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Localization to chromosomes of structural genes for the major protease inhibitors of barley grains

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Summary. Wheat-barley chromosome addition lines were compared by isoelectric focusing of protein extracts to identify chromosomes carrying loci for the major immunochemically distinct protease inhibitors of barley grains. Structural genes for the following inhibitors were localized: an inhibitor of both endogenous α-amylase 2 and subtilisin (ASI) on chromosome 2, two chymotrypsin/subtilisin inhibitors (CI-1 and CI-2) on chromosome 5 (long arm) and the major trypsin inhibitor (TI-1) on chromosome 3.

Key words: Addition line – Barley – Isoelectric focusing – Protease inhibitors – Structural gene

Introduction

Barley grains contain five major immunochemically distinct protein inhibitors of microbial and/or animal serine proteases (Boisen et al. 1981; Boisen and Djurtoft 1982; Mikola and Suolinna 1969, 1971; Yoshikawa et al. 1976). These inhibitors represent a relatively large part of the water/salt-soluble protein. For several reasons it might be desirable to increase or reduce the grain content of individual inhibitors, either by conventional breeding or by gene manipulation: 1) Some of the inhibitors are rich in lysine and to some extent behave like storage proteins (Kirsi 1973; Hejgaard and Boisen 1980); 2) Grain protease inhibitors may have a protective function against invading microorganisms or insect pests (Ryan 1981); 3) Inhibitors may affect protein utilization in monogastric animals (Pedersen and Boisen 1982); and 4) Inhibitors of endogenous enzymes may have regulatory or protective functions during grain development or germination (Mikola and Enari 1970; Mundy et al. 1983).

A series of disomic wheat addition lines containing homologous pairs of one of six identified barley chromosomes added to the complete hexaploid set of wheat chromosomes has been constructed (Islam et al. 1981). Zymogram analyses of such addition lines have enabled the simple and unambiguous chromosome localization of structural genes for a number of barley enzymes (Powling et al. 1981), and some of the isozymes were found useful as chromosome markers in further studies (Hart et al. 1980).

The present communication describes how the wheat-barley addition lines produced by Islam et al. (1981) were used for the identification of chromosomes carrying loci for the now characterised barley protease inhibitors. Separation of wheat and barley inhibitors was based on isoelectric focusing and detection of specific enzyme inhibition by a negative-staining technique (Bruhn and Djurtoft 1977; Hejgaard 1981). Addition lines containing barley chromosome 5 have not been obtained. The presence of loci for two of the barley inhibitors on this chromosome was confirmed by use of trisomic barley lines.

Materials and methods

Plant material

Wheat (*Triticum aestivum* L. cv. 'Chinese Spring') – barley (*Hordeum vulgare* L. cv. 'Betzes') chromosome addition lines were obtained from Dr. A.K.M.R. Islam, Adelaide, Australia. A wheat with a translocated short arm of barley chromosome 5 was also obtained. The kernels studied were harvested from plants tested by chromosome counting and Giemsa C-band staining.

Seeds of 'Shin Ebisu' barley trisomic for chromosome 5 were obtained from T. Tsuchiya, Colorado, USA. A trisomic plant was selected by chromosome counting and used as the female parent in a cross with an unknown barley. A trisomic

F_1 plant was selected and self-pollinated, and 14 F_2 kernels were used for the detection of gene dosis effects. Three of the plants were identified as trisomics (15 chromosomes), the rest were normal with 14 chromosomes.

Extraction

Two grains were crushed with a pair of pliers and extracted with 400 µl water by vigorous shaking for 1 h at room temperature in 9×70 mm capped plastic tubes also containing a 5 mm (d.) glass ball. After centrifugation for 10 min at 5,000 G, 25 µl samples of the supernatant solution were applied for isoelectric focusing. In some experiments, 50 µl extracts were treated with 10–25 µl purified monospecific antibody preparations for 15 min and centrifuged before application of the supernatant sample (Hejgaard 1981).

