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Purification and Characterization of Barley Dipeptidyl Peptidase IV[1]

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Barley (*Hordeum vulgare* L.) storage proteins, which have a high content of proline (Pro) and glutamine, are cleaved by cysteine endoproteases to yield peptides with a Pro next to the N-terminal and/or C-terminal amino acid residues. A peptidase cleaving after Xaa-Pro- at the N terminus of peptides was purified from green barley malt. It was identified as a serine-type dipeptidyl peptidase (DPP), based on inhibitor studies, and the nature of the cleavage product. It is a monomeric glycoprotein with an apparent molecular mass of 105 kD (85 kD after deglycosylation), with a pI of 3.55 and a pH optimum at 7.2. Substrate specificity was determined with a series of fluorogenic peptide substrates with the general formula Xaa-Pro-AMC, where Xaa is an unspecified amino acid and AMC is 7-amino-4-methylcoumarin. The best substrates were Xaa = lysine and arginine, while the poorest were Xaa = aspartic acid, phenylalanine, and glutamic acid. The K_m values ranged from 0.071 to 8.9 μM, compared with values of 9 to 130 μM reported for mammalian DPP IVs. We discuss the possible role of DPP IV in the degradation of small Pro-containing peptides transported from the endosperm to the embryo of the germinating barley grain.

The mobilization of storage proteins (hordeins) during barley (*Hordeum vulgare* L.) germination involves the action of both endo- and exopeptidases. Hordeins are water-insoluble proteins containing domains of Pro- and Gln-rich repeated sequences. Pro residues confer unique structural constraints on the peptide chain and significantly influence their susceptibility to proteolytic cleavage (Yaron and Naider, 1993). Cys endoproteases EP-A and EP-B and the Ser carboxypeptidases I, II, and III play a major role in hordein degradation (Koehler and Ho, 1990; Dal Degan et al., 1994; Davy et al., 1998). A study of EP-A and EP-B specificity has shown that many of the hordein peptides released by their action have a Pro residue in the second and/or penultimate position (Davy et al., 1998). However, peptides containing Pro in the penultimate position are very poor substrates for the barley Ser carboxypeptidases I, II, and III (Breddam and Sørensen, 1987). Germinating barley grains must contain peptidases with Pro specificity, since Pro is the most abundant free amino acid accumulating during germination (Higgins and Payne, 1981).

There are two Pro-specific dipeptidyl peptidases (DPPs), DPP II and DPP IV, which can be distinguished by their substrate specificity, since only DPP II can cleave Xaa-Pro-Pro. While both accept the substitution of Pro by Ala at P_1, the cleavage rate with DPP IV is much slower. DPP IV (EC 3.4.14.5) was first identified in rat liver (Hopsu-Havu and Glenner, 1966) and was subsequently purified from various mammalian tissues and from bacteria (Cunningham and O'Connor, 1997). DPP IV activity has also been reported in insects, yeast, and plants (Kreil et al., 1980; Bordallo et al., 1984; Benešová et al., 1987; Stano et al., 1994a, 1994b). It is a Ser peptidase that releases dipeptides from the N terminus of peptides or proteins, with a preference for substrates having Pro (and to a lesser extent Ala or Hyp) as the second residue (McDonald and Barrett, 1986). DPP IV is involved in a number of biological processes, such as intestinal assimilation, immunological activation of B and T-cells, and recycling of Pro-containing peptides (Cunningham and O'Connor, 1997).

In plants, DPP IV activity has been reported in poppy (Benešová et al., 1987; Stano et al., 1997) and gherkin seedlings (Stano et al., 1994b) and in ginseng callus (Stano et al., 1994a), and is suggested to play a role in the mobilization and/or utilization of storage proteins during germination. This paper describes the first (to our knowledge) purification of a plant DPP IV and the characterization of its biophysical and biochemical properties, including substrate specificity using Xaa-Pro-AMC (where Xaa is an unspecified amino acid and AMC is 7-amino-4-methylcoumarin) substrates and various peptides.

MATERIALS AND METHODS

Plant Material

Air-dried (45°C) green barley (*Hordeum vulgare* L.) malt was obtained from Carlsberg A/S (Copenhagen).

