Plant Physiology, January 2000, Vol. 122, pp. 137–145, www.plantphysiol.org © 2000 American Society of Plant Physiologists

Prediction of Protein Cleavage Sites by the Barley Cysteine Endoproteases EP-A and EP-B Based on the Kinetics of Synthetic Peptide Hydrolysis[1]

Anne Davy, Mikael Blom Sørensen[2], Ib Svendsen, Verena Cameron-Mills*, and David J. Simpson

Carlsberg Research Laboratory (A.D., M.B.S., V.C.-M.), Department of Chemistry (I.S.), and Department of Physiology (D.J.S.), Carlsberg Laboratory, Gamle Carlsbergvej 10, DK–2500 Valby, Denmark

Hordeins, the natural substrates of barley (*Hordeum vulgare*) cysteine endoproteases (EPs), were isolated as protein bodies and degraded by purified EP-B from green barley malt. Cleavage specificity was determined by synthesizing internally quenched, fluorogenic tetrapeptide substrates of the general formula 2-aminobenzoyl-P_2-P_1-P_1'-P_2' 1-tyrosine(NO_2)-aspartate. The barley EPs preferred neutral amino acids with large aliphatic and nonpolar (leucine, valine, isoleucine, and methionine) or aromatic (phenylalanine, tyrosine, and tryptophan) side chains at P_2, and showed less specificity at P_1, although asparagine, aspartate, valine, and isoleucine were particularly unfavorable. Peptides with proline at P_1 or P_1' were extremely poor substrates. Cleavage sites with EP-A and EP-B preferred substrate sequences are found in hordeins, their natural substrates. The substrate specificity of EP-B with synthetic peptides was used successfully to predict the cleavage sites in the C-terminal extension of barley β-amylase. When all of the primary cleavage sites in C hordein, which occur mainly in the N- and C-terminal domains, were removed by site-directed mutagenesis, the resulting protein was degraded 112 times more slowly than wild-type C hordein. We suggest that removal of the C hordein terminal domains is necessary for unfolding of the β-reverse turn helix of the central repeat domain, which then becomes more susceptible to proteolytic attack by EP-B.

Proteases play a central role in the mobilization of energy reserves in the germinating barley (*Hordeum vulgare*) grain, providing the embryo with a supply of amino nitrogen in the form of free amino acids and short peptides. Multiple representatives of the Ser protease, Asp protease, metalloprotease, and Cys protease families have been detected in the germinating barley grain, where the Cys proteases are the most abundant (Zhang and Jones, 1995). Two Cys endoproteases (EPs) EP-A and EP-B are expressed in the scutellum epithelium and aleurone tissue of the germinating grain and are secreted into the starchy endosperm (Marttila et al., 1993). Enzymatic studies of purified EP-A and EP-B showing in vitro digestion of the barley hordein polypeptides provide evidence for their role in storage protein degradation during germination (Koehler and Ho, 1990). The hordein polypeptides, comprising sulfur-poor and sulfur-rich families, share the distinctive properties of solubility in alcohol-water mixtures and domains of Pro/Gln-rich repeated sequences (Shewry and Tatham, 1990). Detailed characterization of EP-A and EP-B has largely been based on the cleavage of non-native substrates (Jones and Poulle, 1990; Zhang and Jones, 1996), which may not provide a direct insight into the specific enzymatic properties associated with degradation of the hordein polypeptides.

As a first step in dissecting the proteolytic events within the germinating grain, we studied the proteolytic cleavage of a single recombinantly expressed member of the sulfur-poor C hordein polypeptide family by EP-A and EP-B (Davy et al., 1998). The two EPs showed cleavage site specificity, with a strong preference for Phe, Leu, or Val in the P_2 position. (The substrate positions are denoted P_i,. . . . ,P_2, P_1, P_1',P_2',. . . . ,P_j', in correspondence with the binding subsites S_1, etc., according to Schechter and Berger [1967].)

Conformational studies of members of the hordein polypeptides and the closely related wheat prolamins indicate that intermolecular disulfide bonds between sulfur-rich hordein polypeptides and extensive hydrogen bonding between the repetitive domains of the sulfur-poor hordeins are involved in their assembly into protein bodies in the endosperm vacuole of the developing grain (Tatham and Shewry, 1995; Shimoni and Galili, 1996). Studies in wheat suggest that a NADP/thioredoxin system together with specific proteases are required for the mobilization of the prolamin storage proteins (Kobrehel et al., 1992). We have now investigated the ability of EP-B to hydrolyze the hordein polypeptides in their native state in barley protein bodies and the requirement for a reducing agent.

Synthetic fluorogenic peptides, based on native substrate sequences, provide a powerful tool to examine the cleavage site specificity of EP-B and EP-A. We have exploited this technology by synthesizing a series of peptide substrates to examine systematically the substrate preference of barley Cys EPs at the P_2 and P_1 sites and compare them with papain, a well-characterized plant Cys EP. We then examined the ability of synthetic substrate cleavage rates to predict the cleavage of wild-type and mutated C hordein

[1] A.D. is in receipt of a studentship from the Danish Academy of Technical Sciences.

[2] Present address: Laboratoire de Reproduction et Développement des Plantes, Unité Mixte de Recherche 9938 Centre National de la Recherche Scientifique/Institut National de la Recherche Agronomique/Ecole Normale Supérieure, 46, Allée d'Italie, F–69364 Lyon cedex 07, France.

* Corresponding author; e-mail verena@biobase.dk; fax 45–3327–4766.

polypeptides. Since barley Cys EPs are believed to mediate the release of β-amylase from the bound form during germination, we have selected this enzyme as an additional native substrate. Our investigations reveal that the amino acid residues at the P_2 and P_1 sites define the cleavage sites of barley Cys EPs and that elimination of these sites from a protein substrate can drastically reduce its rate of hydrolysis.

MATERIALS AND METHODS

Purification of EP-A and EP-B

EP-A and EP-B were purified from air-dried (45°C) green malt, provided by Carlsberg A/S, Copenhagen, and titrated as previously described (Davy et al., 1998). Papain (twice recrystallized), Brij 35, and buffers were purchased from Sigma (St. Louis). The purity of papain was established by SDS-PAGE and N-terminal amino acid sequencing, which revealed a single protein species.

