Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 8140–8144, September 1991
Plant Biology

C-terminal processing of barley α-amylase 1 in malt, aleurone protoplasts, and yeast

(multiple forms/posttranslational modification/carboxypeptidase/heterologous expression/SH groups)

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Communicated by Diter von Wettstein, June 14, 1991 (received for review April 17, 1991)

ABSTRACT **C-terminal processing of low pI barley α-amylase (AMY1) results in multiple forms in malt, aleurone protoplasts, and transformed yeast. Expression of an AMY1 cDNA in yeast thus leads to four secreted forms with distinct pI values between 4.7 and 5.1 and essentially identical M_r. AMY1-1 and AMY1-2 lacking the C-terminal Arg-Ser are generated by carboxypeptidase *in vitro* from AMY1-3 and AMY1-4, respectively. *In vivo* processing is due to the *KEX1*-encoded yeast carboxypeptidase. AMY1-2 and AMY1-4 are fully active, whereas AMY1-1 and AMY1-3 retain 3–4% activity toward *p*-nitrophenyl maltoheptaoside and have one fewer SH group, due to reaction with glutathione. AMY1-1–AMY1-4 are indistinguishable from malt AMY1 with respect to Ca^{2+}-, substrate-, and β-cyclodextrin-binding as well as recognition by three monoclonal antibodies and limited proteolysis by proteinase K. Transient AMY1 precursors present in barley aleurone protoplasts were trapped by addition of serine carboxypeptidase inhibitors, indicating that endogenous carboxypeptidase participates in the maturation of AMY1 during germination. Three pairs of precursor/mature AMY1 forms are recognized, presumably corresponding to the three genes encoding AMY1. Malt carboxypeptidase II can convert *in vitro* the precursors isolated from protoplasts into processed enzyme, and AMY1 from malt accordingly lacks the C-terminal heptapeptide. This report thus demonstrates posttranslational protein modification by carboxypeptidase in higher plants.**

The aleurone cell layers surrounding the starchy endosperm in barley grains produce many hydrolases that are delivered to the endosperm to provide nutrients for the growing embryo (1). In particular, the hormonally regulated synthesis of digestive enzymes during germination has attracted interest (see refs. 1 and 2), and barley α-amylases have been widely used as a model for gene expression and protein secretion in cereals. A major issue is to elucidate how a multitude of forms with similar size and slightly different pI values arise in the two isozyme families, comprising low pI barley α-amylase (AMY1) and high pI α-amylase (AMY2) encoded by three and six genes, respectively (3, 4). Posttranslational modification occurs in addition to multigene expression. After removal of the signal peptide, the N terminus of AMY2 is modified into pyroglutamate (5). AMY1 members are converted to forms of lower pI. In aleurone protoplasts of Himalaya barley, modification processes involving phosphorylation, sulfation, fatty acid acylation, N-glycosylation, or limited proteolysis by thiol endoproteases (6, 7) have been excluded.

We have reported recently that expression of an AMY1 cDNA in yeast results in four secreted AMY1 species (8) that have been isolated in the present study and shown to differ in (*i*) C-terminal sequence, (*ii*) content and reactivity of SH groups, and (*iii*) enzymatic activity. The lower pI forms AMY1-1 and -2 are generated *in vitro* by carboxypeptidase digestion from AMY1-3 and AMY1-4, respectively. Like AMY1 produced in yeast, precursor forms from malt and from aleurone protoplasts are C-terminally truncated by a serine carboxypeptidase.

EXPERIMENTAL PROCEDURES

Yeast Fermentation. DBY746 harboring pBAL7 (8), an AMY1 expression-plasmid carrying clone E (9) between promoter and terminator of the yeast *PGK* gene in the vector pMA91, was grown in double-strength synthetic complete medium lacking leucine (8) in a 150 l Chemap fermentor. Cells were removed in a Westphalia CSA 8-06-476 disc centrifuge, and AMY1 was isolated (see below) from the filtrated supernatant (Pall AB1Y0057P filter).

