Endopeptidases of triticale seeds

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Abstract

The changes in endopeptidase activity in different parts of germinating triticale cv. Malno were investigated. Haemoglobin, gliadin, azocasein and azoalbumin were used as substrates. During the first day of germination the activity of haemoglobin hydrolyzing endopeptidases predominated while after the second day, mainly in the endosperm, a rapid increase in endopeptidases activity preferring gliadin hydrolysis was observed. In all the investigated tissues azocaseinolytic activities increased with the successive days of germination. Similar changes were observed using azoalbumin with one exception: in the embryo axis this activity decreased with the progression of germination. Separation of endopeptidases on the *DEAE Sepharose CL-6B* reveals three activity peaks in extract from dry seeds and four peaks in extract from 3 d germinated seeds. The obtained peaks differed in substrate specificity and in sensitivities to class-specific inhibitors.

Additional key words: aspartyl, cysteine, hydrolytic specificity, serine endopeptidases, *Triticosecale*.

Introduction

During germination the embryos of cereal seeds use nitrogen accumulated in protein bodies in the aleurone layer and starchy endosperm. The mobilization of seed reserves is a gradual process controlled by plant growth regulators (Bethke *et al.* 1996). The structure of reserve proteins, particularly the N-sequence of the end of reserve proteins is responsible for the initiation of the first stage of the reserve protein degradation process, *i.e.* the limited proteolysis. The second stage is determined by the *de novo* synthesis of the secreted hydrolases both in the aleurone layer of the starchy endosperm (Fincher 1989, Bethke 1998) and in the *epithelium* directly adhering to the endosperm (Fincher 1989). In the final stage, the released hydrolases degrade the endosperm reserve proteins. Products of this hydrolysis are transported to the embryo and utilized further by the developing root and shoot tissues.

In cereal seeds two groups of endopeptidases seem to play a significant role. The aspartyl proteinases participate in the accumulation of reserve proteins in the forming seeds (Nguyen *et al.* 1995b). During the seed development the reserve proteins are mostly synthesized as pre-proteins and aspartyl proteinases probably participate in their maturation directly prior to their "storage" (Multu and Gal 1999). Their relatively high activity is also observed, besides carboxypeptidase activities, during the first stage of germination (Bielawski *et al.* 1994). With the onset of germination activity of aspartyl proteinases decreases concomitantly with the marked increase in the activity of cysteine proteinases (Multu and Gal 1999). It may point that aspartyl proteinases initiate degradation of reserve proteins mainly, preceding *de novo* synthesis of cysteine proteinases, responsible for degradation of reserve proteins (Voigt *et al.* 1997). On such a physiological role of cysteine proteinases indicate their substrate specificity towards the prolamines, the main reserve protein in cereal seeds: barley hordeine (Zhang and Jones 1996) and wheat gliadin (Bottari *et al.* 1996). To our best knowledge, the most studies completed so far clarified the specificity and the role of various endopeptidases in germinated grains of barley and wheat, while the proteinases of seed of remaining cereal species including triticale remain unknown.

The aim of the present work was investigation of changes of the endopeptidase activities in various parts of germinating triticale against different substrates including gliadin. The obtained data together with the results of the

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Abbreviations: *p*-CMB - *p*-chloromercuribenzoate; DFP - diisopropylfluorophosphate; EDTA - ethylenediaminetetraacetic acid; 2-ME - 2 mercapto-ethanol; TCA - trichloroacetic acid.
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separation of endopeptidases from dry and germinating seeds and with the determined effect of specific inhibitors on enzyme activity allow giving an initial understanding of the role of various proteinases in protein degradation during triticale germination.

Materials and methods

Plants: Triticale (*Triticosecale* cv. Malno) mature seeds were germinated at 22 °C and relative humidity of almost 100 %. After 48 and 72 h the seeds were divided into the coleoptile, seminal roots, scutellum and endosperm. Dry seeds and seeds after 24 h germination were divided into embryonic axis and endosperm only.

Extraction: Plant tissues were homogenized in 0.1 M acetate buffer, pH 5.2, containing 5 mM 2-ME (m/v 1:5). Then the homogenate was extracted for 30 min in the ice bath and centrifuged at 15 000 *g* for 20 min. The obtained supernatant was dialysed in 0.01 M acetate buffer with 5 mM 2-ME at 4 °C overnight.