Protein Body Isolation and Degradation by EP-B

Protein bodies were isolated from developing endosperm from 10 barley (*Hordeum vulgare* cv Alexis) spikes harvested 20 DPA using a modification of the published method (Cameron-Mills, 1980). The homogenized endosperms were resuspended in 20 mL of buffer A (20 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES], pH 7.6, 100 mM NaOAc, and 5 mM $MgCl_2$). The suspension was layered onto a 5-mL 1.75 M Suc cushion made up in buffer A and centrifuged for 2 min at 500*g*. Material banding on top of the Suc cushion was collected and resuspended in buffer B (20 mM HEPES, pH 7.6, 100 mM NaOAc, 5 mM EDTA, and 0.25 M Suc). This suspension was loaded onto a Percoll step gradient in a 10-mL Corex tube (2 mL each, 1.08 and 1.13 g/m^3) and centrifuged at 9,000 rpm in an SS34 rotor. The protein bodies banding on top of the 1.13 g/m^3 layer were collected in 250 μL and stored at −20°C.

Protein bodies were diluted 10 times in water, and 6 μL of the diluted suspension was incubated at 25°C with 10 μL of assay buffer (50 mM NaOAc, pH 4.5, and 0.05% Brij 35), 4 μL of EP-B 44 nM solution, and different concentrations of β-mercaptoethanol. A 10-μL aliquot was taken after 2 and 24 h and immediately mixed with 3 μL of sample buffer containing SDS kept at 80°C on a heating block and heated for 3 min. The samples were run on 12% acrylamide Tris-Gly gels under reducing conditions, according to the method of Laemmli (1970) on a mini-gel apparatus (Protean II, Bio-Rad Laboratories, Hercules, CA) and stained with Coomassie Brilliant Blue G250 according to the method of Neuhoff et al. (1988).

Synthesis of Internally Quenched Fluorogenic Substrates

Fluorogenic substrates were of the general formula Abz-$(Xaa)_n$-Tyr(NO_2)-Asp-OH, where Abz (2-aminobenzoyl) is the fluorescent group, 3-nitrotyrosine (Tyr[NO_2]) is the quencher, Xaa is any of the genetically encoded amino acids, and $n = 4$ to 8 (Meldal and Breddam, 1991). Peptides were synthesized on a solid support ($Pega_{1900}$) by manual multiple column peptide synthesis (Meldal et al., 1993) in a 20-column library synthesizer (Meldal, 1994). $Pega_{1900}$ resin was derivatized with a base-labile hydroxymethyl benzoic acid linker using *O*-benzotriazol-1-yl-*N,N,N'*, I*N'*-tetramethyl uronium tetrafluoroborate (TBTU) (Knorr et al., 1989).

The C-terminal residue 9-fluorenylmethyloxycarbonyl (Fmoc)-Asp(*t*-butyl)-OH was attached to the resin by esterification with 1-mesithylenesulfonyl-3-nitro-1,2,4-triazol and *N*-methylimidazol (Blankemeyer-Menge et al., 1990). The resin was washed with dimethylformamide (DMF) and deprotected with 20% (v/v) piperidine in DMF as previously described (Meldal et al., 1993). The columns were washed with DMF, and Fmoc-Tyr(NO_2)-OH was preactivated with TBTU, and 4-ethyl morphine for 5 min before addition to each column. The peptide was assembled using Fmoc-Xaa-pentafluorylphenyl esters (3 equivalents) with 3,4-dihydro-4-oxo-1,2,3,-benzotriazo-3-yl-OH catalyst (1 equivalent), and 20% (v/v) piperidine was used for deprotection. The amino acid side chain protections used were trityl (Asn, Cys, Gln, and His), *t*-butyl (Asp, Glu, Ser, Thr, and Tyr), *t*-butyloxycarbonyl (Lys), and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Arg). The last residue added was *t*-butyloxycarbonyl-Abz-O-3,4-dihydro-4-oxo-1,2,3,-benzotriazo-3-yl (3 equivalents).

The resins were washed with DMF, deprotected, washed with DMF and dichloromethane, and dried by airflow. The protection groups were cleaved off using a mixture containing 87.5% (v/v) trifluoroacetic acid, 2.5% (v/v) ethanedithiol, 5% (v/v) thioanisole, and 5% (v/v) water, and the resins were washed with 95% (v/v) acetic acid, 5% (v/v) diisopropylethylamine in DMF, DMF, and finally dichloromethane. The resins were dried by airflow, and the peptides were cleaved off with 0.1 M NaOH for 2 h and washed out with water. The solutions were neutralized with 0.1 M HCl and lyophilized. The peptides were dissolved in DMF and the insoluble sodium chloride was removed. Purity and identity were confirmed by HPLC, amino acid analysis, and matrix-assisted laser-desorption ionization time of flight mass spectroscopy.

Determination of Enzyme Activity and Kinetic Constants

The enzymatic hydrolysis of the peptide substrates was followed on a luminescence spectrometer (LS 50, Perkin-Elmer, Foster City, CA). The assay, titration, and determination of kinetic constants for EP-A, EP-B, and papain were performed as previously described (Davy et al., 1998). The activity of EP-A and EP-B was assayed with Abz-Phe-Arg-Gln-Gln-Tyr(NO_2)-Asp from pH 2.5 to 7.5 in 100 mM citrate-phosphate containing 2 mM Cys and 2 mM β-mercaptoethanol with 5 μL of EP-B (0.24 μM) or 5 μL of EP-A (0.135 μM).

Heterologous Expression and Purification of β-Amylase

The entire coding sequence of barley β-amylase (accession no. X52321) was amplified by PCR from the plasmid

pCβC51 (Kreis et al., 1987) with tailed primers (5′-GACGACGACAAGATGGAGGTGAACGTG-3′, 5′-GAGGAGAAGCCCGGTTTACATGGTGGC-AGG-3′). The amplified sequence was purified and inserted in-frame into the expression vector pET-32 LIC (Novagen, Madison, WI) by ligation-independent cloning according to the supplier's recommendations. The resulting plasmid (pET32-βAmy) contains an open reading frame of 2,079 bp, encoding a fusion protein of 77 kD comprising the 60-kD β-amylase fused with an N-terminal 17-kD tag (thioredoxin·Tag, His·Tag, and S·Tag), which facilitates solubilization and purification of the expressed protein.