Preparation and Cultivation of Barley Aleurone Protoplasts. For protoplast preparation, 50 dry seeds (*Hordeum vulgare* cv. Himalaya; either the 1985 or 1988 harvest at Washington State University, Pullman) were deembryonated, cut through the ventral grove, preimbibed for 10 min in 2 ml of H_2O, sterilized in 4 ml of 0.11% NaOCl for 20 min, washed four times in H_2O, and finally imbibed for 60 hr in 10 ml of H_2O containing 1.5 mg of Cefotaxime (Calbiochem). Isolated aleurone layers were treated for 2 hr with 3% cellulase R-10 (Yacult Honsha, Tokyo) in aleurone protoplast medium [APM; 1× Gamborg B5 (Flow Laboratories)/2% (wt/vol) glucose/10 mM arginine/20 mM $CaCl_2$/10 mM Mes, pH 5.4/mannitol to a final osmotic pressure of 700 mosmol] and incubated in a fresh cellulase solution for 16 hr. The protoplasts were released by repeated pipet suction and purified by discontinuous gradient centrifugation on 55% Percoll. Protoplasts (≈200,000) were cultivated at room temperature for 48 hr in 1 ml of APM medium/0.015% Cefotaxime [and when required 1 μM GA_3 (Sigma)]. In parallel experiments, the serine carboxypeptidase inhibitors bacitracin, phenylmethylsulfonyl fluoride, or benzylsuccinic acid (all 5 mM) had been added, and pH was adjusted to 5.4. Malt carboxypeptidase II (2 μM) was used in other experiments. For enzyme isolation protoplasts were harvested by centrifugation for 5 min at 50 × *g*, resuspended in 200 μl of 20 mM NaOAc, pH 5.5/1 mM $CaCl_2$ and for enzyme isolation lysed by freezing in liquid nitrogen followed by maceration with a pistil (Kontes).

α-Amylase Purification. Supernatant of 100 liters of yeast culture was concentrated by ultrafiltration to 10 liters (DDS-RO GR81P membrane; 20-kDa cut-off; Danisco, Copenhagen) and then to 300 ml (Millipore Pellicon PTGC0005; 10-kDa cut-off), and AMY1 was isolated by affinity chromatography on β-cyclodextrin(= cycloheptaamylose)-Sepharose (5 × 8 cm) (8). The AMY1 forms were subsequently

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Abbreviations: AMY, barley α-amylase; AMY1, low pI AMY; AMY2, high pI AMY.

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separated on CM-fractogel 650M (2.6 × 25 cm) by gradient elution (2 × 1.5 liters) from 10 mM NaOAc, pH 4.3/1 mM $CaCl_2$ (buffer A) to 10 mM NaOAc, pH 5.3/1 mM $CaCl_2$/0.1 M NaCl (buffer B) at a flow rate of 30 cm/hr. Selected pools were rechromatographed by using the above resin (1.6 × 10 cm) and conditions. The AMY1-1 through AMY1-4 fractions obtained were concentrated in 20 mM NaOAc, pH 5.5/1 mM $CaCl_2$ (Amicon YM10 membrane) and stored at −20°C. AMY1 from barley green malt cv. Triumph was purified as described (5, 10).

[^{35}S]Methionine-labeled AMY (≈0.5 nmol) was isolated on β-cyclodextrin-Sepharose (0.9 × 1 cm) from the lysate (200 μl) of protoplasts cultured for 48 hr in the presence of gibberellic acid (GA_3) and [^{35}S]methionine at 40 μCi/ml (1 Ci = 37 GBq). After a wash with 20 mM NaOAc, pH 5.5/1 mM $CaCl_2$/bovine serum albumin at 0.1 mg/ml, AMY was eluted in 1 ml of the buffer containing β-cyclodextrin at 8 mg/ml and concentrated 10-fold by centrifugation (Millipore Ultrafree-MC concentrator) before isoelectric focusing.

α-Amylase Assays. Phadebas tablets (Pharmacia) were used in the routine assay (8). The activity of recombinant AMY1 toward 0.5% soluble potato starch (Sigma) from pH 3 to 8 was determined at 37°C as described (8).

Isoelectric Focusing. Isoelectric focusing from pH 4 to 6.5 was done (Ampholine PAG plates or Phast gels; Pharmacia) according to the manufacturers' recommendations. Protein was stained with Coomassie brilliant blue while activity was detected by soaking in 1% soluble starch (Merck)/20 mM NaOAc, pH 5.5/1 mM $CaCl_2$ at 37°C for 4 min followed by 0.025% I_2/0.4% KI (Ampholine PAG plates) or 0.25% I_2/4% KI (PhastGels).