Determination of endopeptidase activity: Endopeptidase activities were determined according to Casano *et al.* (1989) using 0.7 % azocasein or azoalbumin in 0.1 M acetate buffer at pH 5.2 with 5 mM 2-ME. The endopeptidase activity with haemoglobin and gliadin was done according to Kołaczkowska *et al.* (1983) with the following modifications: the incubation mixture contained 0.02 - 0.2 cm^3 of extract supplied to the volume of 1 cm^3 with 2 % haemoglobin in 0.1 M acetate buffer, pH 3.6, or 1 % gliadin in 0.1 M acetate buffer, pH 4.3. Both buffers contained 5 mM 2-ME. After 2 h incubation at 37 °C the reaction was interrupted by 0.5 cm^3 12 % TCA and after 10 min it was centrifuged at 15 000 *g* for 20 min.

Ion-exchange chromatography: The column (40 cm long, 2.0 cm i.d.) formed with *DEAE Sepharose CL-6B* was equilibrated with 50-mM acetate buffer at pH 4.6 with 5 mM 2-ME. The enzyme extracts were brought to 80 % saturation with $(NH_4)_2SO_4$, dialyzed overnight against the equilibrating buffer and then sample of 100 mg protein was loaded onto the column. After the nonadsorbed proteins were washed from the column with equilibration buffer, the adsorbed materials were eluted with a linear 360 cm^3 gradient of NaCl (0→0.4 M) in the same buffer. Fractions of 6 cm^3 were collected at flow rate of 50 cm^3 h^{-1} and those containing maximum endopeptidase activity were concentrated with an *Amicon* (*Millipore Corporation*, Bedford, USA) ultrafiltration cell.

Electrophoresis and gelatin-agarose assay: Electrophoretic analysis of concentrated active fractions (20 - 80 µg protein) was carried out under the nondenaturating conditions according to Laemmli (1970), using 7.5 % polyacrylamide slab gel. Directly after electrophoresis, the polyacrylamide gel was placed with flat surface down onto the gelatin-agarose plate prepared according to Miller and Huffaker (1981) using 0.1 M acetate buffer (pH 5.2) instead 0.2 M K-phosphate buffer (pH 6.0). The both plates were incubated at 37 °C for 4 h. After incubation the substrate plate was stained with the Amido Black (*Sigma*, St. Louis, USA).

Results and discussion

The endopeptidase activity changes in different parts of dry and germinating triticale seeds were examined using haemoglobin or gliadin (Fig. 1) and azocasein or azoalbumin (Fig. 2) as substrates. The obtained results showed that after 24 h germination the gliadin endopeptidase activity is significantly lower than the haemoglobin degrading activity particularly in the endosperm. It points to various substrate preferences of endopeptidases from different parts of the seed. Similar observations were made by Galleschi *et al.* (1989) who reported that aspartyl proteinases from germinating ×*Hyanaldoticum sardoum* were inactive against gliadin although they hydrolyzed haemoglobin. Similarly, the aspartyl proteinases from barley seeds did not hydrolyze hordein, the main reserve protein in barley (Kervinen *et al.* 1993) whereas wheat aspartyl proteinase hydrolyzed gliadin (Belozersky *et al.* 1989).

On the second day of germination the gliadin degrading activity rapidly increased particularly in the endosperm (Fig. 1*B*). It may indicate the involvement of these endopeptidases in degradation of prolamines, the main reserve protein of starchy endosperm of cereal seeds. Jacobsen and Varner (1967) were the first to demonstrate that protease active against wheat gliadin are cysteine proteinases synthesized *de novo* in barley aleurone layers. Similar tendences in cysteine proteinases activity in germinated triticale grains (Nguyen *et al.* 1995a) and, in mRNA of main barley cysteine proteinase (EPB) responsible for the degradation of reserve prolamines (Mikkonen *et al.* 1996) were observed. Within the first day of germination the EPB mRNA was synthesized in the *epithelium* and since the second day in the aleurone layer of the starchy endosperm, whereas the EPB mRNA in the aleurone on the first day of

germination was absent.

On the second and third day an increasing gliadinolytic activity in the embryonic axis/scutellum was observed. It may mean that endopeptidases present in these parts of the seed participate in degradation of prolamines accumulated in the starchy endosperm. The obtained data also confirm numerous reports that the site of synthesis of these endopeptidases is the *scutellum* and alurone of the germinating cereal seeds (Fincher 1989, Bethke *et al.* 1998). A non-significant activity decrease in the *scutellum* on the fourth day corresponds with the decreased mRNA level coding the EPB in the barley epithelium after the second day after the onset of germination (Mikkonen *et al.* 1996).

