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The cysteine endoproteases (EP)-A and EP-B were purified from green barley (Hordeum vulgare L.) malt, and their identity was confirmed by N-terminal amino acid sequencing. EP-B cleavage sites in recombinant type-C hordein were determined by N-terminal amino acid sequencing. Tetrapeptide substrates of the general formula 2-aminobenzoyl-P2-P1-P1′,P2′-tyrosine(NO2)-aspartic acid, in which cleavage occurs between P1 and P1′, showed that the cysteine EPs preferred phenylalanine, leucine, or valine at P2. Arginine was preferred to glutamine at P1, whereas proline at P2, P1′, or P1′ greatly reduced substrate kinetic specificity. Enzyme cleavage of C hordein was mainly determined by the primary sequence at the cleavage site, because elongation of substrates, based on the C hordein sequence, did not make them more suitable substrates. Site-directed mutagenesis of C hordein, in which serine or proline replaced leucine, destroyed primary cleavage sites. EP-A and EP-B were both more active than papain, mostly because of their much lower K_m values.

Two EPs known to play a central role in the breakdown of barley (Hordeum vulgare L.) endosperm storage proteins (hordeins) are Cys EPs designated EP-A (Koehler and Ho, 1990a) and EP-B (Koehler and Ho, 1988). They are secreted by the scutellum and aleurone layer into the starchy endosperm during germination in response to GA_3 (Koehler and Ho, 1990b; Marttila et al., 1995). EP-B has an apparent molecular mass of 30 kD, is identical to MEP-1 (Phillips and Ho, 1990b; Marttila et al., 1995). EP-A and EP-B have been shown to digest hordein (Phillips and Wallace, 1989), and has a possible homolog with an apparent molecular mass of 31 kD (Zhang and Jones, 1996). The substrate specificity of this EP-B has been determined from cleavage patterns of small proteins such as hordothiolins, which are stored as compact protein bodies within the vacuoles of endosperm cells, comprise four major classes: B, C, D, and γ. Purification of the hordein polypeptides reveals that they are complexed in larger aggregates by intermolecular disulfide bonds between Cys residues present in B, D, and γ hordein. Circular dichroism spectroscopy and small-angle x-ray-scattering studies of purified hordein polypeptides and synthetic oligopeptides indicate that C and D hordeins are rod-shaped molecules in an extended β-turn helix structure (Halford et al., 1992; I’Anson et al., 1992). The ability to express a recombinant C hordein and to refold it to its native conformation (Tamas et al., 1994) provides the first opportunity, to our knowledge, to study the degradation of a native substrate by a barley CYS EP. We have used this system to determine the Cys EP cleavage sites in C hordein. Furthermore, we have designed internally quenched, fluorogenic peptide substrates (Meldal and Breddam, 1991) based on the C hordein cleavage sequences to determine substrate specificity and the effect of increasing substrate length.

MATERIALS AND METHODS

Air-dried (45°C) green malt was obtained from Carlsberg A/S, Copenhagen, Denmark.

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1 This work was supported by the Danish Academy of Technical Sciences (A.D. received a studentship). This is Adapting barley for Industrial Needs publication no. 159.

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Abbreviations: Abz, 2-aminobenzoyl; AMC, 7-amido-4-methylcoumarin; CBZ, benzoylcarbonyl; E-64, trans-epoxysuccinyl-l-leucylamido-(4-guanido)butane; EP, endoprotease; Fmoc, fluoren-9-ylmethoxycarbonyl; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; Tyr(NO2), 3-nitrotyrosine; Xaa, unspecified amino acid.
Chemicals
E-64, β-mercaptoethanol, papain (2× recrystallized), N-CBZ-Phe-Arg-AMC, Cys, and buffers were purchased from Sigma.