pET32-βAmy was transformed into BL21 (DE3); 0.5-L cultures (Luria-Bertani + 100 mg/L ampicillin) were started from single colonies, grown with vigorous agitation at 37°C until OD_{600} = 0.7, and subsequently transferred to 20°C upon overnight induction of expression with 1 mM isopropylthiogalactoside. The tagged β-amylase was purified under native conditions by metal-affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Valencia, CA) as recommended by the manufacturer. Cells were resuspended in 50 mM phosphate buffer, pH 8.0, 300 mM NaCl, lysed by sonication (3 × 30 s), and the supernatant was loaded onto the Ni-NTA matrix after centrifugation at 10,000*g* for 20 min. The column was washed with approximately 10 volumes of the loading buffer, followed by 50 mM phosphate buffer, pH 6.0, and 300 mM NaCl until the OD_{280} of the eluate was below 0.1. The β-amylase was eluted with 50 mM NaOAc buffer, pH 5.2, and 300 mM NaCl, and the purified fusion protein was treated with enterokinase using the S Tag rEK Purification Kit (Novagen) to remove the N-terminal tag. The purified protein was stored at −20°C following the addition of glycerol to a final concentration of 10%. β-amylase activity was assayed using the Betamyl kit (Megazyme International, Bray, County Wicklow, Ireland) according to the manufacturer's instructions.

Effect of EP-B on β-Amylase

Four micrograms of β-amylase in 20 μL of 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, buffer was incubated overnight with 10 μL of EP-B assay buffer and 20 μL of 44 nM EP-B at 4°C. The samples (10 μL of tag-β-amylase, β-amylase, and β-amylase treated with EP-B) were run on 12% acrylamide high-Tris gels under reducing conditions according to the method of Fling and Gregerson (1986). Twenty microliters of β-amylase treated with EP-B was subjected to N-terminal sequencing, as previously described (Davy et al., 1998).

Site-Directed Mutagenesis of a C Hordein Gene

Mutations were introduced into a C hordein genomic sequence using an in vitro mutagenesis system (Altered Sites II, Promega, Madison, WI). The C hordein coding sequence in λ-hor1-17 (Entwistle et al., 1991), cloned in a pET-3d expression vector (Tamas et al., 1994), was excised with *Bam*HI and *Xba*I restriction enzymes and cloned into the polylinker of the plasmid pALTER-1. The following five antisense mutagenic oligonucleotides, with mutated nucleotides shown in italics and bold, were designed to anneal to the C hordein coding sequence introducing mutations into the encoded protein: 1,5′-GGTGATTGC-***G***ACTCTTGGC-3′; 2,5′-TATGGCTGCTGC***GA***ATATGATT-GTTGT-3′; 3,5′-ACGGTTGCTGC***GA***TGGAAATGGTTT-3′; 4,5′-GGTTGCTGG***G***AAATTATTTG-3′; 5,5′-TGTTGGCGG-***G***ATGGTTGTTG-3′. Mutant plasmids transformed into JM101 were purified and their nucleotide sequence determined by dideoxy chain termination using the Ampli Cycle Sequencing kit (Perkin-Elmer) and a sequencer (model 373A, Applied Biosystems, Foster City, CA).

Following successive rounds of mutation, a mutated C hordein gene in pALTER-1, encoding a C hordein polypeptide with 12 amino acid substitutions, was selected for expression studies. The mutant C hordein gene was amplified by PCR and cloned into the pET-32 LIC expression vector supplied by Novagen, using the following tailed sense and antisense primers: pET32Chor-s 5′-GACGACGACAAGATGAGGCAACTAAACCCT-3′ and pET32Chor-as 5′-GAGGAGAAGCCCGGTCTAGACCATACTCCA-3′. The sequence of the mutant C hordein gene in pET32 LIC was confirmed by sequencing.

C Hordein Expression and Purification

Wild-type C hordein was expressed and purified as previously described (Davy et al., 1998). The mutated C hordein coding sequence cloned in the pET 32 expression vector, was expressed in BL21 (DE3) host cells as a fusion protein having N-terminal His-tag and S-tag domains. Five-hundred milliliters of Luria-Bertani medium containing 100 μg/mL ampicillin was inoculated with a single colony of transformed BL21 cells and incubated at 37°C; when the OD_{600} reached 0.6, cells were induced with isopropylthiogalactoside at 0.4 mM and incubated for 4 h at 37°C. The cells were harvested by centrifugation at 4,000g for 20 min and resuspended in lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 10 mM imidazole). After incubation with lysozyme (1 mg/mL) on ice for 30 min, the cells were disrupted with a sonicator and centrifuged at 10,000*g* for 30 min at 4°C. The supernatant was loaded onto Ni-NTA agarose (Qiagen) and eluted under native conditions according to the supplier's instructions. The eluted protein was further purified with the S-Tag rEK Purification Kit (Novagen). The bound C hordein was eluted from the S-Tag agarose column by cleavage of the S-Tag with enterokinase, according to the supplier. The purified mutated C hordein was freeze-dried and stored at −20°C until used.

Assay of C-Hordein and Mutant C-Hordein Degradation by EP-B

Purified wild-type C hordein was dissolved in EP-B assay buffer to a concentration of 1 μg/μL. Twenty-seven microliters of C hordein solution was incubated with 115 μL of assay buffer and 2 μL of 0.27 μM EP-B solution at 40°C. Purified mutant C hordein was dissolved in assay buffer to 0.2 μg/μL and 90 μL of the solution was incu-

bated with 4.6 μL of assay buffer and 1.3 μL of 0.27 μM EP-B solution at 40°C. To follow the degradation process, 16-μL aliquots were taken at different time points and immediately mixed with 4 μL of sample buffer containing SDS kept at 80°C on a heating block and incubated for 3 min. The mutant C hordein degradation was repeated with the same amount of substrate and 15 times more EP-B. The samples were run on 16% (w/v) acrylamide high-Tris gels under reducing conditions according to the method of Fling and Gregerson (1986), and stained with Coomassie Brilliant Blue G250 according to the method of Neuhoff et al. (1988). The level of C hordein was quantified by photographing the gels with a digital camera and using ImageQuant software to integrate pixel densities.