***In Vitro* Conversion of AMY1.** AMY1-1–AMY1-4 (5 μM) were treated with 0.5 μM yeast carboxypeptidase Y or malt carboxypeptidase II in 20 mM NaOAc, pH 5.5/1 mM $CaCl_2$ at room temperature for 2 hr. The conversion was monitored by isoelectric focusing. [^{35}S]Methionine AMY (≈5 μM) isolated from protoplast lysate was incubated for 2 hr at room temperature with malt carboxypeptidase II (1 μM). After silver staining according to the manufacturer's instructions the isoelectric focusing gel was autoradiographed (Amersham Hyperfilm-MP RPN-6).

***In Vivo* Conversion of AMY1 in Yeast.** A *Hin*dIII fragment of pBAL7 (8) comprising the AMY1 coding region sandwiched between the yeast *PGK* promoter and terminator was inserted into Yep24 (pBAL91). Strains M204-8C (a *leu2 his3 ade1 ura3 kex1*::LEU2) and T109-3C (α *leu2 his3 ura3*) carrying pBAL91 were cultivated in double-strength synthetic complete medium lacking uracil (1 liter; shake flask). AMY1 was purified on β-cyclodextrin-Sepharose (0.9 × 2 cm) as described above and subjected to isoelectric focusing.

Sequence and Amino Acid Analyses. N-terminal sequences were determined on an Applied Biosystems model 470A sequenator equipped with an on-line model 120A phenylthiohydantoin analyzer. C-terminal sequencing was done essentially as described (11). Aliquots (20 μl) of AMY1 samples (≈100 μM, determined by amino acid analysis) in 50 mM Mes, pH 6.3/1 mM EDTA were removed at time intervals, and 3 μl of 1 M HCl was added to stop the reaction. Malt carboxypeptidase II (0.5 μM) was used for AMY1-3 and AMY1-4 and yeast carboxypeptidase Y (0.5 μM) for AMY1-1 and AMY1-2. For malt AMY1, yeast carboxypeptidase Y (10 μM) was used in 0.5% SDS. The amino acid analyses were done on an LKB model Alpha Plus analyzer with norleucine as internal standard. Amino acid compositions of AMY1 forms were obtained on 0.2–0.5 nmol of protein hydrolyzed for 24 hr (triplicate) or 72 hr (duplicate) in 6 M HCl at 110°C using a Durrum model D-500 analyzer. Valine, isoleucine, and leucine were determined after 72 hr, while serine and threonine contents were extrapolated to zero time of hydrolysis.

Limited Proteolysis. Recombinant AMY1, malt AMY1, or malt AMY2 (45 μM) were incubated with proteinase K (4.5 μM) at room temperature for 24 hr essentially as described (12).

$^{45}Ca^{2+}$ Exchange. AMY1 contains one Ca^{2+} equivalent (13), which exchanged by overnight dialysis of AMY1 from malt or yeast (100 μM) against 50 mM Hepes, pH 7.4/0.1 mM $CaCl_2$ (200 μCi of $^{45}Ca^{2+}$/mmol of Ca^{2+}) in 100 mM KCl. The radiolabeled AMY1 (4 μg per lane) was subjected to isoelectric focusing, and the gel was immediately dried and autoradiographed.

ELISA. Five monoclonal antibodies (25-7-G5-B11, 29-8-I6-D9, 46-1-G4-D5, 46-3-D4-G8, and 24-10-G5-B4) against malt AMY1, isolated and characterized by Xingzhi Wang (Carlsberg Laboratory), were used with horseradish peroxidase-conjugated secondary antibodies (p161, Dakopatts, Glostrup, Denmark) for ELISA of recombinant AMY1 forms.

RESULTS AND DISCUSSION

Purification and Characterization of Recombinant AMY1 Forms. AMY1 (≈100 mg) was recovered from the yeast culture supernatant using β-cyclodextrin-Sepharose. The individual forms AMY1-1–AMY1-4 with pI values increasing from 4.7 to 5.1 were further purified on CM-fractogel (Fig. 1) to homogeneity as verified by isoelectric focusing (Fig. 2). From 100 liters of culture supernatant ≈6, 8, 3, and 20 mg were prepared of AMY1-1, AMY1-2, AMY1-3, and AMY1-4, respectively.