Synthesis of Internally Quenched Fluorogenic Substrates
Fluorogenic substrates were of the general structure Abz-(Xaa)n-Tyr(NO2)-Asp-OH, where the fluorescent group is Abz, the quencher is Tyr(NO2), Xaa is any of the genetically encoded amino acids, and n = 4 to 11 (Meldal and Breddam, 1991). Peptides were synthesized on solid support (Pega1900 resin) using an Applied Biosystems 432A peptide synthesizer. Normal amino acids were introduced under (1-H-benzotriazol-1-yl)-1,2,3,3-tetramethyluronium hexafluorophosphate activation. The fluorogenic amino acids were incorporated using Fmoc-Tyr(NO2) and t-butyloxy carbonyl-Abz-O,3,4-dihydro-4-oxo-1,2,3-benzo triazo-3-yl prepared as previously described (Meldal and Breddam, 1991). During each cycle the Fmoc group was removed by piperidine prior to the addition of each new amino acid. The final product was cleaved from the resin by treatment with 95% trifluoroacetic acid, washed with 95% acetic acid, concentrated in a rotary evaporator, washed with diethyl ether, and freeze dried. Purity and identity were confirmed by HPLC, amino acid analysis, and MALDI-TOF MS.

EP-A and EP-B were isolated from green malt by the modification of published methods (Phillips and Wallace, 1989; Koehler and Ho, 1988, 1990a). The 35 to 75% (NH₄)₂SO₄ fraction was applied to an S-200HR column. The active fractions were pooled and dialyzed overnight against 20 mM NaC₂H₃O₂, pH 4.5, 2 mM Cys, and 2 mM β-mercaptoethanol. The initial fluorescence (I₀) was measured. Values were confirmed by measuring Iₗₜ after the addition of subtilisin and/or trypsin to the same reaction mixture. The kₗₒ/Kₗₜ values were determined using the relation:

which is valid at low substrate concentrations (sₜ ≪ Kₗₜ) for systems that obey Michaelis-Menten kinetics.

SDS-PAGE and Blotting
SDS-PAGE was performed under reducing conditions in a mini-gel apparatus (Protean II, Bio-Rad) using 16% acrylamide, high-Tris gels according to the method of Fling and Gregerson (1986). Protein-containing bands were visualized by staining with 0.03% Coomassie brilliant blue R250 dissolved in 10% TCA and 40% methanol, or by silver staining. For N-terminal amino acid sequencing, proteins were blotted onto a PVDF membrane (Immobilon-P, Millipore) using a semidy electroblotter (Aricos, Ølstykke, Denmark) and the Tris/6-amino hexanoic acid buffer system recommended by the manufacturer. Bands were visualized by staining for a few seconds in Coomassie brilliant blue (see above), and were then cut out and subjected to N-terminal amino acid sequencing with a model 470A sequenator coupled to a model 120A phenylthiohydantoin analyzer (both Applied Biosystems). Total proteolysis of C hordein was carried out at 40°C for 60 min, and the products were separated by HPLC before analysis by N-terminal amino acid sequencing or MALDI-TOF MS.

Determination of Enzyme Activity and Kinetic Constants
Enzymatic hydrolysis of the peptide substrates was followed by observation of the change in substrate fluorescence upon addition of enzyme with a luminescence spectrofluorimeter (emission at 420 nm, 10-nm slit; excitation at 320 nm, 10-nm slit; model LS50, Perkin Elmer) at 25°C. The substrates were dissolved in dimethylformamide at concentrations of 50 to 200 μM, and 10 μL of the substrate was added to a cuvette containing 2.4 mL of assay buffer (50 mM NaC₂H₃O₂, pH 4.5, 2 mM Cys, and 2 mM β-mercaptoethanol). The initial fluorescence (I₀) was measure and the enzyme solution (final concentration, Eₜ) was added. The concentration of active enzyme was determined by titration with E-64 according to the method of Barrett and Kirschke (1981). At each of the three substrate concentrations (sₜ), the initial velocity for substrate cleavage (vₒ) was determined from the initial slope of the curve (emission versus time). The hydrolysis was allowed to proceed overnight and final fluorescence for total cleavage (Iₗₒ) was measured. Values were confirmed by measuring Iₗₜ 24 h after the addition of subtilisin and/or trypsin to the same reaction mixture. The kₗₒ/Kₗₜ values were determined using the relation:

The cleavage site for each substrate was determined by amino acid sequencing of the hydrolysis products obtained after the incubation of 1 μL of a concentrated solution of substrate with 1 μL of diluted enzyme in 45 μL of assay buffer for 3 h at 25°C.
Papain was activated in 100 mm sodium phosphate, pH 7.0, containing 10 mm Cys, 1 mm EDTA, and 0.01% Brij 35 for 5 min at 4°C. Assays were carried out in 50 mm sodium phosphate, pH 6.5, 2 mm Cys, 1 mm EDTA, and 0.08% Brij 35, as described by Ménard et al. (1990). N-CBZ-Phe-Arg-AMC was dissolved in dimethylformamide to make a stock at 7.7 mm, and assays were run at 25°C in a final volume of 2 mL, using an excitation at 360 nm and an emission at 460 nm. $k_{cat}/K_m$ values were calculated as above using 10-nm slits and substrate concentrations of about 200 nm, whereas $K_m$ values were calculated from Hanes plots of $s$ versus $s/v$ (where $s$ = substrate concentration and $v$ = initial rate) using 5-nm slits and substrate concentrations between 1 and 150 μM. The spectrofluorimeter was calibrated with known concentrations of AMC.

Nucleotide Sequencing and Site-Directed Mutagenesis

The nucleotide sequences of the C hordein coding sequence obtained from Tamas et al. (1994) and the EP-A cDNA clone were determined by dideoxy chain termination using the TaQ Dye Deoxy Terminator Cycle Sequencing kit and a sequencer (model 373A, Applied Biosystems). Site-directed mutagenesis of the primary cleavage site near the N terminus was carried out by splice-overlap PCR using the appropriate forward primer.

RESULTS


A protein with an apparent molecular mass of 30 kD was isolated in pure form from green barley malt after extraction in 50 mm NaC$_2$H$_3$O$_2$ buffer containing 2 mm Cys and 2 mm β-mercaptoethanol, and subsequent column chromatography. Its proteolytic activity during purification was followed by fluorometric assay with the internally quenched peptide substrate Abz-AFRFAA-Tyr(NO$_2$)PPD. This activity was inhibited by the Cys endopeptidase inhibitors E-64 and leupeptin. Its identity as EP-B was established on the basis of its N-terminal amino acid sequence, which was identical to that reported by Koehler and Ho (1990a). A second peak of protease activity, which eluted after EP-B on SP Sepharose, was purified by elution from Q Sepharose. It had an apparent molecular mass of 37 kD, and was identified as EP-A from the agreement of its N-terminal amino acid sequence with the published sequence (Koehler and Ho, 1988).

Determination of Primary Cleavage Sites

Pure C hordein, expressed in E. coli and folded in vitro to its native conformation (Tamas et al., 1994), was used as a substrate to determine EP-B cleavage sites. Recombinant C hordein was incubated for different times with purified EP-B and C hordein sequence organized to show its intrinsic octameric repeats (PQQXPaaPQQ), where Xaa = F, L, Y, I, S, etc., together with primary (\(\psi\)) and secondary (\(\phi\)) cleavage sites deduced from partial and total digestion with EP-B.

Figure 1. Coomassie blue-stained gel of the time course of recombinant C hordein digestion by purified EP-B. Numbers above each lane are minutes of incubation at 40°C. A similar gel was used to electroblot proteolytic bands onto PVDF for N-terminal amino acid sequencing to determine the original cleavage site in C hordein. C, Control (no EP-B); M, molecular mass markers.

Figure 2. Amino acid sequence of the recombinant C hordein deduced from the nucleotide sequence, with the major cleavage sites shown in bold and the scissile bond indicated by an asterisk. The sequence differs from that published by Entwistle (1988), which contains a tandem repeat of the sequence PQQASPLQPQ.

Figure 3. C hordein sequence organized to show its intrinsic octameric repeats (PQQXPaaPQQ), where Xaa = F, L, Y, I, S, etc., together with primary (\(\psi\)) and secondary (\(\phi\)) cleavage sites deduced from partial and total digestion with EP-B.
EP-B. Several discrete bands could be seen within the first 0.5 min of incubation, and the C hordein was fully degraded within 40 min (Fig. 1). The experiment was repeated on a larger scale, and the separated cleavage products were blotted onto a PVDF membrane for N-terminal amino acid sequencing to determine the sites within the C hordein sequence where EP-B cleaved most rapidly. The five major polypeptides had one of two N-terminal sequences: QPYPQNPYLPQKPFPVQ or QPFHTPQQYF-PYLP. Comparison of these sequences with that of C hordein (Fig. 2) revealed that initial EP-B cleavage sites occurred after Q-20 and Q-37. The size distribution of the cleavage products predicts at least two further primary cleavage sites closer to the C terminus, to account for the smaller polypeptides with N termini identical to the larger ones.