Chemicals

4-(2-Aminoethyl)benzenesulfonyl fluoride (AEBSF), Brij 35, diprotin A and B, dimethyl sulfoxide (DMSO), *trans*-epoxysuccinyl-L-leucylamido-(4-guanido)butane (E-64), Gly-Pro-AMC, leupeptin, Lys-Ala-AMC, 3-(*N*-morpholino)-propanesulfonic acid (MOPS), 2-(*N*-morpholino)-ethanesulfonic acid (MES), β-mercaptoethanol, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), and Pro-AMC were

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purchased from Sigma-Aldrich (St. Louis). Lys-Pro-AMC, Z-Gly-Pro-AMC, and Z-Phe-Ala-AMC were purchased from Bachem (Bubendorf, Switzerland). Other chemicals were dimethylformamide (DMF) (Fluka, Buchs, Switzerland), AMC (ICN, Costa Mesa, CA), endo-β-N-acetylglucosamidase H (Endo-Hf, New England Biolabs, Beverly, MA), and [^{14}C]-diisopropyl fluorophosphate (DFP) (NEN-DuPont, Boston).

Enzyme Activity

Peptidase activity was measured during purification using the fluorogenic substrate Lys-Pro-AMC. Enzymatic hydrolysis of the substrate was followed by the change of fluorescence due to the liberation of AMC upon addition of enzyme using a luminescence spectrofluorimeter (LS50, Perkin-Elmer Applied Biosystems, Foster City, CA) with an excitation wavelength of 380 nm (10-nm slit), and emission wavelength of 460 nm (10-nm slit) at 25°C. Substrates were dissolved in DMF and diluted in water to 40 to 200 μM, except for Lys-Pro-AMC, which was dissolved and diluted in DMSO to avoid formylation of the Lys ϵ-amino group (Olesen and Breddam, 1998). Ten microliters was added to a cuvette containing 2 mL of assay buffer (50 mM MOPS, pH 7.2, and 2 mM β-mercaptoethanol). The initial fluorescence was measured and enzyme solution was added. At each of the substrate concentrations, the initial velocity for substrate cleavage 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 was measured. k_{cat} and K_m values were calculated from Hanes plots using substrate concentrations between 0.1× and 5× K_m. Enzyme concentration was determined from protein concentration using a molecular mass of 105 kD. The spectrofluorimeter was calibrated with known concentrations of AMC.

Enzyme Purification

All operations were carried out at 4°C. Green barley malt (500 g) was ground in a coffee grinder before mixing with 4 L of buffer (0.1 M $NaC_2H_3O_2$, pH 4.5, and 2 mM β-mercaptoethanol) and stirring for 2 h. After 20 min of centrifugation at 17,000*g*, the supernatant was concentrated to 300 mL and diafiltered against buffer A (20 mM $NaC_2H_3O_2$, pH 5.0, and 2 mM β-mercaptoethanol) using a spiral-wound cartridge concentrator with a 10,000-D filter (CH2RS, Amicon, Beverly, MA).

The sample was loaded onto an S Sepharose (Pharmacia, Uppsala) column (26 mm/40 cm) equilibrated with 20 mM $NaC_2H_3O_2$ (pH 5.0) containing 2 mM β-mercaptoethanol. The flow-through was collected, concentrated to 300 mL, and diafiltered against buffer B (20 mM Bis-Tris, pH 6.5, and 2 mM β-mercaptoethanol) as described above. The sample was loaded onto a Q Sepharose (Pharmacia) column (26 mm/20 cm) equilibrated with buffer B. Bound material was eluted with 400 mL of a linear gradient of 0 to 500 mM NaCl. Fractions (10 mL) containing peptidase activity were pooled.

The sample (100 mL) was concentrated to 3 mL on a pressurized cell and loaded onto a Superdex 200 column (16 mm/50 cm) equilibrated with buffer B. The active fractions (6 × 2.5 mL) were pooled. The active pool (15 mL) was loaded onto a Source 15Q (Pharmacia) column (16 mm/5 cm) equilibrated with buffer B. After elution with a 150-mL linear gradient of 0 to 500 mM NaCl, the active fractions (6 × 2.5 mL) were pooled. The sample (15 mL) was concentrated to 0.5 mL using a concentrator (10,000 D molecular mass cutoff) (Vivaspin, Vivascience, Binbrook, UK) and loaded onto a Superdex 200 column (10 mm/28 cm) equilibrated with buffer B.