Isoelectric focusing

Isoelectric focusing of cereal grain extracts in 74×2.7 mm polyacrylamide gel rods has been described in detail previously (Hejgaard 1981). In all experiments the gel contained 2% spacer ampholyte and, depending on the pH range, the following combinations of ampholyte and electrode solutions were used: pH 3–10: Pharmalyte (Pharmacia, Uppsala, Sweden), 1% phosphoric acid (anode) and 0.01 M sodium hydroxide (cathode); pH 4–6: Ampholine (LKB, Bromma, Sweden), 0.01 M glutamic acid (anode) and 0.01 M histidine (cathode); pH 6–8: Ampholine, 0.01 M glutamic acid (anode) and 0.01 M ethanol amine (cathode); pH 8–10: Pharmalyte, 0.01 M N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid (HEPES) (anode) and 0.025 M ethylene diamine (cathode). After electrophoresis for 3 h at 175 V (25 °C), gels were stained for inhibition of trypsin, chymotrypsin or subtilisin, exactly as described by Bruhn and Djurtoft (1977) and Hejgaard (1981).

Immunoelectrophoresis

Quantitative determination of inhibitor CI-1 was made by rocket immunoelectrophoresis (Hejgaard and Boisen 1980). Inhibitor CI-2 was determined by immunodiffusion (Jonassen 1980). Diluted samples of barley extract were used for relative standardization in both assays.

Results and discussion

When grain extracts of wheat-barley addition lines were subjected to isoelectric focusing followed by staining for protease inhibition, relatively complex patterns of molecular forms of wheat and barley inhibitors with different isoelectric points were often obtained (Figs. 1–4). It has previously been shown how zones formed by inhibitors with similar antigenic properties could be identified by treatment of barley or wheat extracts with monospecific antibodies prior to isoelectric focusing (Hejgaard 1981). In the present study, monospecific antibodies towards the five major barley inhibitors were used to identify the corresponding barley inhibitor zones as well as zones formed by immunochemically related (homologous) wheat inhibitors. The result of such an experiment is shown in Fig. 1. Antibodies towards the barley α-amylase/subtilisin inhibitor are seen to absorb the barley inhibitor and the homologous wheat inhibitor, while the position and staining intensity of the wheat chymotrypsin/subtilisin inhibitor zone is unaffected by the treatment.

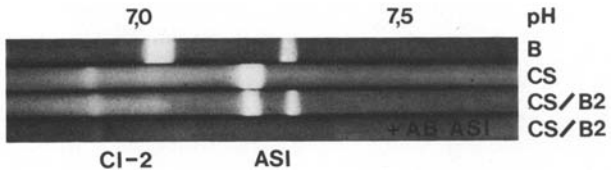

Fig. 1. Isoelectric focusing, pH 6–8: separation and immunochemical identification of the α-amylase/subtilisin inhibitor (*ASI*) in barley and wheat. The gels were stained for inhibitors of subtilisin A. *B* 'Betzes' barley. *CS* 'Chinese Spring' Wheat. *CS/B 2* 'Chinese Spring'-'Betzes' addition line carrying barley chromosome 2. *CI-2* Chymotrypsin/subtilisin inhibitor CI-2 (only one molecular form of barley and one of wheat origin appear in the pH-range depicted here). *ASI* α-Amylase/subtilisin inhibitor of barley and wheat. *+Ab ASI* Indicates that the extract was treated ("absorbed") with monospecific antibodies towards the subtilisin inhibitor ASI prior to immunoelectrophoresis. *pH* Approximate pH-scale indicates isoelectric points of molecular forms of the different inhibitors

For clarity the five different barley inhibitors will be discussed separately, although information about loci for more than one inhibitor often could be extracted from the same experiment.

α-Amylase/subtilisin inhibitor (ASI)

This relatively specific inhibitor of subtilisin-like bacterial proteases was first purified and characterized by Yoshikawa et al. (1976). Recently, it was found that this protein also inhibits an entirely unrelated enzyme, the endogenous α-amylase 2 (Mundy et al. 1983). The molecular weight is near 20,000, and amino acid sequence studies have shown that the inhibitor is homologous with the well-characterized soy bean Kunitz inhibitor (Hejgaard et al. 1983). Treatment with monospecific antibodies showed that this inhibitor is represented by one zone originating from barley and one from wheat in the focusing patterns (Fig. 1). Isoelectric focusing of grain extracts of all addition lines clearly shows that the structural gene for this inhibitor is located on barley chromosome 2 (Fig. 2). The designation *Isa 1* (*i*nhibitor of *s*ubtilisin and *a*mylase) is proposed for this inhibitor locus.