The N-terminal sequence of all four recombinant forms, His-Gln-Val-Leu-Phe, was identical to that of AMY1 purified from malt (5). The amino acid composition showed higher contents of arginine and serine in AMY1-3 and AMY1-4 than in AMY1-1 and AMY1-2, indicating that the C-terminal dipeptide Arg-Ser was absent in the two forms of lower pI. C-terminal sequencing, using either yeast carboxypeptidase Y or malt carboxypeptidase II, confirmed that AMY1-4 and AMY1-3 matched the entire cDNA coding region, whereas AMY1-2 and AMY1-1 both lacked Arg-Ser (Fig. 3). Different reactivity toward dithionitrobenzoate at pH 8.0 for AMY1-1/AMY1-3 versus AMY1-2/AMY1-4 suggested that the two AMY1 pairs differ in oxidation state of cysteine residues. AMY1-1/AMY1-3 thus had two free SH groups, one of which

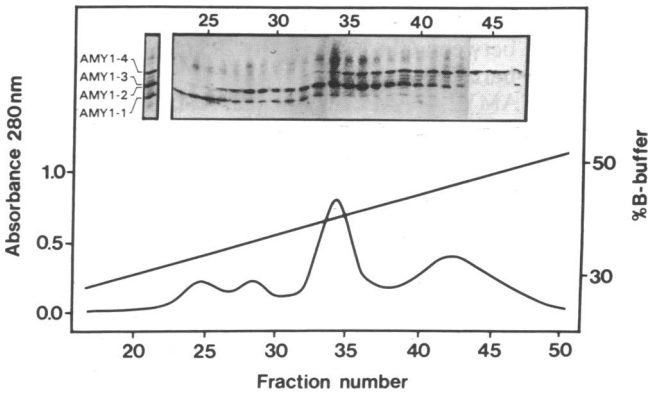

FIG. 1. Purification of recombinant AMY1 forms. Recombinant AMY1 purified by affinity chromatography on β-cyclodextrin-Sepharose was subjected to CM-fractogel 650M chromatography, as described. Purification was followed by isoelectric focusing in a PhastGel (*Inset*), which was silver stained. Fractions 33–37 were pooled and rechromatographed to obtain pure AMY1-2 and AMY1-3. Fractions 22–25 and 44–49 were pooled as pure AMY1-1 and AMY1-4, respectively. A linear gradient of buffer A (10 mM NaOAC, pH 4.3/1 mM $CaCl_2$) and buffer B (10 mM NaOAC, pH 5.3/1 mM $CaCl_2$/0.1 M NaCl) was used. The percentage of buffer B is indicated.

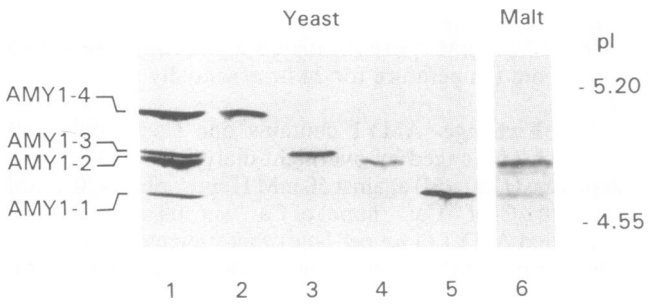

FIG. 2. Isoelectric focusing of purified recombinant AMY1 forms. Lanes: 1, unfractionated, recombinant AMY1; 2, purified AMY1-4; 3, AMY1-3; 4, AMY1-2; 5, AMY1-1; and 6, AMY1 isolated from malt is shown after Coomassie staining. Approximately 0.2 μg of protein was applied per lane.

reacted very slowly. In comparison AMY1-2/AMY1-4 had three SH groups, and malt AMY1 had two SH groups, all of intermediate reactivity. Higher glutamate/glutamine and glycine (Table 1) and lower SH contents and lower pI values of AMY1-1/AMY1-3 were found to be due to the covalent attachment of approximately one glutathione per molecule. Thus, reduction of AMY1-1 and AMY1-3 with dithiothreitol converted them into the fully active forms AMY1-2 and AMY1-4, respectively (data not shown).