Agarose isoelectrofocusing plates (IsoGel, FMC, Rockland, ME) covered the range from pH 3.0 to 10.0. The active fractions were pooled, concentrated, and electrofocused in six lanes for 15 min at 1 W followed by 1 h at 10 W using 1 M NaOH (cathode solution) and 0.5 M acetic acid (anode solution) on a flat-bed multiphor II (Pharmacia) at 12°C. The outer lanes were Coomassie (Neuhoff et al., 1988) stained together with the marker lanes comprising pI markers from Pharmacia. The rest of the lanes were sliced into 1-mm bands and extracted with 500 μL of assay buffer. Extracts with active protein were pooled and stored at 4°C.

Active fractions from the Q Sepharose column were pooled and adjusted to 4 M NaCl before loading onto a Phenyl Sepharose CL4B (Pharmacia) column (26 mm/11 cm) previously equilibrated with buffer B containing 4 M NaCl. The proteins were eluted stepwise with 4, 3, 2, 1, and 0 M NaCl.

Protein Determination, SDS-PAGE, and Blotting

Protein concentration was determined with protein assay reagent (Bio-Rad, Hercules, CA) or, at low concentrations, with NanoOrange (Molecular Probes, Eugene, OR). The Superdex 200 column was calibrated with the following protein standards (Boehringer Mannheim, Mannheim, Germany): aldolase (158 kD, 45 mL), β-galactosidase (116 kD, 48 mL), bovine serum albumin (67 kD, 63 mL), ovalbumin (45 kD, 75 mL), and chymotrypsinogen A (25 kD, 98 mL). SDS-PAGE was performed under reducing conditions in a Protean II mini-gel apparatus (Bio-Rad) using 10% (w/v) acrylamide high-Tris gels according to the method of Fling and Gregerson (1986). Proteins were visualized by silver staining (Bloom et al., 1987). For N-terminal sequencing, proteins were blotted onto polyvinylidene fluoride (PVDF) membrane (Immobilon P, Millipore, Bedford, MA) using a semidry electroblotter (Aricos, Ølstykke, Denmark), as previously described (Davy et al., 1998).

Endo-Hf Treatment

A sample of the pure DPP IV equivalent to 20 to 40 ng was denatured and treated with 1 unit of Endo-Hf (Boehringer Mannheim) for 2 h according to the manufacturer's instructions. The samples were analyzed by SDS-PAGE followed by silver staining.

Determination of pH Optimum

The peptidase activity was assayed with Lys-Pro-AMC over the pH range 5.5 to 9.0. The buffers used were 50 mM

MES (pH 5.5–6.5), 50 mM MOPS (pH 6.0–7.0), and 50 mM *N,N′*-bis(2-hydroxyethylglycine) (Bicine) (pH 6.5–9.0), all containing 2 mM β-mercaptoethanol.

DFP Labeling

The peptidase preparation (3 μg) was incubated with 10 μL of [^{14}C]DFP (160 mCi mmol^{-1}) for 1 h at 25°C in the dark. After incubation, the mixture was separated on a 10% (w/v) SDS-PAGE gel and electroblotted onto PVDF membrane as described above. The membrane was exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, CA) for 12 d and scanned using a phosphor imager (Storm 860, Molecular Dynamics). ^{14}C-labeled protein molecular mass markers were from Amersham (Birkerød, Denmark).

Degradation of Peptide Substrate by DPP IV

Each substrate (50–100 pmol) was incubated with 1 μL of the purified DPP IV preparation for 15 h. Cleavage sites were determined by N-terminal sequencing of the degradation products.

Synthetic Substrates

t-Butyloxycarbonyl (Boc)-Pro-AMC was synthesized by the phosphorous oxychloride anhydride method and purified as previously described (Alves et al., 1996). Boc-Pro (10 mmol) and AMC (9 mmol) were dissolved in pyridine, which was dried over KOH pellets and distilled just before use. This solution was cooled to −15°C, and phosphorous oxychloride (11 mmol) was added dropwise under vigorous stirring. After 30 min, crushed ice and water was added, followed by ethyl acetate. The organic phase was washed successively with cold 0.5 N HCl, water, and 5% (w/v) $NaHCO_3$ and then dried over Na_2SO_4. After removal of the solvent, the material was triturated with diethyl ether. Purification of Boc-Pro-AMC on silica-gel column chromatography was performed using chloroform and chloroform-methanol mixtures for elution.

