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Chromosomal location of a barley malt endopeptidase gene

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Summary

Extracts of disomic wheat-barley addition lines were tested for the presence of a barley malt endopeptidase (MEP-1) by employing isoelectric focusing (IEF) and western blotting. The blots were probed with polyclonal antibodies raised against MEP-1 purified from the endosperms of 5-day-old germinated barley seedlings. The endopeptidase was detected in the Betzes barley cultivar and the addition line containing the full genome of the wheat cultivar Chinese Spring plus a chromosome 3 pair from Betzes barley. The endopeptidase was not expressed in Chinese Spring nor the addition lines containing other Betzes chromosome pairs. The endopeptidase was detected in a ditelosomic addition line containing the long arm of Betzes chromosome 3. We have concluded that the gene coding for MEP-1 (*Cep-B*) is located on the long arm of Betzes chromosome 3.

1. Introduction

The degradation of endosperm proteins is required for germination of barley seeds and the production of quality malt. The isolation of the main endopeptidase from barley extracts (Jones & Poulle, 1988; Phillips & Wallace, 1989; Koehler & Ho, 1990) has prompted research into the action of barley endopeptidases during germination. A cysteine endopeptidase with an approximate M_r of 30000 has been detected in all three laboratories.

The development of the disomic addition lines containing the complete compliment of wheat chromosomes (cv. Chinese Spring) and a pair of barley (cv. Betzes) chromosomes 1, 2, 3, 4, 6 or 7 provides the opportunity to investigate the gene content of barley chromosomes (Islam *et al.*, 1981; Soliman & Allard, 1989). In this study we have used IEF, western blotting and immunological techniques to identify the barley chromosome containing the gene for the malt endopeptidase (MEP-1) isolated by Phillips & Wallace (1989). The ditelosomic addition lines (Islam, 1983) have also been examined to assign the gene to a barley chromosome arm.

2. Materials and methods

Wheat-barley disomic addition lines 1, 2, 3, 4, 6, 7, and ditelosomic addition lines 3L and 3S were supplied by A. K. M. R. Islam (Department of Plant Science in the above Institute). Seeds were surface sterilized in 5% (v/v) sodium hypochlorite solution and germinated for 6 days in the presence of gibberellic acid (7 ppm; 90% total gibberellins supplied by Sigma Chemical Co. MO, USA) after which the roots and coleoptiles were removed.

Single seeds were mashed with a knitting needle and extracted with acetate buffer, 50 mM; 2-mercaptoethanol, 1 mM; pH 4·5 (3 ml/g f. wt). The homogenate was centrifuged 10 min at 12000 *g* and 5 μl aliquots were loaded onto vertical isoelectric focusing (IEF) gels. The purified malt endopeptidase (MEP-1) sample was isolated according to Phillips & Wallace (1989).

Vertical IEF was performed, using a Mighty Small II SE 250 unit (Hoefer, San Francisco), at 4 °C. Gels (0·75 mm) were prefocused at 5 W, 500 V for 30 min and run at 20 W, 700 V for 4 h. The pH range was achieved by mixing LKB ampholytes (Pharmacia, Sweden), pH 3·5–10, pH 4–6 and pH 3–3·5 in a ratio of 1:2:3, respectively. The anode solution was 0·02 M-NaOH and the cathode solution was 0·02 M acetic acid.

The protein bands were electro-transferred to a PDVF membrane (Immobilon-P supplied by Millipore MA, USA) in 0·7% acetic acid for 1 h at 100 V. The membrane was blocked overnight in 3% gelatin in 20 mM Tris, 0·5 M-NaCl, 0·1% Tween 20; pH 7·5 (TST) then incubated for 1 h with polyclonal anti-

bodies, raised against MEP-1 in a rabbit (Phillips & Wallace, 1989), diluted 1:1000 with TST. After two 5 min washes with TST the membrane was incubated for another hour with goat anti-rabbit antibodies conjugated with alkaline phosphatase (Bio-Rad, Australia) diluted 1:1000 with TST. The membrane was sequentially washed 2 × 5 min in TST, 5 min in 20 mM Tris, 0·5 M-NaCl and 5 min in 100 mM Tris, 100 mM-NaCl, 5 mM-$MgCl_2$ pH 9·5. The colour reaction was produced by incubation for 5 min with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) supplied by Promega (WI, USA) and diluted to the manufacturers specifications. The membranes were then rinsed with TST and finally with distilled water before air drying.

The N-terminal sequence of MEP-1 (200 pmol) was determined by Edman degradation. The amino acids were separated by HPLC on an Applied Biosystems 475A protein sequencer. The analysis was done at the Department of Biochemistry, University of Adelaide.