RESULTS

EP-B Hydrolysis of Native Hordein Polypeptides

Protein bodies were isolated from developing barley endosperm, following homogenization of the tissue and centrifugation on Suc and Percoll step gradients. The prolamin fraction of barley, comprising the alcohol-soluble storage proteins of the grain, is composed of four polypeptide classes localized in the protein bodies. SDS-PAGE of the protein body polypeptides (Fig. 1) revealed sulfur-rich B and γ-hordeins with M_rs of 36,000 to 42,000, sulfur-poor C hordeins with M_rs of 45,000 to 70,000, and HMW D hordein with a M_r of 90,000.

The polypeptide composition of the protein bodies following a 2-h incubation with purified EP-B showed a rapid hydrolysis of D hordein. The degradation of C, B, and γ-hordein was strongly enhanced by the addition of the reducing agent β-mercaptoethanol at concentrations of 10 mM and above. However, all of the hordein polypeptides in the protein body were accessible to EP-B degradation in the absence of disulfide reduction, judging by the appearance of low-molecular-mass peptides of between 20 and 30 kD after a 2-h incubation and almost complete hordein degradation after 24 h. Two minor polypeptides were detected in the protein body preparation after the 24-h incubation, suggesting that they are poor substrates for EP-B hydrolysis.

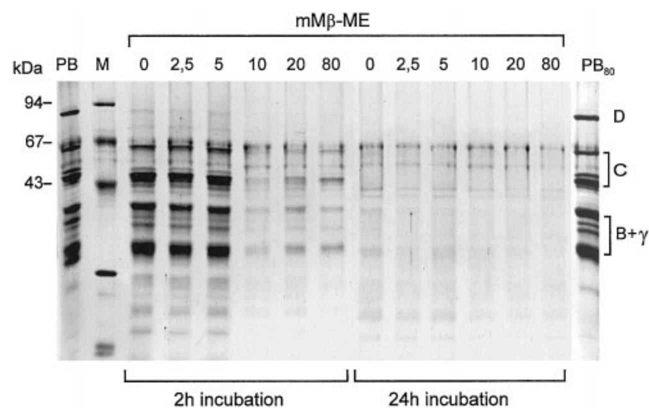

Figure 1. SDS-PAGE showing degradation of hordein in isolated protein bodies by purified EP-B. Samples were incubated at 20°C for 2 or 24 h, with varying amounts of added β-mercaptoethanol (β-ME), the concentration of which (in mM) is shown above each lane. Protein body controls (PB) are with 0 and 80 mM added β-mercaptoethanol after 24 h of incubation without EP-B. Molecular mass markers (M) are in kD. The positions of B, C, and D hordeins are indicated on the right.

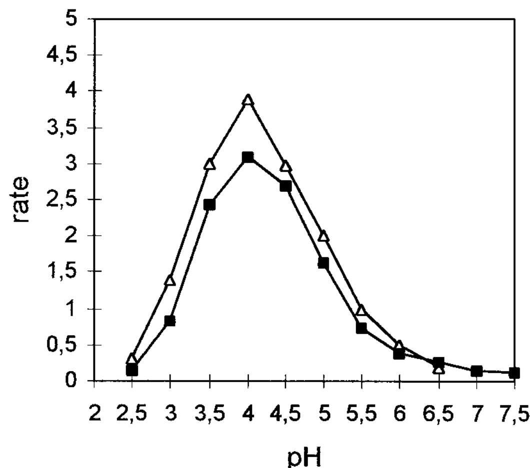

Figure 2. pH optima for EP-A and EP-B were determined by measuring the rate of hydrolysis of Abz-Phe-Arg-Gln-Gln-Tyr(NO_2)-Asp from pH 2.5 to 7.5 in 100 mM citrate-phosphate containing 2 mM Cys and 2 mM β-mercaptoethanol. △, EP-B; ■, EP-A.

Systematic Study of Cys EP Substrate Specificity Using Synthetic Substrates

A series of synthetic fluorogenic substrates were synthesized to make a systematic analysis of the importance of the residue at the P_2 and P_1 positions. Substrates with 19 different amino acid residues at the P_2 position were synthesized with the general sequence Abz-Xaa-Arg-Gln-Gln-Tyr(NO_2)-Asp. Previous studies had shown that this sequence with Phe in the P_2 position, based on an EP-B cleavage site in C hordein, was the best synthetic substrate for barley EPs (Davy et al., 1998). Peptide cleavage by the Cys EPs EP-A, EP-B, and papain was assayed at three substrate concentrations under pseudo-first-order conditions ($\ll K_m$), and the concentration of active enzyme was determined by titration with trans-epoxysuccinyl-L-leucylamido-(4-guanido)butane. The pH optimum for EP-A and EP-B was 4.0 (Fig. 2), but assays were performed at pH 4.5 to reduce protonation of the amino group of Abz, which decreases its fluorescence (Meldal and Breddam, 1991). The derived second-order kinetic constants k_{cat}/K_m for each substrate are shown in Table I.

The order of preference for the amino acid residue at the P_2 site in the substrate was very similar for the two barley EPs, EP-A and EP-B, where neutral amino acids with large aliphatic and nonpolar (Leu, Val, Ile, and Met) or aromatic (Phe, Tyr, and Trp) side chains were present in substrates giving the highest k_{cat}/K_m values. Substrates with small, polar, or charged side chains in the P_2 position had the lowest k_{cat}/K_m values. While the substrate preference of papain at the P_2 site largely reflected that of EP-A and EP-B, the range of k_{cat}/K_m values displayed by papain was

Table I. *Kinetic constants (k_{cat}/K_m) for the hydrolysis by plant Cys endoproteases of substrates with substitution at the P_2 position*

P_2 P_1 ↓ P_1' P_2' [a]	k_{cat}/K_m		
Xaa-Arg ↓ Gln-Gln	EP-A	EP-B	Papain
	mM^{-1} s^{-1}		
Leu	5,220 ± 290	16,300 ± 510	45.4 ± 0.8
Phe	6,280 ± 230	8,580 ± 100	294 ± 4
Tyr	2,670 ± 150	5,650 ± 190	303 ± 16
Trp	549 ± 8	1,200 ± 120	28.0 ± 3.2
Val	1,200 ± 40	983 ± 33	49.5 ± 1.0
Met	962 ± 42	875 ± 30	126 ± 0.8
Ile	404 ± 6	532 ± 4	53.4 ± 1.5
Cys	81 ± 2	89 ± 5	51.6 ± 2.6
Arg	49 ± 3	57.9 ± 1.2	1.45 ± 0.01
His	9.2 ± 0.3	52.3 ± 1.6	4.52 ± 0.06
Thr	7.6 ± 0.5	16.8 ± 0.8	9.57 ± 0.18
Asp	17 ± 2	16.6 ± 0.8	0.20 ± 0.007
Glu	47 ± 4	14.6 ± 1.2	0.70 ± 0.01
Gln	10.6 ± 0.4	11.4 ± 1.5	5.44 ± 0.20
Ala	10.1 ± 0.4	9.7 ± 0.3	12.7 ± 0.3
Lys	14.3 ± 0.5	9.0 ± 0.7	0.96 ± 0.02
Asn	7.6 ± 0.2	14.6 ± 1.2	1.08 ± 0.02
Gly	1.9 ± 0.4	7.2 ± 0.4	0.21 ± 0.02
Ser	4.8 ± 0.2	3.3 ± 0.1	3.90 ± 0.05

[a] ↓ indicates cleavage site.