Sequencing of peptides after exhaustive C hordein cleavage by EP-B followed by HPLC revealed three relatively abundant peptides with the same C-terminal sequence: \( \text{QPLPQPQQPFR-199} \), indicating cleavage after R-199. Most of the initial C hordein cleavage products can be explained as being the result of attack by EP-B at one or more of these specific cleavage sites (Fig. 2). In view of the nonrandom proteolytic degradation of C hordein by EP-B, the initial cleavage sites may be defined as primary cleavage sites.

Determination of Secondary Cleavage Sites

After 40 min, EP-B cleaved C hordein into fragments too small to be resolved by SDS-PAGE, and these products were separated by HPLC for subsequent identification by MS and/or amino acid sequencing. This allowed us to identify 44 fragments that ranged from 2 to 26 residues, and, thus, the position of a further 35 cleavage sites in the C hordein polypeptide (Fig. 3). The C hordein sequence has been shown to emphasize the octameric repeat PQQPXaaPQQ, in which Xaa is usually a hydrophobic residue such as Phe, Val, Leu, or Tyr. These secondary cleavage sites are listed in Table II and the most common one, PQ\(_2\)QP, is a reflection of the high Pro and Gln content of C hordein.

Cleavage of Mutated C Hordeins by EP-B

To investigate whether the major cleavage sites were determined by primary sequence or secondary structure, site-directed mutagenesis was used to change the sequences around two C hordein cleavage sites (L-11 \(\rightarrow\) S and L-19 \(\rightarrow\) P), together with the introduction of a novel putative cleavage site by the substitution of Q-15 \(\rightarrow\) R (Fig. 4). The expressed mutated C hordein DNA was purified and incubated with EP-B, and the primary cleavage prod-

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**Table 1.** Second-order kinetic constants \(k_{cat}/K_m\) for plant cysteine endoproteases

<table>
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<tr>
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<tr>
<td>XQIFQ (\downarrow) QPQZD</td>
<td>609 ± 29</td>
<td>2650 ± 132</td>
<td>166 ± 6</td>
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<tr>
<td>XQIFQ (\downarrow) QPQSZPPD</td>
<td>149 ± 3</td>
<td>45 ± 8</td>
<td>98 ± 5</td>
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<tr>
<td>XQIFQ (\downarrow) QPQSZPPD</td>
<td>63 ± 2</td>
<td>150 ± 10</td>
<td>333 ± 9</td>
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<tr>
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<td>4400 ± 290</td>
<td>8110 ± 573</td>
<td>319 ± 9</td>
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<td>230 ± 4</td>
<td>365 ± 16</td>
<td>219 ± 13</td>
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<td>XQIFQ (\downarrow) QPQSZPPD</td>
<td>180 ± 11</td>
<td>160 ± 5</td>
<td>312 ± 11</td>
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<td>6500 ± 700</td>
<td>53 ± 2</td>
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<td>XQIFQ (\downarrow) QPQSZPPD</td>
<td>300 ± 8</td>
<td>1340 ± 17</td>
<td>125 ± 5</td>
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<td>XQIFQ (\downarrow) QPQSZPPD</td>
<td>930 ± 19</td>
<td>7130 ± 460</td>
<td>182 ± 10</td>
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<tr>
<td>XQIFQ (\downarrow) QPQSZPPD</td>
<td>84 ± 1</td>
<td>540 ± 36</td>
<td>208 ± 3</td>
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<tr>
<td>XQIFQ (\downarrow) QPQSZPPD</td>
<td>nd (^a)</td>
<td>nd</td>
<td>0.32 ± 0.007</td>
</tr>
<tr>
<td>XQIFQ (\downarrow) QPQSZPPD</td>
<td>nd</td>
<td>nd</td>
<td>0.15 ± 0.004</td>
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<td>XQIFQ (\downarrow) QPQSZPPD</td>
<td>nd</td>
<td>2.8</td>
<td>1.10 ± 0.11</td>
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<tr>
<td>XQIFQ (\downarrow) QPQSZPPD</td>
<td>nd</td>
<td>2.5</td>
<td>2.88 ± 0.08</td>
</tr>
</tbody>
</table>

\(^a\) X, 2-Aminobenzyol; Z, 3-nitrotyrosine. \(^b\) nd, Not detected; rate too low.
products were separated by SDS-PAGE and sequenced. Cleavage occurred between Q-16 and S-17, indicating that mutation of the two primary cleavage sites had greatly reduced their suitability as EP-B substrates, whereas the introduction of L-15 created a new primary cleavage site. In a second mutant C hordein (P-22→S), there was no effect on the cleavage pattern with EP-B (Fig. 4).