The Fmoc-aminoacyl-Pro-AMCs were synthesized by solution-phase peptide synthesis with *O*-benzotriazo-1-yl-*N,N,N,N′*-tetramethyl uronium tetrafluoraborate as the coupling reagent. After the final deprotection with piperidine followed by trifluoroacetic acid (TFA), the peptides were purified by semi-preparative HPLC using an Econosil C_{18} column (10 μM, 22.5 × 250 mm) and a two-solvent system: (A) TFA:H_2O (1:1,000) and (B) TFA:acetonitrile:H_2O (1:900:100). The column was eluted at a flow rate of 5 mL min^{-1} with a 10% (or 30%) to 50% (or 60%) gradient of solvent B for 30 or 45 min. Analytical HPLC was performed using a binary HPLC system (Shimadzu, Kyoto) with a UV-VIS detector (SPD-10AV, Shimadzu) and a fluorescence detector (RF-535, Shimadzu) coupled to an Ultrasphere C_{18} column (5 μm, 4.6 × 150 mm) eluted with solvent systems A_1 (H_3PO_4/H_2O, 1:1,000) and B_1 (ACN/H_2O/H_3PO_4, 900:100:1) at a flow rate of 1.0 mL min^{-1} and a 10% to 80% gradient of B_1 over 20 min. The HPLC column eluates were monitored by their A_{220} and by fluorescence emission at 395 nm following excitation at 325 nm. Substrate concentration was determined by amino acid analysis (Biochrom 20, Pharmacia Biotech, Cambridge, UK), and confirmed by measurement of AMC fluorescence after total hydrolysis of each peptide.

RESULTS

Purification of DPP IV

Lys-Pro-AMC was used as a substrate to identify a peptidase that would attack Xaa-Pro-peptide fragments. This enabled us to detect activity in a crude malt extract. After loading the extract onto an S Sepharose column, the peptidase was detected in the flow-through with a 35-fold purification. The flow-through was concentrated and loaded onto a Q Sepharose column. The peptidase was

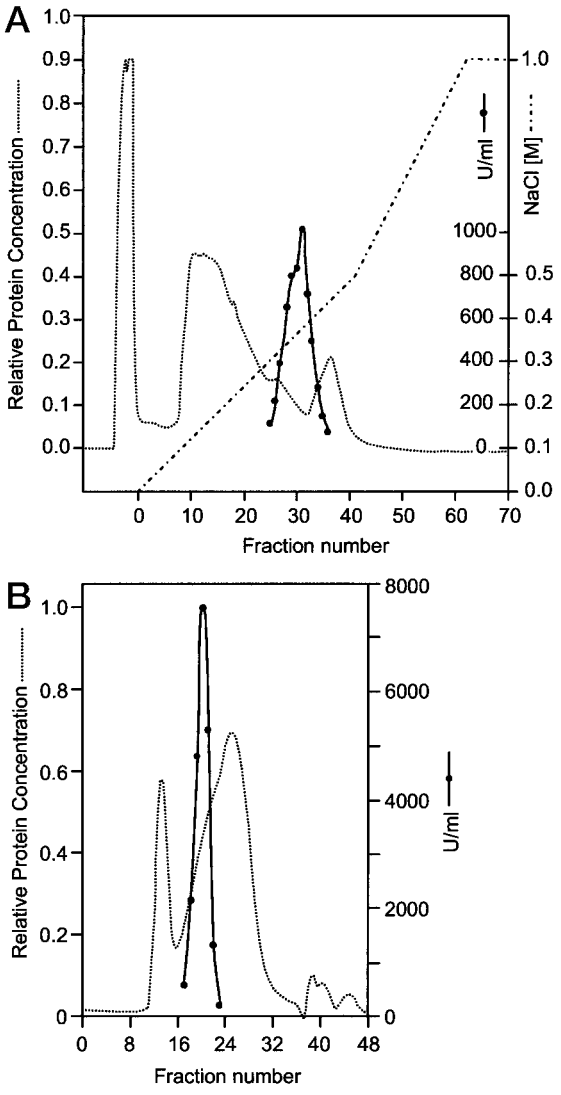

Figure 1. A, Elution profile from the Q Sepharose column. Peptidase activity, detected by the cleavage of Lys-Pro-AMC, elutes at a 0.35 M salt concentration. B, Elution profile from the first Superdex 200 gel filtration column. Peptidase activity has an elution volume corresponding to a molecular mass of 105 kD.