Isoelectric focusing and autoradiography (data not shown) indicated that the AMY1 forms readily exchanged Ca^{2+} for $^{45}Ca^{2+}$. Also their structural integrity was maintained at the β-cyclodextrin binding site, as seen from the retention on β-cyclodextrin-Sepharose. Moreover, three monoclonal antibodies (25-7-G5-B11, 46-1-G4-D5, and 46-3-D4-G8) directed against malt AMY1 reacted with AMY1-1–AMY1-4, whereas two others (24-10-G5-B4 and 29-8-I6-D9) probably recognized a different malt AMY1 isozyme. The recombinant AMY1 forms were not more susceptible to limited proteolysis with proteinase K than malt AMY1. This result indicates that anomalous modification of cysteine residues in yeast does not destabilize or change the overall conformation of the protein. However, several cysteine residues are near functional groups (14), and their modification with glutathione is responsible for the low enzymatic activity of AMY1-1 and AMY1-3. Although proteolysis of AMY1 was slower than that of AMY2, fragments produced from both were of ≈30 kDa and 14 kDa (data not shown) and found in AMY2 to arise by cleavage between Gln-294 and His-295 (12) within a loop of the $(\beta\alpha)_8$-barrel domain (14).

Although AMY1-4/AMY1-2 and malt AMY1 were equally active on *p*-nitrophenyl maltoheptaoside, the activity of AMY1-3/AMY1-1 was 3–4% due to a low k_{cat} (Table 2). The pH optimum for starch hydrolysis was between pH 4 and pH

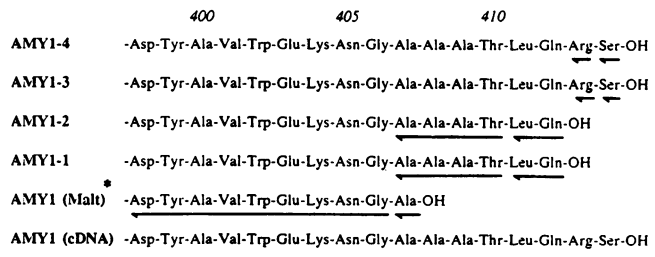

FIG. 3. C-terminal sequence of AMY1 forms. C-terminal sequences of AMY1-1–AMY1-4 from yeast and AMY1 from malt were determined by enzymatic digestion with yeast carboxypeptidase Y or malt carboxypeptidase II. The C-terminal sequence of nontruncated AMY1 is deduced from clone E cDNA (9). Residue numbering starts at the N-terminal histidine (5, 9). Arrows indicate the observed order of release of amino acids from the C terminus. *, C-terminal sequence inferred from the initial release of one alanine per molecule.

Table 1. Amino acid composition of AMY1 forms

Amino acid	AMY1-1	AMY1-2	AMY1-3	AMY1-4	AMY1 cDNA*	AMY1 Malt
Asx	56.8	55.0	56.2	55.9	54	55.3
Thr	17.9	17.8	17.9	18.0	17	17.5
Ser	20.5	20.4	21.4	21.8	21	21.3
Arg	13.9	13.8	14.8	15.0	15	13.8
Glx	26.3	24.8	26.9	25.2	25	26.2
Gly	46.9	45.6	48.3	46.8	44	47.6
Ala	47.6	47.8	49.1	48.2	47	43.7
Val	29.4	29.6	30.1	29.9	28	28.2
Ile	21.2	21.3	21.8	21.7	23	21.9
Leu	24.6	24.7	24.9	25.0	25	23.8
Phe	14.8	14.7	14.4	14.6	15	14.1
His	12.6	12.9	13.2	12.8	13	12.5
Lys	22.1	22.1	22.5	22.3	22	19.8
Pro	17.1	17.0	16.8	17.0	17	ND

Amino acid composition of AMY1 forms was determined as described. Cysteine, methionine, tyrosine, and tryptophan contents are not reported. ND, not determined.
*Data deduced from refs. 5 and 9.

6 for malt AMY1 (8) and all four recombinant forms (data not shown). In summary, the structural differences between the four AMY1 forms produced by clone E expression in yeast consist in loss of (*i*) the C-terminal Arg-Ser in AMY1-1/AMY1-2 and of (*ii*) free SH group(s) in AMY1-1/AMY1-3 due to reaction with glutathione, which, perhaps, accounts for their low activity (Table 2) and pI (Fig. 2).

