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The expression of serine carboxypeptidases during maturation and germination of the barley grain

(protease/storage protein/hordein/aleurone/processing enzyme)

FLORENCE DAL DEGAN*†, ANNE ROCHER*, VERENA CAMERON-MILLS†‡, AND DITER VON WETTSTEIN*

*Department of Physiology, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby, Denmark; and †Weissheimer Research Laboratory, Schaarstrasse 1, D-56626 Andernach, Germany

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ABSTRACT **cDNA clones encoding three additional serine carboxypeptidases (Ser-CPs) have been isolated from a gibberellic acid-induced barley aleurone cDNA library. The three deduced Ser-CPs belong to the two-chain subfamily of Ser-CPs; they are synthesized as precursors with a putative signal peptide, propeptide, and linker peptide between the A and B chains. Their identification provides the proof for the existence of more than three Ser-CPs in cereal grains, and, based on their sequences, they may exhibit new substrate specificities. The expression of these and of the three previously isolated Ser-CPs from barley grains (CP-MI, CP-MII, and CP-MIII) has been investigated by Northern and Western analysis and RNA PCR. CP-MII is the only Ser-CP to be expressed and accumulate in the developing grain and is stored in its active form in the mature grain. All six Ser-CPs are expressed *de novo* in the germinating grain, in the scutellum, and/or in the aleurone. Furthermore, at least CP-MI, CP-MII, and CP-MIII are secreted into the endosperm. In addition, all Ser-CPs (except CP-MI) are also expressed in the roots and shoots of the growing seedling. This enzyme family thus appears to be ubiquitous in the barley plant, which suggests that Ser-CPs play additional roles besides their participation in the mobilization of storage proteins.**

During germination of cereal seeds, the scutellum and the aleurone layer, surrounding the starchy endosperm, synthesize hydrolases in response to the phytohormone gibberellic acid (GA_3) released by the embryo. Many of these enzymes are secreted into the endosperm and mobilize storage macromolecules to support the growth of the seedling (1). In particular, the breakdown of storage proteins is effected by the concerted action of endoproteases and exoproteases. Serine carboxypeptidases (Ser-CPs) are the major exopeptidases found in germinating cereal seeds. Enzymatic assays with various N-blocked dipeptide substrates reveal that Ser-CP activity is present in the resting barley grain and increases during germination. Chromatographic separation of protein extracts from germinating barley (2) or wheat (3) seeds yields five fractions exhibiting different Ser-CP activities, suggesting the existence of five Ser-CPs. However, only two two-chain barley (CP-MI and CP-MII), one single-chain barley (CP-MIII), and one two-chain wheat (CP-WII) Ser-CPs have been isolated and characterized from germinating seeds (4–6) and have partially different substrate preferences.

Hammerton and Ho (7) established that aleurone layers isolated from mature barley seeds synthesize a Ser-CP. In response to GA_3, the synthesis is enhanced and the protein is secreted. This Ser-CP is assumed to be CP-MIII, based on substrate preference and inhibition by mercuric ions. Two genes encoding Ser-CPs homologous to CP-MIII, isolated from wheat (8) and rice (9), are also expressed in the aleurone during germination. In contrast, expression of CP-MI has only been detected in the scutellum of germinating barley seeds (10).

We have endeavored to study the temporal and tissue specificity of the synthesis of each Ser-CP, in order to obtain information as to their function in the cereal seed. Several previous investigations used activity assays to detect Ser-CPs, but unfortunately these cannot distinguish the individual Ser-CPs, since there is no absolutely specific substrate for any of these enzymes. In this report, we provide proof for the existence of additional Ser-CPs in barley seeds by the isolation of cDNA clones§ encoding three additional Ser-CPs. We have studied the expression of each Ser-CP in the different tissues of the developing and germinating barley grain by using cDNA probes. Specific antibodies raised against CP-MI, CP-MII, and CP-MIII have enabled us to follow the fate of each of these enzymes, in particular their secretion into the endosperm.

MATERIALS AND METHODS

Plant Material. Barley plants (*Hordeum vulgare* L. cv. Alexis) were grown in growth chambers (16 h light, 15°C/8 h dark, 10°C). Mature seeds for germination studies were dehusked, surface sterilized, and germinated in Petri dishes on two layers of 3MM sterilized filter paper plus 6 ml of sterile water. The dishes were sealed with Nescofilm (Nippon Shoji Kaisha, Osaka), and the seeds were allowed to germinate for 1–5 days in the dark at 10°C.

Carboxypeptidase cDNA Probes. Partial cDNA clones encoding CP-MI (667 nt, from A-chain residue 213 to B-chain residue 115), CP-MII (375 nt, B-chain residues 31–159), and CP-MIII (480 nt, residues 76–235) were synthesized by RNA PCR (Perkin–Elmer/Cetus) from poly(A)$^+$ RNA from scutellum of 2-day-germinated embryos. The oligonucleotide primers (Applied Biosystems 394 DNA/RNA synthesizer) were based on the nucleotide sequence of CP-MI (11) or the protein sequences of CP-MII and CP-MIII (12), incorporating inosine in redundant codons. RNA PCR fragments were cloned in pUC18, and their identity was established by sequencing.

cDNA Library Screening. A 24-h GA_3-treated barley aleurone cDNA library of 1×10^5 λZAPII primary clones, kindly provided by R. Leah (13), was screened at 20°C below the t_m with the CP-MII (*Cxp;2*) partial cDNA probe. From 40 positive clones, 15 were characterized and 3 were sequenced by Erase-a-Base (Promega) and PCR cycle sequencing on an Applied Biosystems 373 DNA sequencer.