Table II. *Kinetic constants (k_{cat}/K_m) for the hydrolysis by plant Cys endoproteases of substrates with substitution at the P_1 position*

P_2 P_1 ↓ P_1' P_2' [a]	k_{cat}/K_m		
Leu-Xaa ↓ Gln-Pro	EP-A	EP-B	Papain
	mM^{-1} s^{-1}		
Arg	4,150 ± 120	9,230 ± 440	124 ± 6
Met	1,600 ± 60	7,500 ± 330	152 ± 4
Leu[b]	4,600 ± 210	7,270 ± 410	62.6 ± 2.6
Phe	1,490 ± 80	7,270 ± 320	66.3 ± 3.6
Tyr[b]	5,300 ± 230	6,360 ± 260	14.3 ± 1.1
Gln	1,170 ± 75	5,960 ± 100	64.6 ± 1.1
Thr[b]	6,020 ± 260	5,930 ± 110	54.5 ± 1.4
Lys[b]	11,400 ± 380	5,920 ± 200	80.5 ± 1.9
Glu	822 ± 38	5,340 ± 120	6.86 ± 0.04
Gly	600 ± 34	4,260 ± 130	232 ± 1
Ala	527 ± 8	3,950 ± 130	79.3 ± 0.6
Ser	824 ± 41	3,350 ± 140	39.0 ± 1.1
Cys	340 ± 14	2,490 ± 130	128 ± 2
His	875 ± 60	1,320 ± 40	17.3 ± 0.4
Trp	830 ± 10	1,220 ± 50	18.2 ± 0.3
Asn	212 ± 5	838 ± 10	16.1 ± 0.5
Asp	213 ± 3	690 ± 23	0.50 ± 0.02
Val	174 ± 3	588 ± 31	3.51 ± 0.07
Ile	72 ± 3	372 ± 21	2.64 ± 0.02
Pro	ND[c]	ND	2.9 ± 0.2

[a] ↓ indicates cleavage site. [b] Determined from Hanes-Woolf plots (see Table III). [c] ND, Not detected (<3).

significantly smaller than that of the barley EPs. The preference of papain for Phe or Tyr at P_2 was much greater than for other aromatic or nonpolar residues. It was not possible to test Pro in the P_2 position of this substrate due to quenching of Abz fluorescence when Pro is immediately adjacent (Ito et al., 1998). Another substrate (Abz-Gln-Pro-Gln-Gln-Pro-Tyr(NO_2)-Asp had previously been synthesized and was a very poor substrate for EP-B (Davy et al., 1998).

A further 20 substrates were synthesized with amino acid substitutions at the P_1 position of the general sequence Abz-Leu-Xaa-Gln-Pro-Tyr(NO_2)-Asp, based on the best substrate from the P_2 series. A Pro residue (instead of Gln) was placed at P_2' to direct cleavage between Xaa and Gln, since it was known that cleavage would not occur with Pro at P_1' (Davy et al., 1998). The cleavage site of the substrates was confirmed by N-terminal sequencing. The k_{cat}/K_m values of the different substrates for EP-A and EP-B were not strongly influenced by the residue at the P_1 position, with the exception of Pro, where cleavage was not detectable (Table II), and the range of k_{cat}/K_m values was much less than found for the P_2 substrate series. The best substrates for EP-B contained nonpolar or charged residues at P_1 (Arg, Met, Leu, Phe, Tyr, Gln, Thr, Lys, and Glu), while those with small residues (Ala, Gly, Ser, and Cys) were slightly poorer, and Asn, Asp, Val, and Ile gave the lowest k_{cat}/K_m values.

The substrate preference profile for EP-A at P_1 was similar to that of EP-B, except that there was a clear preference for positively charged residues (Lys and Arg). For four residues (Lys, Thr, Tyr, and Leu), K_m values were so low that k_{cat}/K_m values were calculated from Hanes-Woolf plots (Table III). In general, k_{cat}/K_m values for a particular substrate were lower for EP-A compared with EP-B, particularly for Ala, Cys, Glu, and Ile. In contrast, papain showed a preference for Gly at P_1, followed by Met, Cys, and Arg. When the k_{cat}/K_m values for EP-A and EP-B were expressed as a ratio of the corresponding values for papain, substrates with Asp, Glu, and Tyr at P_1 were seen to be significantly better substrates for EP-A and EP-B than papain (Table II). Similarly, substrates with Leu at P_2 were better substrates for EP-A and EP-B than papain (Table I).

The substrate preferences seen from the results with synthetic peptides, particularly at P_2, should provide a basis for predicting cleavage sites in native protein substrates. This concept was tested with β-amylase and site-specific mutants of C hordein.