Table I. *Purification of DPP IV from barley*

Step	Protein	Activity	Specific Activity	Yield	Purification
	mg	*units*[a]	*units/mg*	*%*	*fold*
Extract-diafiltration	2,275	85,000	37.4	100	1
S Sepharose	60	79,500	1,330	93.5	35
Q Sepharose	1.72	56,650	32,900	66.6	880
Superdex 200	0.32	55,000	172,000	64.7	4,600
Source 15Q	<0.04[b]	38,525	>963,000	45.3	>26,000
Superdex 200	<0.03[b]	33,290	>1,110,000	39.2	>30,000
IEF-purified enzyme	<0.01[b]	16,000	>1,600,000	18.8	>43,000

[a] One unit of activity corresponds to an increase of 1 unit of fluorescence min^{-1} μL^{-1}. [b] Estimated using the NanoOrange kit (Molecular Probes).

eluted at 0.35 M NaCl (Fig. 1A), with a further 25-fold purification (Table I). The first Superdex 200 column chromatography was also effective, giving a further 5-fold purification (Fig. 1B; Table I). This step was followed by a Source 15Q column and a second, smaller Sephadex 200 column. The last step was isoelectric focusing, which removed several contaminating proteins. Activity measurements of extracts from 1-mm IEF gel slices localized the peptidase to a Coomassie-stained band focused at a pI of 3.55 (Fig. 2A). SDS-PAGE of the active sample revealed two protein bands (Fig. 2B), one with an apparent molecular mass of 60 kD and the other of 105 kD.

Since the two proteins co-migrated on anion-exchange and gel filtration columns and had the same pI, a hydrophobic interaction column chromatography step was used to separate the peptidase from its contaminant. A Phenyl Sepharose CL4B column chromatography step was added after the Q Sepharose column. The active fractions eluting at 3 M NaCl were pooled and loaded onto a Superdex 200 gel filtration column. SDS-PAGE of the active fractions showed a single protein band with an apparent molecular mass of 105 kD (Fig. 2C). Although the 105-kD peptidase was purified by hydrophobic interaction column chromatography, this resulted in a 50% yield reduction.

Biochemical and Biophysical Properties of Barley DPP IV

The purified peptidase had an apparent molecular mass of 105 kD on SDS-PAGE, which was consistent with the molecular mass deduced from its elution from a calibrated gel filtration column, indicating that it was a monomer. The Coomassie-stained band was smeared, suggesting that the peptidase was glycosylated. This was confirmed by incubation of the peptidase with Endo-Hf, resulting in a reduction in apparent molecular mass to 85 kD (data not shown). The de-glycosylated protein was still enzymatically active. The 105-kD protein was blotted onto PVDF membrane for protein sequencing but was N-terminally blocked.

The peptidase had a pH optimum of 7.2 (Fig. 3), and all analyses were performed at this pH. Its activity was inhibited by the Ser protease inhibitors PMSF and AEBSF, but not by the Cys protease inhibitors E-64 and leupeptin, the Asp inhibitor pepstatin A, or the metalloprotease inhibitor EDTA (Table II), indicating that the peptidase belongs to the Ser class of proteases. This was confirmed by reacting the preparation containing the peptidase and a 60-kD contaminant with [^{14}C]DFP, a Ser protease inhibitor that binds covalently to the active site. This labeled a single band at 105 kD (Fig. 2B). The peptidase activity was also inhibited

