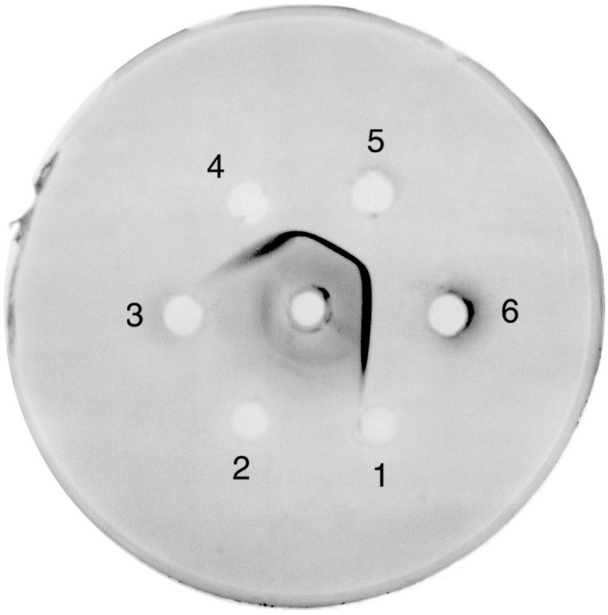

Fig. 3. Ouchterlony immunodiffusion of oat CBP and wheat CBF-1 against anti-CBF-1 IgG (15 µg in centre well); 1, 2, 3 = CBP (oat), 0.8, 4 and 20 µg, respectively, 4, 5, 6 = CBF-1 (wheat), 0.8, 4 and 20 µg, respectively.

As shown in Table 1, cytokinin-binding activities of oat CBP and wheat CBF-1 expressed by K_d of complexes formed between the two CBPs and different compounds exhibiting cytokinin activity were very similar. Generally, BA-type cytokinins and the cytokinin-active aromatic urea derivatives (*N*-phenyl-*N*′-1,2,3-thidiazol-5yl urea [thidiazuron] and 1-phenyl-3-(thiazolyl)-2-thiourea [PTTU]) had higher affinity to both CBPs than isoprenoid cytokinins. There was stronger binding of N^6-(2-isopentenyl)adenosine (iPR) than of the other tested isoprenoid cytokinins to both CBPs, and oat CBP exhibited somewhat higher binding of most of tested compounds, especially of BA-type cytokinins and urea derivatives. Similar differences in affinities of isoprenoid- and BA-type cytokinins to the two CBPs were found when the BA-Sepharose affinity chromatography step in protein purification was omitted (results not shown). This indicates that low affinities of isoprenoid cytokinins to the two CBPs did not result from a selective isolation of BA-specific CBPs.

Table 1. Dissociation constants of the complexes formed between oat CP and wheat CBF-1 and various compounds exhibiting cytokinin activity.

Compound	CBP ($K_d \times 10^7$ *M*)	CBF-1 ($K_d \times 10^7$ *M*)
N^6-(2-hydroxybenzyl)adenosine	4.5	6.1
BA	4.6	5.9
Thidiazuron	8.8	8.3
N^6- (3-hydroxybenzyl)adenosine	14.3	15.1
PTTU	15.5	13.5
N^6-benzyladenosine	18.5	19.4
N^6-(2-isopentenyl)adenosine	170.0	75.2
dihydrozeatin	330.0	> 2000
trans-Zeatin	= 1000	> 1000
trans-Zeatin riboside	> 2000	> 2000

Discussion

The apparent molecular weight of CBP from wheat grains determined by gel filtration by different authors varied between 120 and 180 kDa (Polya and Davis 1978, Moore 1979). Our estimate of 133 kDa for wheat CBF-1 is near to the 155 kDa reported by Erion and Fox (1981). Interestingly, apparent molecular weights of purified native CBPs from other plant materials, i.e. barley leaves (Romanov et al. 1986, Kulaeva et al. 1998), maize shoots (Romanov et al. 1990), tobacco leaves (Yoshida and Takegami 1977, Momotani and Tsui 1992), mung bean seedlings (Nagata et al. 1993, Fujimoto et al. 1998) and tobacco callus (Kobayashi et al. 2000) were found much lower (4 to 67 kDa) than those of oat CBP and wheat CBF-1 (Fig. 1). The only exception is soluble CBP from etiolated mung bean seedlings where an apparent molecular weight of about 200 kDa was reported (Sakai and Kamei 1992).