C-Terminal Processing of Barley β-Amylase by EP-B

The C-terminal cleavage by barley EPs, which releases barley β-amylase from a bound form, was investigated with expressed recombinant barley β-amylase and EP-B. The barley β-amylase coding sequence, cloned in the plasmid pET32-βAmy, was expressed as a 77-kD fusion pro-

Table III. *Kinetic constants for the hydrolysis by EP-A of substrates with substitution at the P_1 position*

Residue	K_m	k_{cat}	k_{cat}/K_m
	μM	s^{-1}	mM^{-1} s^{-1}
Lys	1.06	12.10	11,400
Thr	0.21	1.28	6,020
Tyr	0.29	1.52	5,300
Leu	0.26	1.18	4,600

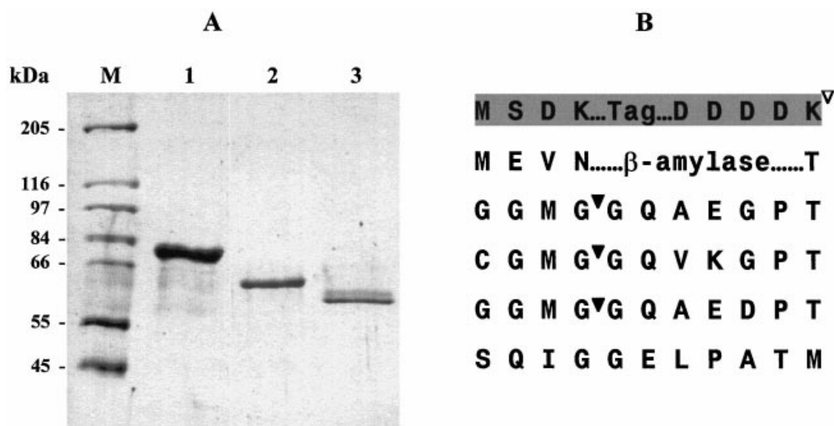

Figure 3. A, SDS-PAGE of N-tag-β-amylase, β-amylase, and β-amylase treated with EP-B. N-Tag-β-amylase was expressed and purified from *E. coli* (lane 1), and the N-terminal tag was cleaved with enterokinase (lane 2) and then incubated with purified EP-B (lane 3). B, Partial sequence of β-amylase formatted to emphasize the 11-residue domain, repeated four times in the C-terminal 45 residue extension, showing cleavage sites of enterokinase (▽) and EP-B (▼). The latter were determined by amino acid sequencing of the products released from β-amylase after incubation with EP-B.

tein, with an N-terminal tag of 17 kD fused to the 60-kD β-amylase to facilitate purification.

The expressed β-amylase fusion protein (approximately 0.8 μg) was analyzed by SDS-PAGE (Fig. 3A) following purification (lane 1), N-terminal tag cleavage with enterokinase (lane 2), and incubation with EP-B for 16 h at 4°C (lane 3). Following enterokinase treatment, the β-amylase migrated as a single 60-kD polypeptide, indicating complete cleavage of the fusion protein. N-terminal amino acid sequencing confirmed the correct N-terminal sequence of the 60-kD β-amylase: MEVN (Fig. 3B). The 60-kD β-amylase was further cleaved by EP-B, giving polypeptides of approximately 56 to 58 kD. N-terminal amino acid sequence analysis of the peptides released by EP-B digestion revealed that the EP-B cleaved the sequence MG ↓ GQ at three sites located near the C terminus of the 60-kD β-amylase polypeptide (Fig. 3B), accounting for the digestion products seen by SDS-PAGE. The absence of additional peptide sequences indicates that only the C-terminal domain of the native 60-kD β-amylase is accessible to proteolytic attack, and that the three sites cleaved by EP-B are a reflection of EP-B substrate specificity.

Figure 4. Deduced amino acid sequence of wild-type recombinant C hordein (left), showing primary (⬇) and secondary (↓) cleavage sites. The sequence has been formatted to emphasize the repeat domain. The sequence on the right shows the mutant C hordein after removal of the primary cleavage sites (⇓). Point mutations are indicated by shading and include some residues not at primary cleavage sites due to multiple primer annealing events.

Mutant C Hordein Polypeptide with Reduced Susceptibility to EP-B Degradation

The expression of a C hordein polypeptide in *Escherichia coli* and its purification and folding into a native conformation enables the proteolytic cleavage of a single hordein substrate to be analyzed. EP-B initiates C hordein proteolysis by cleavage at a few primary sites, followed by cleavage at a large number of secondary sites, to yield peptides of four to 15 residues (Davy et al., 1998). To investigate the importance of the primary cleavage sites for C hordein degradation, a mutant C hordein polypeptide was expressed in which the primary EP-B cleavage sites were eliminated by substituting a Ser residue at the P_2 position (see Table I). Five mutagenic primers were used to mutate the C hordein nucleotide sequences encoding the five primary cleavage sites. Because of the highly repetitive C hordein gene sequence, some multiple primer annealing events were unavoidable. The amino acid sequence of the mutant C hordein selected for expression is shown alongside the wild-type protein in Figure 4, with mutations at all five cleavage sites. The additional mutations induced by primer 5 were not localized at primary cleavage sites.

The purified mutant and wild-type C hordein polypeptides were incubated with EP-B and samples were taken over 40 min for SDS-PAGE analysis. The wild-type

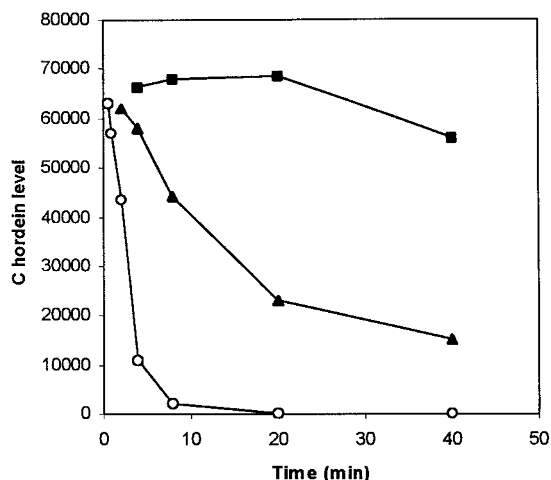

Figure 5. Time course of recombinant C hordein degradation by EP-B. Wild-type C hordein (○) is degraded rapidly, with intermediate products. Mutant C hordein, lacking the primary cleavage sites, is only slightly degraded after 40 min with the same amount of EP-B as for wild type (■), and requires 15 times more EP-B (▲) before significant degradation can be seen. No intermediate degradation products were seen.

polypeptide was initially cleaved at the primary cleavage sites, releasing a characteristic set of peptide fragments, followed by complete degradation within 40 min. The mutant polypeptide remained largely intact throughout the incubation. Degradation by EP-B was monitored by quantifying the Coomassie-stained full-length C hordein protein in SDS-PAGE (Fig. 5). Digestion of the mutant polypeptide following the addition of 15-fold more units of EP-B indicated that the mutant is susceptible to EP-B hydrolysis. Wild-type C hordein was degraded 7.5 times faster than the mutant form, with 1/15 as much EP-B, so that the mutant was degraded 112 times slower than wild-type C hordein. The absence of detectable discrete cleavage products suggested that the mutant C hordein was randomly cleaved at secondary cleavage sites.