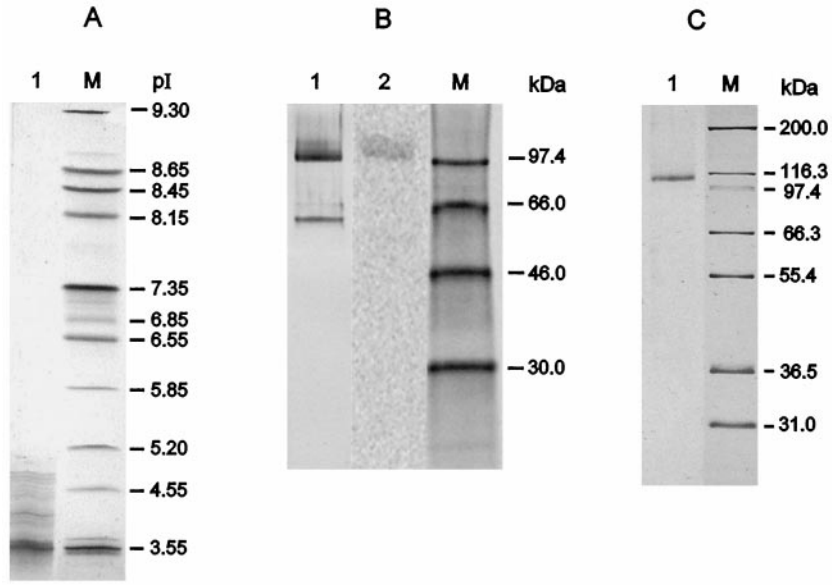

Figure 2. A, Isoelectric focusing of an active fraction from the second Superdex 200 gel filtration column (lane 1) and pI markers (lane M) stained with Coomassie Blue. B, SDS-PAGE of silver-stained, partially purified barley DPP IV after elution from the second Sephadex 200 (lane 1). A duplicate fraction (3 μg of protein) labeled with [^{14}C]DFP was separated by SDS-PAGE (lane 2), with ^{14}C-labeled protein molecular mass markers (lane M), then blotted onto PVDF membrane and analyzed by phosphor imaging. C, SDS-PAGE of silver-stained, IEF-purified barley DPP IV (0.5 μg) with an apparent molecular mass of 105 kD.

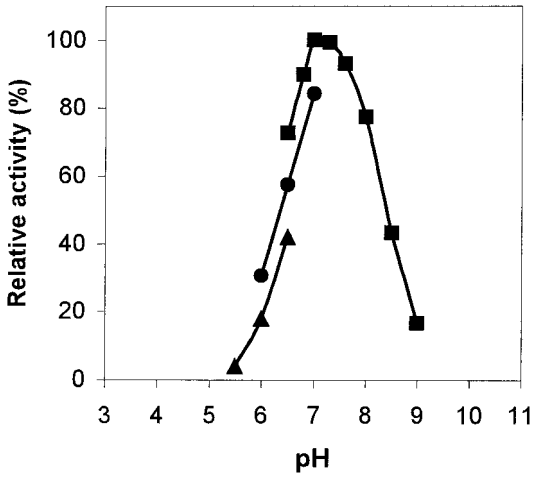

Figure 3. pH curve for barley DPP IV, with maximum activity normalized to 100%. Assays were performed in 50 mM MES (pH 5.5–6.5) (▲), 50 mM MOPS (●) (pH 6–7), and 50 mM Bicine (■) (pH 6.5–9.0). All buffers contained 2 mM β-mercaptoethanol.

by diprotin A and B, which are specific inhibitors of DPP IV (Umezawa et al., 1984).

Substrate Specificity of Barley DPP IV

Cleavage of Lys-Pro-AMC was used to monitor peptidase activity during the purification of DPP IV. Detection of peptidase activity with this substrate requires cleavage of the Pro-AMC bond, which can be achieved by a prolyl endopeptidase or a DPP or a combination of an aminopeptidase P and an iminopeptidase (Cunningham and O'Connor, 1997). The purified barley peptidase showed no activity on Pro-AMC or on N-terminally blocked substrates such as Z-Phe-Ala-AMC or Z-Gly-Pro-AMC, indicating that it had neither aminopeptidase or endopeptidase activity. When the peptidase was incubated with Lys-Pro-AMC, no free amino acids could be detected, indicating that a dipeptide was released. These results show that the activity is due to a DPP, either DPP II or DPP IV, both of which cleave after Pro. These can be distinguished by their substrate specificity, since only DPP II can cleave Xaa-Pro-Pro. In addition, while DPP II and DPP IV both accept the substitution of Pro by Ala at P_1, the cleavage rate with DPP IV is much slower (Cunningham and O'Connor, 1997). The barley peptidase was incubated with various peptides and their cleavage sites were determined by N-terminal sequence analysis (Table III). The results showed that the purified enzyme was a DPP, releasing dipeptides sequentially from the N terminus of peptide substrates. The enzyme did not attack the N-terminal Arg-Pro-Pro- sequence in RPPGFSPFR (bradykinin), which indicates that it is not a DPP II. This lack of cleavage of the internal Pro-Phe bond in bradykinin demonstrated the absence of endopeptidase activity.