**Determination of EP Specificity by Using Synthetic Substrates**

Several synthetic, internally quenched fluorogenic substrates were synthesized, the sequence of which was based on the determined major cleavage sites of C hordeins, i.e. LQ↓SP, LQ↓QP, VQ↓QP, FO↓QP, and FR↓QQ, with the general sequence Abz-P2-P1-P19-P29-Tyr(NO2)-D. In addition to these, longer substrates based on the C hordein sequence, with residues extending to P6 and P59, were also synthesized to investigate the importance of these residues in determining substrate kinetics. Each peptide substrate was assayed at three different concentrations under pseudo-first-order conditions (Km, kcat) with EP-A, EP-B, and papain, which had been titrated with E-64 to determine the concentration of the active enzyme. The second-order kinetic constant kcat/Km was calculated for each substrate (Table I). It can be seen that substrates with Phe at P2 had the highest values for kcat/Km followed by Val and Leu. Extension of the substrate by the addition of residues from P3 to P6 and P39 to P59 usually decreased the kcat/Km. The cleavage of the same substrates by papain was measured and in several cases, in contrast to EP-A and EP-B, increasing substrate length gave increasing kcat/Km values (Table I).

**DISCUSSION**

Whereas Cys EPs such as EP-A and EP-B are known to contribute to storage-protein degradation in the germinating barley grain, the cleavage site specificity on native substrates has not, to our knowledge, previously been studied. A C hordein polypeptide, expressed as a recombinant protein, was selected as a substrate for EP-B, because the fidelity of its refolding to the native conformation has been demonstrated (Tamas et al., 1994). C hordein proteolysis was initiated by cleavage at a limited number of sites, followed by cleavage to smaller peptides consisting of 2 to 15 residues. The initial cleavage sites characteristically had hydrophobic residues (F, L, V, and I) at P2. To study the kinetics of cleavage at these sites, we synthesized synthetic, internally quenched, fluorogenic substrates based on their sequence. EP-A and EP-B belong to the papain-type group of Cys EPs (each have 51% sequence identity to papain), and the same substrates were used with papain for comparison. The most important residue for papain-
type Cys EP specificity is that at P₂, which is usually large and hydrophobic (Berger and Schechter, 1970).

For substrates of the type Abz-XaaQQP-Tyr(NO₂)D (in which Xaa = F, L, V, P, or S), the order of decreasing activity for EP-B and EP-A was L > F > V > S > P, whereas for papain, the order was F > L > V > S > P (Table I). Substrates with P or S at P₂ were particularly poor substrates, which was confirmed by site-directed mutagenesis of the C hordein polypeptide, where cleavage at LQ ↓ SP and LQ ↓ QP was greatly reduced after mutagenesis to SQ ↓ SP and PQ ↓ QP, respectively (Fig. 4). This indicates that cleavage of C hordein by EP-B is mainly determined by the primary sequence at the cleavage site. These results are in broad agreement with those reported for EP-B cleavage of hordothionins, with cleavage at sites containing L or V at P₂ (Jones and Poulle, 1990). Cleavage of other polypeptides by a presumptive isoform of EP-B yielded similar results with W, F, L, I, V, Y, and A at P₂ (Zhang and Jones, 1996). Most of the B₁ hordein-cleavage sites predicted by these authors have Pro at P₁, which would make them extremely poor EP-B substrates.