***In Vitro* Processing of Recombinant AMY1 Forms.** AMY1-2 is generated *in vitro* by incubation of AMY1-4 with either malt carboxypeptidase II (Fig. 4) or yeast carboxypeptidase Y (data not shown), and similarly AMY1-1 is produced from AMY1-3. No change in pI occurred when either AMY1-2 or AMY1-1 is treated with the two carboxypeptidases. Malt carboxypeptidase II prefers basic and yeast carboxypeptidase Y large hydrophobic side chains (15). Accordingly, processing of AMY1-3 and AMY1-4 with a penultimate arginine is efficiently catalyzed by malt carboxypeptidase II. C-terminal processing of heterologous proteins secreted from yeast has previously been found for human atrial natriuretic peptide (16), human epidermal growth factor (17), and leech hirudin HV2 (18). Two of these (16, 17) possess a basic residue at either the penultimate or the terminal position. Apparently a carboxypeptidase specific for basic residues operates in the secretory pathway, and a likely candidate is the *KEX1*-encoded membrane-bound carboxypeptidase known to remove C-terminal Lys-Arg from α-factor and killer toxin, secreted by certain yeast strains (19). Leech hirudin, however, has no basic amino acid near the C terminus, and its processing, which progressed with culture time, is more likely due to vacuolar carboxypeptidases released by cell lysis (18). Using the *kex1* yeast mutant M204-8C, we have

Table 2. Kinetic properties of AMY1 forms on *p*-nitrophenyl maltoheptaoside

Enzyme	k_{cat}, $10^1 \times s^{-1}$	K_M, $10^3 \times M$	k_{cat}/K_M, $10^4 \times s^{-1} \cdot M^{-1}$
AMY1 (yeast)	45	0.7	64
AMY1-1	1.7	0.5	3.2
AMY1-2	64	0.7	93
AMY1-3	2.3	0.5	4.6
AMY1-4	63	0.7	91
AMY1 (malt)	67	0.7	95

Kinetic parameters toward *p*-nitrophenyl maltoheptaoside were determined at 37°C in a coupled assay using yeast maltase at pH 6.2 in 20 mM 2-morpholinoethanesulfonic acid/1 mM calcium chloride, as described (8).

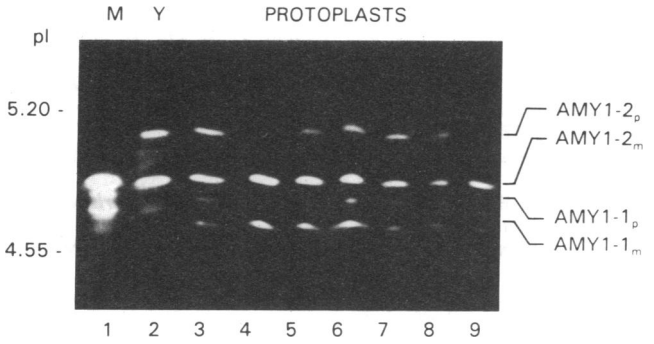

FIG. 4. *In vitro* conversion of AMY1. Isoelectric focusing of AMY1 forms treated with malt carboxypeptidase II (PhastGel, Coomassie stained). Unfractionated, recombinant AMY1 (S) (lanes 1 and 4), AMY1-4 without (lane 2) and with (lane 3) enzyme digestion, and AMY1-3 without (lane 5) and with (lane 6) enzyme incubation. Approximately 0.2 μg of protein was applied per lane.

demonstrated that the *KEX1* gene product is responsible for C-terminal processing of AMY1. AMY1-3 and AMY1-4 are obtained upon AMY1 cDNA expression in this *kex1* strain, whereas all four forms AMY1-1–AMY1-4 are secreted by the closely related wild-type strain T109-3C (to be described in detail elsewhere).