DISCUSSION

The purified barley Cys EP EP-B is able to degrade hordeins in vitro, even when they are presented in the form of insoluble protein bodies. Our results confirm earlier results in planta (Marchylo et al., 1986), which indicated that D hordein is degraded most rapidly during malting. This may be due to its discrete localization in the reticular matrix at the periphery of storage protein bodies (Sørensen et al., 1996), and/or because D hordein is intrinsically a better substrate. The sulfur-rich hordeins, which account for 85% of barley storage proteins, form aggregates that are stabilized by inter- and intramolecular disulfide bonds (Rechinger et al., 1993). The ability of EP-B to degrade both the monomeric C hordein and the aggregated B hordein suggests that protein-to-protein interactions within the protein body are sufficiently weak to allow their rehydration and proteolytic degradation. The addition of β-mercaptoethanol increases the rate of hordein degradation, but is not essential. By reducing hordein disulfide bonds, particularly intermolecular disulfide bonds, β-mercaptoethanol may increase the solubility of the hordeins and their accessibility to EP-B. This function is probably performed during germination by thioredoxin *h* and NADPH (Kobrehel et al., 1992) or by glutathione and Cys (Pheifer and Briggs, 1995), which are secreted by the embryo.

Two series of fluorogenic, internally quenched peptide substrates differing at either P_1 or P_2 were synthesized and used to determine the substrate specificity of EP-A and EP-B, with papain as a reference. Papain is one of the best-studied Cys EPs, but its specificity has been primarily determined from the cleavage products of small polypeptides, rather than by measuring k_{cat}/K_m values for a series of synthetic peptides. The variation in k_{cat}/K_m values at P_2 was 3,300- to 5,000-fold, which confirms that the substrate cleavage site of Cys EPs is primarily determined by the nature of the residue at P_2. In contrast, the range of k_{cat}/K_m values at P_1 indicates a broad specificity in this position, with the exception of Pro, which resulted in very slow cleavage. Furthermore, our results show that Tyr and Phe are the best residues at P_2 for papain, while Gly is almost twice as effective as Arg at P_1. For the substrate series used in this work, all three proteases are able to discriminate between Leu and Ile or Val at P_1 by a factor of 10 to 20, and also between Gln and Asn and Glu and Asp (Table II). Papain does not distinguish between Leu, Val, and Ile at P_2 (Table I), unlike EP-A and EP-B. The substrates VRQQ, which is better for EP-A than EP-B, and LEQP, which is better for EP-B than EP-A, could be used to measure the levels of each of these proteases when both are present in crude extracts of malt (germinating) barley.

These data should also enable cleavage sites to be predicted in protein sequences, not taking secondary structure effects into account. Barley β-amylase has an extension of 45 residues at the C terminus compared with soybean, and this tail is presumably readily accessible to protease attack, since the crystal structure of soybean β-amylase shows the C terminus on the surface of the molecule (Adachi et al., 1998). If the repeat sequence TGGMGGQAEGPT (Fig. 3B) is analyzed for EP-B cleavable sites, taking into account that residues Thr, Glu, Gln, Ala, and Gly at P_2 and Pro at P_1 and P_1', are extremely unfavorable (Table I), then the only predicted cleavage would occur at MG ↓ GQ, which corresponds to the experimentally determined site (Fig. 3B). Cleavage was not detected at two other potential cleavage sites within the C-terminal 45 residues (VK ↓ GP and IG ↓ GE). Other potential EP-B cleavage sites are present in the rest of the β-amylase sequence, but are presumably protected by their tertiary structure, as found for native versus heat-treated lipid transfer protein (Davy et al., 1999).

EP-A and EP-B were generally found to have higher k_{cat}/K_m values for a given synthetic substrate than papain, which is primarily due to lower K_m values, particularly for EP-A (Davy et al., 1998). Four substrates investigated here (LKQP, LTQP, LYQP, and LLQP) have K_m values of 1 μM or less (Table III). The structural basis for this difference is impossible to deduce, since mature EP-A and EP-B are only 50% identical to papain, but they may reflect the way in

which EP-A and EP-B have evolved to degrade the Gln- and Pro-rich repeats found in the hordeins. Compared with papain, EP-A and EP-B show a marked preference for Leu at P_2, and for Asp, Glu, and Tyr at P_1, although Asp and Glu are not common in hordeins. An examination of B hordein sequences (Shewry and Tatham, 1990) shows numerous occurrences of potential EP-A and EP-B primary cleavage sites with Leu, Phe, or Val at P_2 and Arg, Gln, Glu, or Gly at P_1 (LR ↓ TL, LE ↓ AT, VE ↓ GV, FQ ↓ QP, etc.); D hordein (Halford et al., 1992) contains similar sites (LQ ↓ QG, LG ↓ QG, LG ↓ SL, VG ↓ QL, etc.).

In vitro studies have shown that EP-B initiates the degradation of C hordein by cleaving at five primary cleavage sites whose sequences closely mirror its substrate specificity with synthetic substrates. C hordein is predominantly composed of an octameric repeat enriched in Pro and Gln residues which, in the dry grain, is believed to have a rod-shaped structure stabilized by hydrogen bonding between Gln residues. Upon hydration, the octameric repeat domain is proposed to assume an extended structure, with an equilibrium between a β-reverse turn helix and a poly-L-Pro II-like conformation (Tatham and Shewry, 1995). These conformational changes may facilitate access of EP-B to the primary cleavage sites and may explain why hordeins in their native state are so susceptible to proteolytic degradation by EP-B. Four out of the five EP-B primary cleavage sites are located near the N and C termini of the C hordein polypeptide, where the repeat structure is less conserved. Following primary site cleavage, C hordein is rapidly degraded by EP-B at multiple secondary sites. When hydrolysis of the primary sites is prevented, as in our mutated C-hordein, the central domain retains its native conformation and is relatively inaccessible. The absence of discrete fragments upon cleavage of the mutated C-hordein indicates that the initial cleavage occurs at a random secondary site. Our results indicate that the N and C termini stabilize the conformation of the central helical domain, which, upon cleavage of the primary sites, becomes more accessible to cleavage at secondary sites.