Trypsin inhibitors (TI-1 and TI-2)

The major endosperm inhibitor of trypsin (TI-1) has a molecular weight of about 14,500 and was first purified and characterized by Mikola and Suolinna (1969). It may contribute to about 5% of the water-soluble protein in grains of normally cultivated barleys. In the high-lysine mutant 'Risø 1508', this inhibitor is absent or present in only very low amounts (Boisen and

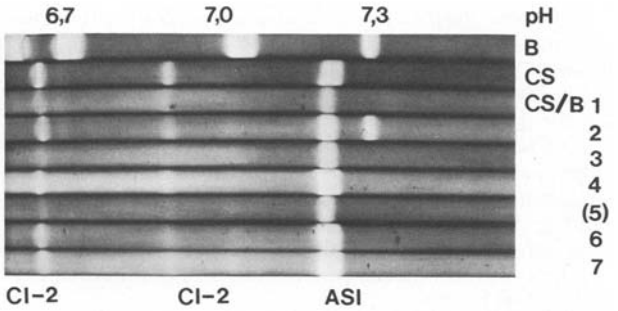

Fig. 2. Isoelectric focusing, pH 6–8: localization of structural genes for the α-amylase/subtilisin inhibitor (*ASI*) and a chymotrypsin/subtilisin inhibitor (*CI-2*). The gel was stained for inhibitors of subtilisin A. (*5*) Brackets indicate that an addition line with this chromosome has not been obtained, and a translocation line with a substituted short arm of barley chromosome 5 was studied. The two major molecular forms of CI-2 in barley were detected in the pH-range studied, cf. Hejgaard (1981). See Fig. 1 for definition of other symbols

Djurtoft 1982). Recently, the inhibitor was found to be homologous with the major wheat inhibitors of animal α-amylases and with a bifunctional α-amylase/trypsin inhibitor of finger millet (Campos and Richardson 1983). The inhibitor is represented by one major zone in isoelectric focusing, while zones from an immunochemically related wheat inhibitor were absent or very faint (Fig. 3). Characterization of the addition lines by isoelectric focusing showed that the locus for this inhibitor is on barley chromosome 3 (Fig. 3). The designation *Itc 1* (*i*nhibitor of *t*rypsin and *c*hymotrypsin) is proposed for the inhibitor locus.

A second trypsin inhibitor (TI-2) also inactivates a *Streptomyces* protease (pronase) component with trypsin-like specificity. The inhibitor is mainly present in the embryo, and two molecular forms have been purified and characterized (Boisen and Djurtoft 1982). The molecular weight is about 16,000, and the inhibitor was unaffected by treatment with pepsin for 2 h at pH 2.0. The inhibitor has an isoelectric point higher than 9, but several attempts to detect the activity of this inhibitor after isoelectric focusing in pH 3–10.5 and pH 8–10.5 ampholyte gradients were unsuccessful. Apparently, the inhibitor has a pI above 10.5 Alternatively, activity was lost at high pH, when approaching the isoelectric point during electrophoresis.

Chymotrypsin inhibitors (CI-1 and CI-2)

The barley grain contains two immunochemically distinct, but 46% homologous, inhibitors of bacterial and fungal alkaline proteases and chymotrypsin. The two inhibitors are 30–40% homologous with potato inhibitor I (Svendsen et al. 1982), which is part of a plant defense system under hormone-like control (Ryan 1981). Both barley inhibitors have monomer molecular weights near 8,000–9,000. They are relatively rich in lysine and contribute a significant fraction of this amino acid in some high-lysine barleys where the grain content of the inhibitors is 5–20 fold higher than in normally cultivated barleys (Jonassen 1980; Hejgaard and Boisen 1980). At least five molecular forms of each inhibitor have been identified and characterized (Boisen et al. 1981; Mikola and Suolinna 1971). Immunochemically related inhibitors are present in wheat (Hejgaard 1981), but fortunately they are present in relatively low amounts in grains of the cultivar 'Chinese Spring' (Figs. 2 and 4). Although strong zones of both barley inhibitors were observed after isoelectric focusing of 'Betzes' barley, no barley chymotrypsin inhibitor zones seemed to be expressed in the addition material (chromosomes 1, 2, 3, 4, 6 and 7) or translocation material (chromosome 5, short arm) studied (Figs. 2 and 4). These results suggest that the structural genes for both inhibitors are located on the long arm of