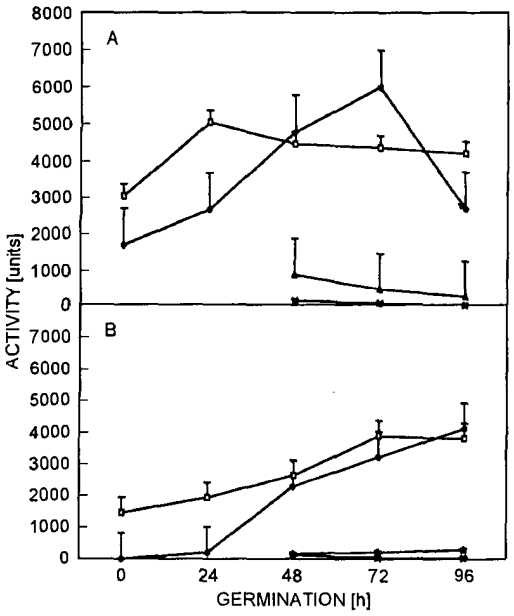

Fig. 1. The changes of endopeptidase activity with haemoglobin (*A*) and gliadin (*B*) as substrates in the endosperm (*rhombs*), the embryonic axis/scutellum (*squares*), seminal roots (*triangles*) and coleoptile (*crosses*) during the germination of triticale seeds. One unit of activity was defined as 0.01 increase of A_{280} after 1 h per g(d.m.). Results are means ± SE of three independent determinations.

In all investigated parts of the seeds, the azocaseinolytic activity increased (Fig. 2*A*) on the successive days of germination. Similar changes in the azoalbumin activity of endopeptidases present in the germinating seeds were observed although activity of this enzyme in embryo axis decreased on the successive days of germination (Fig. 2*B*). It seems that a synthesis of endopeptidases with high substrate specificity towards azocasein and low against azoalbumin takes place in the *scutellum*.

As a result of *DEAE Sepharose CL 6B* separation of endopeptidases from dry seeds and after 3 d of germination (Fig. 3) fraction not adsorbed on the column (*peak A*) was obtained with the endopeptidase activity.

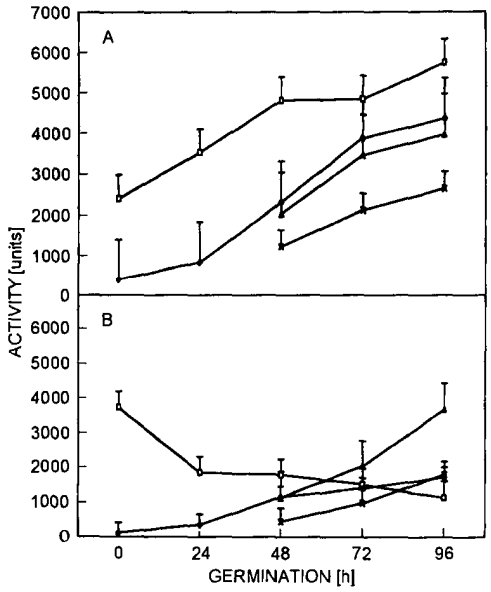

Fig. 2. The changes of endopeptidase activity with azocasein (*A*) and azoalbumin (*B*) as substrates in the endosperm (*rhombs*), the embryonic axis/scutellum (*squares*), seminal roots (*triangles*) and coleoptile (*crosses*) during the germination of triticale seeds. One unit of activity was defined as 0.01 increase of A_{340} after 1 h per g(d.m.). Results are means ± SE of three independent determinations.

Another two peaks of activity eluted by the NaCl gradient for the first extract and three for the second (*B, C* and *D*) were observed. The *peaks B* and *C* are particularly interesting because the former peak corresponded to the main activity in extracts from dry grains, while the latter one to the highest activity in the seeds after three days of germination. It has been shown that the gliadin hydrolyzing activity, which dominates in the 3 d germinating seeds (*peak C*) is nearly twice higher thanazocasein and haemoglobin degrading activity. The endopeptidases activity corresponds to the *peaks B* and *D*

Table 1. Effects of class-specific proteinase inhibitors on the azocasein-hydrolysing activity of triticale seed endopeptidases from fractions obtained by ion-exchange chromatography (see Fig. 3). The enzyme was preincubated in the presence of an inhibitor at the of 30 °C for 1 h. The reaction started by the addition of a substrate and was carried out at the of 37 °C for 2 h.