To study substrate specificity in more detail, several fluorogenic peptides were synthesized with the general formula Xaa-Pro-AMC. Each peptide substrate was assayed at four or more different concentrations, and the K_m, k_{cat}, and k_{cat}/K_m kinetic constants were determined for each substrate (Table IV). The K_m value is a measure of the affinity of the enzyme for its substrate, while the k_{cat} value indicates the number of substrate molecules cleaved per molecule of enzyme per unit of time. The higher the value of k_{cat}/K_m, the better the substrate. Of these substrates, Lys-Pro-AMC and Arg-Pro-AMC were cleaved most rapidly. The rate of hydrolysis of Lys-Pro-AMC was 50-fold greater than that of Lys-Ala-AMC, confirming the identification of the barley peptidase as a DPP IV.

Table II. *Effects of inhibitors on barley DPP IV*

Inhibitor	Final Concentration	Activity
	mM	*% of control*
Control		100
PMSF	1.0	14
AEBSF	1.0	5
Leupeptin	0.1	83
EDTA	10.0	44
E-64	0.01	100
Pepstatin	0.002	100
Diprotin A	0.1	3
Diprotin B	0.1	4

Table III. *Cleavage of peptides by barley DPP IV*

Peptide	Sequence and Cleavage Sites
Substance P	R P↓K P↓Q Q F F G L M
Bradykinin	R P P G F S P F R
des-Pro²-bradykinin	R P↓G F S P F R
β-Casomorphin	Y P↓F P↓G P↓I
–	G P↓R P

Table IV. *K_m and k_{cat} values for the degradation of substrate by barley DPP IV*

Substrate	K_m	k_{cat}	k_{cat/K_m}
	μM	s^{-1}	$s^{-1}\ \mu M^{-1}$
Lys-Pro-AMC	0.071	90	1,280
Arg-Pro-AMC	0.090	71	783
Tyr-Pro-AMC	0.73	99	135
Thr-Pro-AMC	0.93	117	126
Ile-Pro-AMC	0.70	67	96
Ala-Pro-AMC	0.34	23	66
Met-Pro-AMC	2.17	102	47
Ser-Pro-AMC	1.26	58	46
His-Pro-AMC	1.03	41	40
Pro-Pro-AMC	1.10	41	38
Leu-Pro-AMC	1.09	22	21
Asn-Pro-AMC	3.24	35	11
Gly-Pro-AMC	6.88	60	8.7
Gln-Pro-AMC	1.38	10	7.0
Glu-Pro-AMC	2.98	10	3.4
Phe-Pro-AMC	0.65	1.8	2.7
Asp-Pro-AMC	8.88	4.6	0.5
Lys-Ala-AMC	3.74	92	25
Z-Gly-Pro-AMC	ND[a]	ND	ND
Z-Phe-Ala-AMC	ND	ND	ND
Pro-AMC	ND	ND	ND

[a] ND, No cleavage detected.

DISCUSSION

A barley peptidase cleaving after Xaa-Pro at the N terminus of peptides was purified from green barley malt. Based on inhibitor studies and the nature of the cleavage products, it was identified as a Ser-type DPP. There are two types of DPPs that cleave after Pro, DPP II and DPP IV, and they can be distinguished by their substrate specificity and pH optimum (Cunningham and O'Connor, 1997). The barley peptidase was shown to be a DPP IV, since it was unable to cleave Arg-Pro-Pro- and because Lys-Ala-AMC was a poor substrate relative to Lys-Pro-AMC. DPP II is known to cleave substrates of the type Xaa-Pro-Pro-, and substitution of Pro by Ala at P_1 results in substrates with similar cleavage rates (Cunningham and O'Connor, 1997). Barley DPP IV is similar to other DPP IVs in having a low pI (pI 3.55), a neutral pH optimum (7.2), and in being glycosylated. In contrast, DPP II has a pI of 4.8 to 5.0, a slightly acidic pH optimum, and although it is glycosylated, this accounts for only 2% of the mass (Cunningham and O'Connor, 1997).