Resolution of the wheat CBF-1 into 55 kDa polypeptide subunits corresponds to the original estimate of Brinegar and Fox (1985) and supports their conclusion that CBF-1 consists of three identical subunits. The lower apparent molecular weight of oat CBP subunit (47 kDa) reflects the lower apparent molecular weight of the corresponding native protein (Figs 1 and 2). Molecular weights of wheat CBF-1 and oat CBP subunits were about 24 and 29%, respectively, higher than one-third of the molecular weights of the corresponding homotrimeric proteins. Somewhat higher molecular weights of subunits in relation to the corresponding native wheat CBF-1 protein have also been reported by other authors (see Brinegar 1994) and may be caused by differences in protein mobility determined by SDS-PAGE (subunits) and gel filtration (native protein). In any case, the differences in the molecular weights of the two native CBPs and their subunits are small and indicate that they are closely related proteins. The microheterogenity of the oat CBP subunits indicates either the existence of multiple, closely related molecular forms or lower stability of the oat protein during extraction and purification procedures. However, since both oat and wheat plants are hexaploid, microheterogenity can be expected.

Earlier studies using protein extracts from embryos of several cereal species, including oat, showed a lack of immunochemical cross-reactivity with anti-wheat embryo CBF-1 antibody (Brinegar 1994). Using highly purified oat CBP we were able to detect a weak but distinct precipitation line with the same antibody in the Ouchterlony immunodiffusion assay (Fig. 3). This indicates the presence of some similar antigenic determinants in the two CBPs, which again may reflect structural similarities of the conserved 'storage protein' characteristics.

In spite of very low immunochemical cross-reactivity between the oat CBP and the wheat CBF-1 (Fig. 3), both CBPs exhibit very similar binding of different cytokinins and cytokinin groups (Table 1). Remarkable is their very low binding of zeatin-type cytokinins, which have been reported to exhibit high affinity for CBPs from other plant materials (Romanov et al. 1986, 1990). This and the high affinity of BA-type cytokinins for both oat CBP and wheat CBF-1 (Table 1) indicate that these CBPs may function either in signalling or in temporal immobilization of cytokinins bearing an aromatic side chain. Different phenyl derivatives of urea and thiourea that exhibit cytokinin activity have been reported to have relatively high affinities for CBPs from different plant materials (Nagata et al. 1993, Fujimoto et al. 1998). In this respect it is interesting that at least some of the energetically most favourable conformers of *N*,*N*′-diphenylurea exhibit a geometry which is very similar to that of purine cytokinins (Fox 1992). As is shown in Table 1, two different phenyl-substituted derivatives of urea and thiourea (thidiazuron and PTTU) have high affinity to both wheat CBF-1 and oat CBP. Moreover, their K_d values determined by a competition binding assay (Table 1) correlate with the corresponding cytokinin activities in the tobacco callus bioassay and with their inhibition of cytokinin oxidase *in vitro* (Chatfield and Armstrong 1986, Kamínek and Armstrong 1990, Abdelnour-Esquivel et al. 1992, Motyka and Kamínek 1994) apparently in a mixed, predominantly non-competitive manner (Burch and Horgan 1989, Hare and Van Staden 1994).

Low affinities of zeatin-type cytokinins for both CBPs indicate that the CBPs do not function by sequestering isoprenoid cytokinins to prevent their translocation from endosperm to embryo, and induction of premature grain germination (Brinegar and Fox 1987). Actually, *trans*-zeatin and its riboside are transiently accumulated in grains of cereals, including wheat, early after anthesis (see Morris 1997 for review), i.e. much ahead of the accumulation of CBPs (Brinegar et al. 1985). The recent identification of a BA-type cytokinin, N^6-(3-hydroxybenzyl)adenosine, in wheat grains (Kamínek et al. 2000) indicates that CBP may function by regulating the levels of cytokinins with a free aromatic-side chain by their immobilization during grain development, thus preventing premature embryo cell division, and by their release during grain germination where free cytokinins may play a role in the control of growth and development.

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