Inhibitor	[mM]	Relative activity [% of control]			
		A	B	C	D
Control	-	100	100	100	100
Iodoacetamide	1.0	89	100	3	95
p-CMB	0.1	94	88	2	98
$HgCl_2$	0.05	83	82	0	96
Pepstatin A	0.1	80	10	100	90
DFP	1.0	24	96	98	101
EDTA	10.0	100	101	105	100

showed the highest activities against azocasein while those which did not bind on the anion exchanger (*peak A*) were the only ones which preferred haemoglobin. Fractions of these peaks were subjected to electrophoresis under non-denaturing conditions (Fig. 5) and endopeptidases from obtained peaks (except *A* - too low activity against gelatin, data not shown) are seen as multiple bands differ to each other with their electrophoretic mobility.

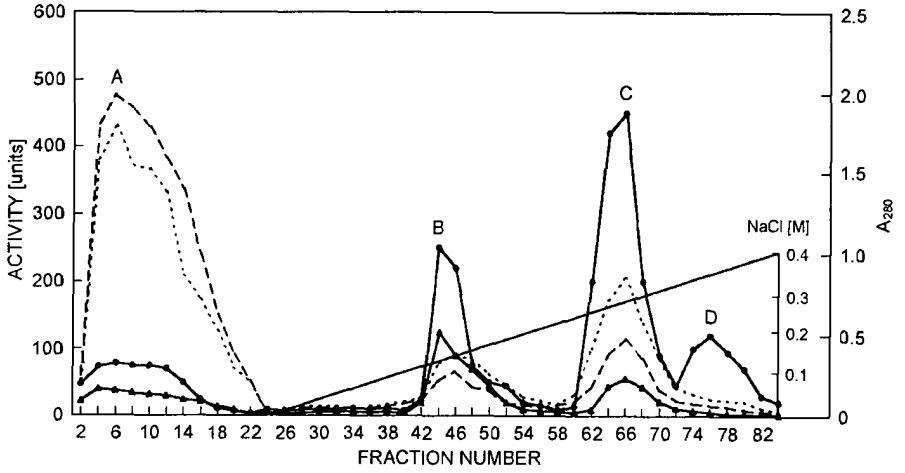

Fig. 3. Separation of dormant and germinated triticale endopeptidases by DEAE-Sepharose CL 6B ion-exchange chromatography. Proteolytic activity was assayed with azocasein. One unit of activity was defined as causing 0.01 increase of A_{340} during 1 h per 1 cm^3 (*triangles* - dormant seeds endopeptidases activities, *circles* - germinated seeds endopeptidases activities, *long-dashed line* - A_{280} (dormant seeds), *short-dashed line* - A_{280} (germinated seeds).

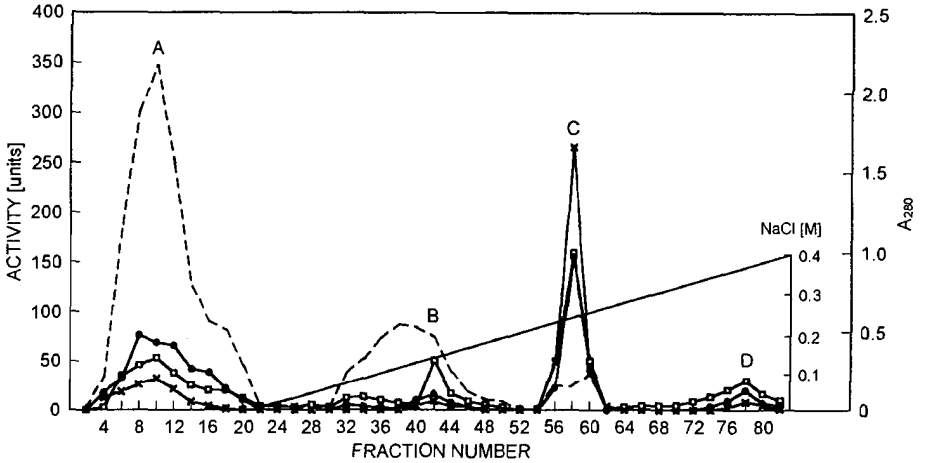

Fig. 4. Separation of germinated triticale endopeptidases by DEAE Sepharose CL 6B ion-exchange chromatography. Activity measurement with azocazein (*squares*), hemoglobin (*circles*), and gliadin (*crosses*) as substrats; *dashed line* - A_{280}. One unit of azocaseinolytic activity was defined as in Fig. 3; haemoglobinolytic and gliadinolytic activity as 0.01 increase of A_{280} after 1 h per 1 cm^3.