ACKNOWLEDGMENTS

We thank Professor Peter Shewry for the gift of the C hordein expression vector and Suksawad Vongvisuttikun for C hordein site-directed mutagenesis and DNA sequencing. We are grateful to Bodil Corneliussen and Lone Sørensen for assistance with amino acid sequencing and matrix-assisted laser-desorption ionization time of flight mass spectroscopy, and to Professor Morten Meldal, Hanne Christiansen, and Kirsten Lilja for help and advice with peptide synthesis and HPLC. Ann-Sofi Steinholz and Nina Rasmussen are thanked for help with some of the figures.

Received July 26, 1999; accepted September 10, 1999.

LITERATURE CITED

Adachi M, Mikami B, Katsube T, Utsumi S (1998) Crystal structure of recombinant soybean beta-amylase complexed with beta-cyclodextrin. J Biol Chem **273:** 19859–19865

Blankemeyer-Menge B, Nimtz M, Frank R (1990) An efficient method for anchoring Fmoc-amino acids to hydroxyl-functionalised solid supports. Tetrahedron Lett **31:** 1701–1704

Cameron-Mills V (1980) The structure and composition of protein bodies purified from barley endosperm by silica sol density gradients. Carlsberg Res Commun **45:** 557–576

Davy A, Svendsen I, Bech L, Simpson D, Cameron-Mills V (1999) LTP does not represent a cysteine endoprotease inhibitor in barley grains. J Cereal Sci **30:** 237–244

Davy A, Svendsen I, Sorensen SO, Sorensen MB, Rouster J, Meldal M, Simpson DJ, Cameron-Mills V (1998) Substrate specificity of barley cysteine endoproteases EP-A and EP-B. Plant Physiol **117:** 255–261

Entwistle J, Knudsen S, Muller M, Cameron-Mills V (1991) Amber codon suppression: the in vivo and in vitro analysis of two C-hordein genes from barley. Plant Mol Biol **17:** 1217–1231

Fling SP, Gregerson DS (1986) Peptide and protein molecular weight determination by electrophoresis using a high-molarity Tris buffer system without urea. Anal Biochem **155:** 83–88

Halford NG, Tatham AS, Sui E, Daroda L, Dreyer T, Shewry PR (1992) Identification of a novel beta-turn-rich repeat motif in the D hordeins of barley. Biochim Biophys Acta **1122:** 118–122

Ito AS, Turchiello RD, Hirata IY, Cezari MH, Meldal M, Juliano L (1998) Fluorescent properties of amino acids labeled with ortho-aminobenzoic acid. Biospectroscopy **4:** 395–402

Jones BL, Poulle M (1990) A proteinase from germinated barley. II. Hydrolytic specificity of a 30-kilodalton cysteine proteinase from green malt. Plant Physiol **94:** 1062–1070

Knorr R, Trzeciak A, Bannwarth W, Gillessen D (1989) New coupling reagents in peptide chemistry. Tetrahedron Lett **30:** 1927–1930

Kobrehel K, Wong JH, Balogh A, Kiss F, Yee BC, Buchanan BB (1992) Specific reduction of wheat storage proteins by thioredoxin *h*. Plant Physiol **99:** 919–924

Koehler SM, Ho T-HD (1990) A major gibberellic acid-induced barley aleurone cysteine proteinase which digests hordein. Plant Physiol **94:** 251–258

Kreis M, Williamson M, Buxton B, Pywell J, Hejgaard J, Svendsen I (1987) Primary structure and differential expression of beta-amylase in normal and mutant barleys. Eur J Biochem **169:** 517–525

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Marchylo BA, Kruger JE, Hatcher D (1986) High-performance liquid chromatography and electrophoretic analysis of hordein during malting for two barley varieties of contrasting malting quality. Cereal Chem **63:** 219–231

Marttila S, Porali I, Ho T-HD, Mikkonen A (1993) Expression of the 30 kD cysteine endoprotease B in germinating barley seeds. Cell Biol Int **17:** 205–212

Meldal M (1994) Multiple column synthesis of quenched solid-phase bound fluorogenic substrates for characterization of endoprotease specificity. Methods **6:** 417–424

Meldal M, Breddam K (1991) Anthranilamide and nitrotyrosine as a donor-acceptor pair in internally quenched fluorescent substrates for endopeptidases: multicolumn peptide synthesis of enzyme substrates for subtilisin Carlsberg and pepsin. Anal Biochem **195:** 141–147

Meldal M, Holm CB, Bojesen G, Jacobsen MH, Holm A (1993) Multiple column peptide synthesis. Int J Pept Protein Res **41:** 250–260

Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivityusing Coomassie Brilliant Blue G-250 and R-250. Electrophoresis **9:** 255–262

Pheifer JH, Briggs DE (1995) Thiols and disulphides in quiescent and germinating barley grains, both dormant and mature. J Inst Brew **101:** 85–93

Rechinger K-B, Simpson DJ, Svendsen I, Cameron-Mills V (1993) A role for γ3 hordein in transport of prolamins to the vacuole in developing barley endosperm. Plant J **4:** 841–853

Schechter I, Berger A (1967) On the size of the active site in proteases. I. Papain. Biochem Biophys Res Commun **27:** 157–162

Shewry PR, Tatham AS (1990) The prolamin storage proteins of cereal seeds: structure and evolution. Biochem J **267:** 1–12

Shimoni Y, Galili G (1996) Intramolecular disulfide bonds between conserved cysteines in wheat gliadins control their deposition into protein bodies. J Biol Chem **271:** 18869–18874

Sørensen MB, Muller M, Skerritt J, Simpson D (1996) Hordein promoter methylation and transcriptional activity in wild-type and mutant barley endosperm. Mol Gen Genet **250:** 750–760

Tamas L, Greenfield J, Halford NG, Tatham AS, Shewry PR (1994) A beta-turn rich barley seed protein is correctly folded in *Escherichia coli*. Protein Exp Purif **5:** 357–363

Tatham AS, Shewry PR (1995) The S-poor prolamins of wheat, barley and rye. J Cereal Sci **22:** 1–16

Zhang N, Jones BL (1995) Characterization of germinated barley endoproteolytic enzymes by two-dimensional gel electrophoresis. J Cereal Sci **21:** 145–153

Zhang N, Jones BL (1996) Purification and partial characterization of a 31-kDa cysteine endopeptidase from germinated barley. Planta **199:** 565–572