DPP IV has mainly been described in mammalian tissues, where it is reported to be a homodimer with a subunit molecular mass in the range of 110 to 130 kD (88 kD after de-glycosylation; Yaron and Naider, 1993). It is found at high concentrations in the cell membranes of the brush border of the small intestine and kidney, where it is believed to be involved in the absorption and recycling of Pro-containing peptides. DPP IV probably contributes to the hydrolysis and assimilation of Pro-containing peptides in the intestine, since DPP IV-deficient rats lost weight when fed gliadin, a Pro-rich protein (Cunningham and O'Connor, 1997). The specificity of mammalian DPP IV has been determined using substrates with the general formula Xaa-Pro-pNA, which had values for K_m and a catalytic efficiency that varied only by a factor of 5, except for Gly, Asp, and Asn at P_2 (Yaron and Naider, 1993). The K_m values ranged from 9 to 130 μM, with Pro-Pro-pNA having the lowest K_m value and Gly-Pro-pNA having the highest. Since k_{cat} values varied only from 30 to 97 s^{-1} (Heins et al., 1988), substrate cleavage rates in mammalian DPP IV were primarily determined by the K_m values.

A detailed study of barley DPP IV substrate specificity indicates a clear preference for a basic residue in P_2. The high specificity toward Lys-Pro-AMC and Arg-Pro-AMC can be explained by their much lower K_m values compared with other substrates. Barley DPP IV shows a much broader range of K_m values (from 0.071–8.88 μM) and cleavage rates than the mammalian DPP IV (Table IV). The K_m values are much lower than those reported for mammalian and prokaryotic DPP IV, but this may be due to the nature of the group at P_1'. Most k_{cat} values are between 22 and 117 s^{-1}, which is similar to mammalian DPP IV (Heins et al., 1988), but Gln-Pro-AMC, Glu-Pro-AMC, Asp-Pro-AMC, and Phe-Pro-AMC had even lower values (Table IV). The range of k_{cat}/K_m values is much greater for barley DPP IV than for mammalian DPP IV (Yaron and Naider, 1993). The lower k_{cat}/K_m value for Lys-Ala-AMC compared with Lys-Pro-AMC (Table IV) is due to the 50-fold higher K_m value when Ala is at P_1.

The function of DPP IV is not clearly established in plants, although its activity has been detected in poppy (Benešová et al., 1987; Stano et al., 1997) and gherkin (Stano et al., 1994b) seedlings and in callus tissue from ginseng (Stano et al., 1994a). DPP IV from these sources had a pH optimum of 8.0 and K_m values for Gly-Pro-pNA of between 300 and 590 μM. DPP IV activity increased with time during germination of cucumber, and was localized mainly to the vascular tissue and epidermis of roots and hypocotyls (Stano et al., 1994b). It is present in developing and germinating poppy seeds, where it is suggested to play a role in the hydrolysis of peptides and proteins (Benešová et al., 1987; Stano et al., 1997).

During germination, barley storage proteins, which are stored in the endosperm, are first degraded by a combination of endo- and exopeptidases secreted from the surrounding aleurone and scutellum tissue (Koehler and Ho, 1990). Based on our knowledge of EP-B specificity and its hordein cleavage pattern, particularly within the repetitive domains, it is known that many of the peptides released by its action have a Pro residue in the second and/or penultimate position (Davy et al., 1998). This is clearly illustrated by the cleavage products of C hordein, where KPFPVQ, QPFPQ, and QPQ are formed. Small peptides of two to five residues accumulate in the endosperm in millimolar concentrations, and are subsequently transported via a specialized uptake system in the scutellar epithelium of the embryo, where they are degraded to free amino acids (Higgins and Payne, 1981; Walker-Smith and Payne, 1983).

The localization of barley DPP IV was not determined in this study. Since the pH of the endosperm is about 5.0 (Mikola and Virtanen, 1980) and DPP IV has almost no activity at this pH (Fig. 3), it is likely that the barley DPP IV is localized in the embryo or the scutellum. Thus, barley DPP IV may be involved in this later step of storage protein mobilization, releasing dipeptides, tripeptides, and amino acids in the scutellum and/or embryo. Although the K_m values for the substrates described here are in the micromolar range, short oligopeptides may have much higher K_m values, as found for rat DPP IV (Kikuchi et al., 1988). The di- and tri-peptides released could then be degraded to free amino acids by aminopeptidases (Sopanen and Mikola, 1975), dipeptidases (Sopanen, 1976), and carboxypeptidases (Breddam and Sørensen, 1987), which are also present in the barley embryo (Dal Degan et al., 1994). The X-Pro dipeptide products of DPP IV cleavage are not substrates for barley malt carboxypeptidase I (Breddam and Sørensen, 1987), but would be cleaved by a prolidase or an aminopeptidase P, with the release of free Pro.

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