Specific proteinase inhibitors (Table 1) allowed the preliminary identification and classification of endopeptidases separated on the *DEAE Sepharose CL-6B* to particular groups of proteinases based on their active site catalytic mechanism. Non adsorbed endopeptidases (*peak A*), were the most sensitive to the inhibitor of serine proteinases, DFP. The inhibitors of cysteine proteinases: iodoacetamide, *p*-CMB, $HgCl_2$ inhibited the endopeptidase activity to a small extent. The pepstatin A, an inhibitor of aspartyl proteinases caused slight 20 % effect while the specific inhibitor of metaloproteinases, EDTA was ineffective. These findings reveal the presence of the serine proteinase which activity dominates in the *peak A*. Endopeptidases eluted from the column at a low NaCl concentration (*peak B*) were insensitive to the of iodoacetamide and EDTA and were inhibited to the small extent by $HgCl_2$ and *p*-CMB. DFP decreased their activity only by a few per cent. A strong inhibition of the endopeptidase activity by pepstatin A points to a significant part of aspartyl proteinases in these fractions. The results presented above confirm previous observations (Kervinen *et al.* 1993, Nguyen *et al.* 1995a,

Multu and Gal 1999) that either activity of aspartyl endopeptidases or contribution to the total endopeptidases activity in the resting seeds is dominant. Thus it should be regarded as the enzymes initiating the seed storage proteins breakdown (Belozersky *et al.* 1989, Voigt *et al.* 1997).

A total inhibition by $HgCl_2$ and nearly total inhibition by *p*-CMB and the iodoacetamide of the activities of endopeptidases eluted by the NaCl gradient (*peak C*) with the simultaneous lack of effect of the remaining inhibitors undoubtedly seems to point to the presence of cysteine proteinases. Since the cysteine proteinase represented the bulk of the activity of the germinating triticale seeds and preferred gliadin as a substrate (Fig. 4, Table 1), it appears to play a key role in the degradation of the storage protein presented in the starchy endosperm. Proteinases with a similar specificity have been isolated from germinating wheat seeds (Shutov and Vaintraub 1987, Bottari *et al.* 1996) and barley seeds (Marttila *et al.* 1995, Zhang and Jones 1996)

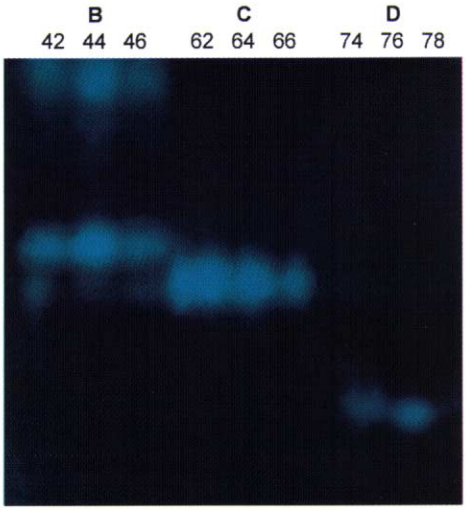

Fig. 5. Degradation of gelatin by endopeptidases from fractions obtained by anion-exchange chromatography of germinated triticale seeds (see Fig. 4) and separated in PAGE as described in Materials and methods.

The effect of inhibitors on the activity of endopeptidases eluted at the highest, nearly 0.5 M concentration of NaCl (*peak D*) is unequivocal. Out of all the inhibitors used only the pepstatin A caused slight but significant effect. These endopeptidases were present only in the extract from seeds after 3 d of germination (Fig. 3) and were characterized by low substrate specificity towards gliadin (Fig. 4). These results do not allow to classify these endopeptidases precisely and to understand their physiological function.

The presented results needs to be for further investigated aiming at identification, characteristics and determination of the functions of particular endopeptidases represented by the four peaks of activity obtained after the separation on *Sepharose CL-6B*.

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