3. Results and discussion

The main malt endopeptidase (MEP-1) that we have studied is a cysteine endopeptidase which accounts for over half of the hordein degrading activity in a 4-day green malt (Phillips & Wallace, 1989). The purified enzyme consists of two species with pI values 4·2 and 4·3. A 30000 M_r endopeptidase (EP-B) characterized by Koehler & Ho (1990) from germinating barley (cv. Himalaya) separates into two bands during IEF with pI values of 4·6 and 4·7. We have recently determined the N terminal amino acid sequence of MEP-1 for twenty residues and found it to be identical to that of EP-B. It is possible that MEP-1 and EP-B are the same enzyme for, although the reported pI values differ, we find a band in Himalaya identical to the endopeptidase in Schooner and Betzes after IEF and immunoblotting. However a complete amino acid sequence of MEP-1 is required for certainty. In the meantime we have adopted the gene symbol *Cep-B* for MEP-1.

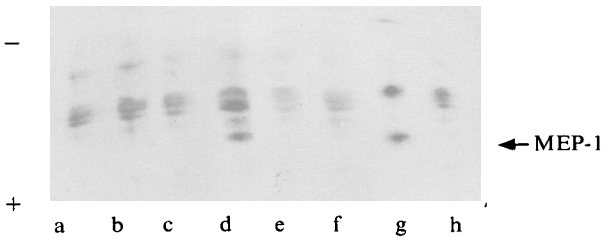

Fig. 1. Immunoblot of crude extracts of the disomic addition lines containing Betzes chromosome 7 (a), 6 (b), 4 (c), 3 (d), 2 (e) and 1 (f), Betzes (g) and Chinese Spring (h).

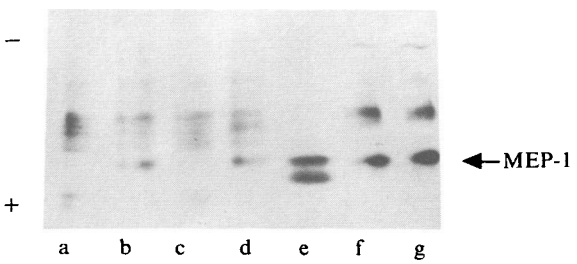

Fig. 2. Immunoblot of crude extracts of Chinese Spring (a), ditelosomic addition lines 3L (b) and 3S (c), disomic addition line 3 (d), purified MEP-1 (e), Schooner (f) and Betzes (g).

Disomic addition lines of Betzes barley chromosomes 1, 2, 3, 4, 6 and 7 were analysed by IEF and immunoblotting (Fig. 1). IEF was chosen over SDS–PAGE as it allowed the MEP-1 band to be differentiated from a wheat protein band of similar size which cross reacted with the polyclonal antibodies. The endopeptidase band is present at pI 4.3 in germinated seeds of Betzes barley and the addition line containing Betzes chromosome 3. Germination in the presence of 7 ppm gibberellic acid enhanced the level of endopeptidase (Jacobsen & Varner, 1967). MEP-1 was not detected in Chinese Spring or any of the other addition lines. It is therefore concluded that the controlling gene is present on Betzes chromosome 3.

The ditelosomic addition lines containing the Betzes chromosome 3 long arm (3L) and short arm (3S) were also analysed by IEF and immunoblotting (Fig. 2). The barley endopeptidase band appears in addition line 3L. Thus *Cep-B* has been assigned to the long arm of Betzes chromosome 3.

The polyclonal antibodies react with two bands in the MEP-1 sample purified from the barley cultivar Schooner, yet in crude extracts of both Betzes and Schooner cultivars only the upper band is detected. The lower band appears only in crude extracts stored at 0 °C for several days (data not shown). It is assumed that the second band which appears in purified MEP-1 (pI, 4·2) is an artefact of isolation or a product of hydrolysis, rather than another gene product.

The antibodies cross react with two other bands in Betzes at pI values of 4·7 and 5·4 and several bands in the Chinese Spring extracts. It is not known if these bands have a similar function to the endopeptidase or only a structural relationship.

A structural gene designated *EpH1* encoding a putative endopeptidase was located in Betzes chromosome 1 (Hart *et al.* 1980). Extracts from 7-day germinated coleoptiles were electrophoresed in horizontal starch gels and the endopeptidase was detected with benzoyl-L-arginine *p*-nitroanilide (BAPNA) and Black K stains. The endopeptidases Ep (Hart *et al.* 1980) and MEP-1 are not related as the latter does not degrade BAPNA (Phillips & Wallace, 1989). Indeed there is no substantiated evidence to suggest that

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enzymes which degrade BAPNA are true endopeptidases (Nishikata, 1984).

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