The effect of a possible secondary structure on $k_{cat}/K_m$ was investigated by increasing the length of the substrate based on the C hordein sequence around the primary cleavage site. In most cases, $k_{cat}/K_m$ values decreased with increased substrate length for EP-A and EP-B (Table I). This was unexpected, since the papain substrate-binding cleft is reported to consist of up to seven subsites (S₁, S₂, S₃, S₄, S₅, S₆, and S₇) (Berger and Schechter, 1970), and one might expect EP-A and EP-B to have the same number of subsites as papain. Similar results with substrate elongation have been reported for subtilisin and were attributed to adverse effects of secondary structure (Meldal and Brendam, 1991). This does not appear to be the case here, because longer substrates were often better substrates for papain, with $k_{cat}/K_m$ values increasing from 2 to 14 times (LQ ↓ QP versus QSYLQ ↓ QYPYQ; VQ ↓ QP versus FPVQ ↓ QPF, and PQ ↓ QP versus QIIFQ ↓ QPQQS), in contrast to EP-A and EP-B. It should be noted that in two of these series, Pro was placed at P₃, which may have been undesirable for EP-A and EP-B.

An examination of the secondary EP-B cleavage sites in C hordein after total digestion revealed that PQ ↓ QP was cleaved 14 times (Table II), although this sequence is not a good substrate (Table I). The residues found at the four different positions (P₂–P₅′) are listed in decreasing order of frequency. Between 9 to 12 amino acids were found at each position, with the total number probably being limited by the amino acid composition and sequence of C hordein (Fig. 2). It is striking, however, that Pro appears at P₂ and P₅′, but not at P₁ or P₄′ (Table II). A list of potential EP-B cleavage sites in C hordein (i.e. those with F, V, or L at P₂) that are not cleaved shows that they all contain Pro at P₁ or P₄′ (Table II). The substrates Abz-LQPQ-Tyr(NO₂)D and Abz-FPQQ-Tyr(NO₂)D were made to measure $k_{cat}/K_m$ values of substrates with Pro at these positions. It was not possible to detect cleavage with EP-A and EP-B, but high concentrations of papain yielded values that were 500 times less for Pro at P₁′ (LQ ↓ FP versus QL ↓ SP) and 2000 times less for Pro at P₁ (FP ↓ QQ versus FR ↓ QQ). Comparable ratios probably exist for EP-A and EP-B.

It is clear from Table I that $k_{cat}/K_m$ values are higher for EP-A and EP-B than for papain. We therefore determined $k_{cat}$ and $K_m$ values for all three enzymes using the substrate CBZ-Phe-Arg-AMC (Table III). Values obtained for papain of 79 μM (Km) and 45 s⁻¹ (kcat) are close to published values (Gauthier et al., 1993). The high $k_{cat}/K_m$ values for EP-A and EP-B were primarily due to their much lower Km values, indicating a higher affinity of these enzymes for the substrate. Better papain substrates are known, such as Cap-Leu-Arg-AMC (Km = 6 μM, kcat = 55 s⁻¹, and $k_{cat}/K_m = 9166$ mm⁻¹ s⁻¹) (Alves et al., 1996), in which the presence of ε-aminocaproic acid (Cap) at P₁ may cause a drastic reduction in Km. However, Abz-FRQQ-Tyr(NO₂)D and CBZ-FR-AMC appear to be equally good substrates for all three enzymes, even though the residue at P₁′ and particularly P₅′ can enhance substrate suitability considerably (García-Echeverría and Rich, 1992a, 1992b; Lalmanach et al., 1995).

This work has shown that, although EP-A and EP-B are similar proteases (66% amino acid sequence identity), they do exhibit differences in substrate specificity (e.g. LQ ↓ QP). This may be because EP-B can tolerate Pro at P₂, whereas EP-A cannot because there is a relatively smaller difference between EP-A and EP-B with FR ↓ QQ. A detailed study of substrate requirements at P₁ and P₂ and the importance of the primary cleavage sites for subsequent attack at secondary cleavage sites in C hordein will be the subject of future investigations.

ACKNOWLEDGMENTS

We thank Professor Peter Shewry for the gift of the C hordein expression vector, Dr. Finn Lok for providing the EP-A cDNA clone, and Sukswad Vongsvititkun for C hordein DNA sequencing. We are grateful to Dr. Phaedria St. Hilaire, Bodil Cornelussen, and Lone Sørensen for assistance with amino acid sequencing and MALDI-TOF MS, and to Hanne Christiansen and Kirsten Lilja for help and advice with peptide synthesis and HPLC. Ann-Sofi Stein-holz and Nina Rasmussen are acknowledged for making Figure 1.

Received October 31, 1997; accepted February 4, 1998.

Copyright Clearance Center: 0032-0889/98/117/0255/07.

The accession number for EP-A is 297023.

LITERATURE CITED