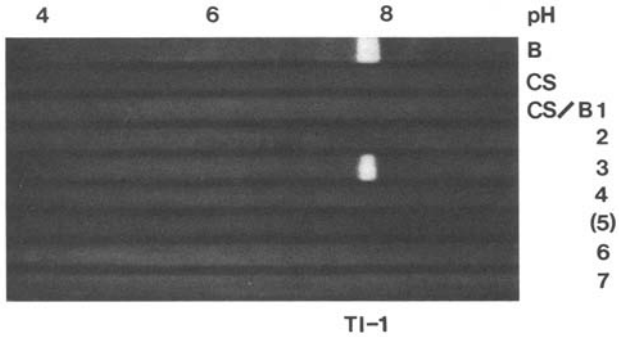

Fig. 3. Isoelectric focusing, pH 3–10: localization of the structural gene for the barley endosperm trypsin inhibitor (*TI-1*). The gel was stained for inhibitors of bovine trypsin. *TI-1* Major molecular forms of the endosperm trypsin inhibitor in barley and wheat. See Figs. 1 and 2 for other symbols

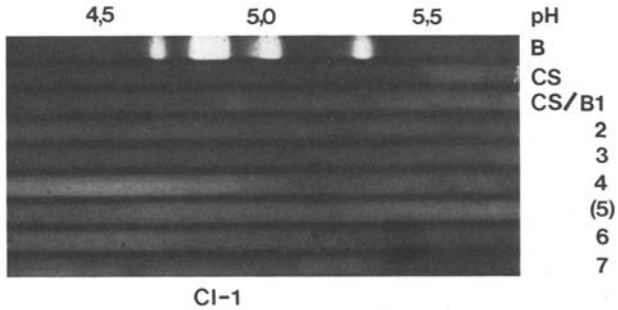

Fig. 4. Isoelectric focusing, pH 4–6: localization of structural gene(s) for a chymotrypsin/subtilisin inhibitor (*CI-1*). The gel was stained for inhibitors of subtilisin A. *CI-1* Four major molecular forms of the barley inhibitor (similar inhibitors are present in wheat but not in detectable amounts, cf. Hejgaard (1981)). See Figs. 1 and 2 for other symbols

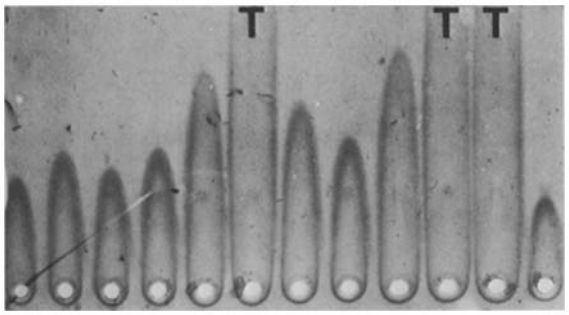

Fig. 5. Estimation of inhibitor CI-1 in extracts of half-kernels by rocket immunoelectrophoresis. F_2 kernels from the same plant were compared. Trisomics carrying an extra chromosome 5 are marked with a *T*. Monospecific antibodies towards barley inhibitor CI-1 (Boisen et al. 1981) were used

barley chromosome 5. To confirm this conclusion from negative evidence, a possible dose response of an extra barley chromosome 5 in trisomic barley lines was investigated. The inhibitor CI-1 could be measured by rocket immunoelectrophoresis (Fig. 5), but due to the low mobility of inhibitor CI-2 during electrophoresis this inhibitor was determined by immunodiffusion ad modum Mancini (not shown). Although the material was limited, both inhibitors were clearly present in higher proportions (50–70%) in extracts of half-kernels trisomic for barley chromosome 5. Studies of wheat-rye addition lines (work in progress) have shown that both loci for the immunochemically related rye CI-1 and CI-2 inhibitors are positioned on rye chromosome 1. Previous studies of the major prolamin storage proteins and other evidence show that barley chromosome 5 and rye chromosome 1 are homoeologous (Lawrence and Shepherd 1981). These observations strongly support the hypothesis that structural genes for both chymotrypsin inhibitors CI-1 and CI-2 are located on barley chromosome 5. Designations *Ica 1* and *Ica 2* (*i*nhibitors of *c*hymotrypsin and *A*spergillus protease) are proposed for these two inhibitor loci.

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