***In Vivo* and *in Vitro* Processing of AMY1 from Barley Aleurone Protoplasts.** Jacobsen, Jones, and coworkers (6, 7) discovered in aleurone protoplasts two transient AMY1 precursors that were converted into molecular forms with a lower pI and showed that the conversion did not involve sulfation, phosphorylation, fatty acid acylation, N-glycosylation, or limited proteolysis by thiol proteases. Our findings on the processing of AMY1 in yeast motivated us to investigate whether C-terminal proteolysis of barley AMY1 occurs *in vivo*. When aleurone protoplasts were cultured in the presence of the serine carboxypeptidase inhibitors bacitracin, phenylmethylsulfonyl fluoride, or benzylsuccinic acid, conversion of the precursor AMY1-2 to mature AMY1-2 and of the precursor AMY1-1 to mature AMY1-1 was clearly suppressed (Fig. 5, lanes 5–7 vs. lane 4). Addition of exogenous malt carboxypeptidase II accelerated the processing (Fig. 5, lane 9 vs. lane 8). The [^{35}S]methionine-labeled precursors of AMY1-1, AMY1-2, and AMY1-3 purified from protoplast lysates were converted *in vitro* by exogenous malt carboxypeptidase to their corresponding mature forms (Fig. 6). The hitherto unreported pair AMY1-3 precursor/mature AMY1-3 (Fig. 6) was more abundant when protoplasts were prepared from grains of the 1988 harvest (Fig. 6) than from grains of the 1985 (Fig. 5) or 1979 (6, 7) harvests. Taken together these observations provide evidence that shortly after synthesis C-terminal truncation catalyzed by serine carboxypeptidase(s) leads to the AMY1 forms of lower pI. In particular, the inhibitory effect of benzylsuccinic acid, which is a specific inhibitor for serine carboxypeptidases (20, 21) strongly suggests a role of such enzymes. Malt AMY1 lacks the C-terminal heptapeptide (Fig. 3), which supports the notion that carboxypeptidases process amylases in the grain during germination. The loss of seven C-terminal residues in malting is expected to confer the same pI shift as the removal of only two in yeast because in both cases the only charged residue removed is Arg-413 (Fig. 3). The present work demonstrates carboxypeptidase-catalyzed trimming of a secreted enzyme in a higher plant. The physiological function of C-terminal truncation of AMY1 remains to be elucidated. C-terminal processing in plants is required for vacuolar targeting of lectins and β-1,3-glucanases (22) and possibly also for mobilization of bound barley β-amylase during seed germination (23). The precursor AMY1 C terminus, however, lacks a putative N-glycosylation site (22) and a repeated protease target sequence (23)—i.e., properties considered significant for processing. Recently, mature barley peroxidase was found to lack the C-terminal part, including a putative N-glycosylation site of its precursor (24).

Large amounts of carboxypeptidases and α-amylases are secreted to the endosperm during germination (1, 2), which may explain why only trace amounts of AMY1 precursors remain in malt extracts. The proportion of mature AMY1 is higher when protoplasts are cultured in the presence of the gibberellin GA_3 (Fig. 5, lane 4 vs. lane 8), possibly reflecting an effect of GA_3 on the amount of active carboxypeptidase. GA_3 is already known to induce expression of a carboxypeptidase I gene in wheat (25). Malt carboxypeptidase II, an abundant enzyme in germinated barley (26, 27) has *a priori* the best specificity fit for the C-terminal Arg-Ser (15, 26). Combined action of several of the five reported malt carboxypeptidases (26) is likely to be responsible for removal of the seven C-terminal amino acids from the AMY1 precursor.

Variations in the amount of the three AMY1 precursor/product pairs in germinating grains from different harvests indicate that they originate from different genes. The synthesis of AMY1-1 requires GA_3 and Ca^{2+}, whereas that of AMY1-2 is less dependent on GA_3 (6). AMY1-3 was found in significant amounts in germinating grains from the 1988, but

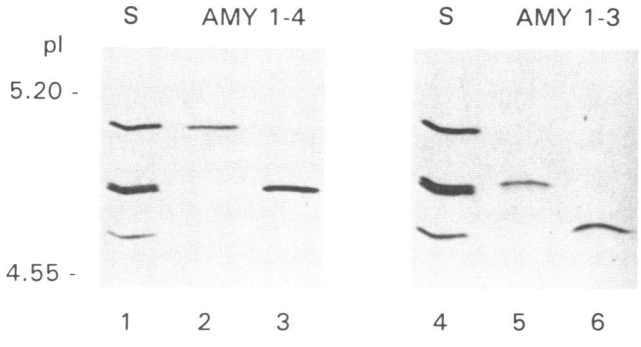

FIG. 5. Inhibition and activation of the AMY1 processing in aleurone protoplasts. Isoelectric focusing (Ampholine PAG plates) of culture medium and lysates from Himalaya barley aleurone protoplasts (grains from the 1985 harvest) stained for amylase activity. Lanes: 1, AMY1 from malt (M); 2, AMY1 from yeast (Y); 3, lysate of protoplasts grown with 1 μM gibberellin A_3 (GA_3); 4–7, supernatants of protoplasts grown with 1 μM GA_3. In lanes 5–7, the serine carboxypeptidase inhibitors bacitracin, phenylmethylsulfonyl fluoride, and benzylsuccinic acid had been added, respectively, to a final concentration of 5 mM. Lanes: 8 and 9, supernatants of protoplasts grown without GA_3. In lane 9 malt carboxypeptidase II was added to 2 μM. One to two μg of AMY was applied per lane.

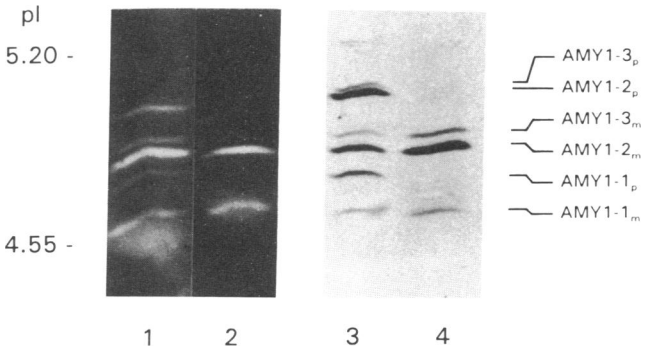

FIG. 6. *In vitro* maturation of AMY1 from aleurone protoplasts. Isoelectric focusing (PhastGel) of AMY purified from lysates of Himalaya barley aleurone protoplasts (grains from the 1988 harvest) visualized by either activity staining (lanes 1 and 2) or autoradiography of the [^{35}S]methionine-labeled proteins (lanes 3 and 4). Samples were incubated without (lanes 1 and 3) or with (lanes 2 and 4) malt carboxypeptidase II. AMY at 0.1–0.2 μg was applied per lane. Subscript p, precursor; subscript m, mature form.

not in those from the 1985, harvest, adding to the observations that the AMY pattern depends on both the growth conditions and cultivar (1, 2). It is proposed that the three AMY1 pairs observed in aleurone cells (Fig. 6) correspond to the earlier reported three structural genes (3, 4) for AMY1 and the two major and one minor mRNA species (2). AMY1-2 is probably encoded by the gene corresponding to clone E cDNA because forms of identical pI are produced upon its expression in *Xenopus* oocytes (28) and yeast (Fig. 5, lanes 2 and 3). Most likely, AMY1 is also C-terminally truncated in *Xenopus* oocytes.

We thank Mss. Sidsel Ehlers and Maj-Britt Rask for expert technical assistance; Mr. Jens Olsen (Danisco A/S–De Danske Spritfabrikker, Copenhagen) for assistance with the large-scale fermentations; Dr. Ib Svendsen and Mss. Bodil Corneliussen and Lone Sørensen for amino acid and N-terminal sequence analyses; and Mss. Ann-Sofi Steinholz and Nina Rasmussen for preparation of photographs and figures, respectively. We are grateful to Drs. Klaus Breddam and Steen Bech Sørensen for the purified CPDY and CPDMII; to Dr. Xingzhi Wang for the five monoclonal antibodies to malt AMY1 and help with ELISA assays; to Dr. John C. Rogers (St. Louis) for clone E; and to Dr. David Y. Thomas (Montreal) for the gift of yeast strains M204-8C and T109-3C. Additionally, we thank Profs. Klaus Bock and Diter von Wettstein for continuous support. F.L.O. held a fellowship from the Research Center for Plant Biotechnology, The Danish Biotechnology Program 1987–1990, and has financial support from the Eureka Project nr. Eu 270 [Adaptation of Barley for Industrial Needs (ABIN)]. This is ABIN publication 63.

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