RNA Extraction. Total RNA was extracted from tissues dissected from developing and germinating barley grains as described in ref. 14. In the case of aleurone layers, polyphe-

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Abbreviations: GA_3, gibberellic acid; Ser-CP, serine carboxypeptidase; CP-M: barley Ser-CP.

‡To whom reprint requests should be addressed.

§The cDNA sequences of *Cxp;2-1*, *Cxp;2-2*, and *Cxp;2-3* have been deposited in the GenBank data base (accession nos. X78876, X78878, and X78877).

nolic pigments, which coprecipitated with the RNA, were removed according to ref. 15.

Northern Blot Analysis. Total RNA samples were separated in 6% formaldehyde gels (16) and blotted onto Hybond N+ membranes (Amersham) according to the manufacturer's instructions. DNA probes were ^{32}P-labeled using a random-primed DNA labeling kit (Promega). The membranes were prehybridized and hybridized at 60°C in 50% (vol/vol) formamide/1% SDS/5× Denhardt's solution/1–2× SSC/sonicated herring sperm DNA (100 μg/ml). The membranes were subsequently washed in 0.1× SSC/1% SDS at 60°C and exposed to x-ray film.

RNA Quantitation by RNA PCR. RNA PCR was performed with a Perkin–Elmer/Cetus kit. The RNA PCR products were analyzed by Southern blotting and hybridized as above with the omission of formamide. Signals were quantified with a PhosphorImager (Molecular Dynamics).

Production of Polyclonal Antibodies to CP-MI, CP-MII, and CP-MIII. Pure CP-MII (5) and partially pure CP-MI (4) were kindly provided by K. Breddam (4, 5). Purification of CP-MI to homogeneity was by FPLC ion-exchange chromatography on a Mono Q column (Pharmacia). CP-MIII was purified according to ref. 6 with the following modifications. After the affinity chromatography step, the purification of CP-MIII to homogeneity was achieved by two subsequent ion-exchange chromatography steps on S Sepharose Fast Flow (Pharmacia) at pH 5.0 and TSK-DEAE (Merck) at pH 6.7, followed by a gel-filtration step on Sephacryl S-200 HR (Pharmacia). Polyclonal antibodies were raised against the purified CP-MI and CP-MIII. The glycans attached to CP-MII proved to be highly immunogenic. Thus, CP-MII was chemically deglycosylated with anhydrous trifluoromethanesulfonic acid according to the manufacturer's instructions (Glycofree deglycosylation kit; Oxford Glycosystems, Abingdon England) prior to immunization. Rabbits were immunized subcutaneously at 4-week intervals with 50–150 μg of pure CP-MI or deglycosylated CP-MII emulsified (1:1, vol/vol) with Freund's adjuvant. Mice were immunized intraperitoneally with 5–15 μg of pure CP-MIII, emulsified as above, at 3-week intervals.

Protein Extraction. Protein was extracted from 10 grains or parts thereof at 4°C for 20 min with 4 ml of a 0.1 M NaOH/1% 2-mercaptoethanol mixture. The homogenates were centrifuged at 15,000 × *g* (15 min), and 4 ml of an acetone/trichloracetic acid mixture (80:20, vol/vol) was added to the supernatant. Proteins were allowed to precipitate for >1 h at −18°C and centrifuged for 15 min at 10,000 × *g*. The protein pellets were washed two times with 2 ml of 80% acetone before solubilization in SDS/PAGE sample buffer.

Western Blotting and Immune Assays. After SDS/PAGE in 12% acrylamide gels and wet Western blotting onto nitrocellulose membranes (Schleicher & Schuell), the membranes were treated with 15 ml of a 1% sodium metaperiodate solution for 15 min and washed with distilled water to eliminate antiserum cross-reactions due to carbohydrates. For immune assays, incubation with the primary antibody, diluted 1:5000 or 1:10,000, and subsequently with alkaline phosphatase-conjugated rabbit anti-mouse or swine anti-rabbit secondary antibody (Dako), diluted 1:2,000, was carried out in 1% skim milk/0.6 M NaCl/0.2% Tween 20 (omitted for incubation with the secondary antibody) in phosphate-buffered saline (pH 7.2) for 2 h at 37°C.

RESULTS

Isolation and Characterization of Three Ser-CP cDNA Clones Encoding Additional Members of the Carboxypeptidase Family in Barley. A cDNA library from poly(A)$^+$ RNA isolated from 24-h GA_3-treated aleurone layers was screened with a CP-MII (*Cxp;2*) partial cDNA probe. Three independent clones of 1252 bp (*Cxp;2-1*), 1580 bp (*Cxp;2-2*), and 1979 bp (*Cxp;2-3*) were sequenced. The *Cxp;2-1*, *Cxp;2-2*, and *Cxp;2-3* cDNAs have an open reading frame extending from base 1 to 972, 1 to 1310, and 85 to 1632 and a 3′ untranslated region of 262, 254, and 328 nt, respectively. *Cxp;2-3* is the only full-length clone, with a 84-nt 5′ leader sequence. None of the clones showed sequence identity with the *Cxp;2* cDNA probe.

Alignment of the three additional protein sequences with those of other Ser-CPs revealed three additional members of the Ser-CP family in barley (Fig. 1). The deduced CP-MII.1, CP-MII.2, and CP-MII.3 amino acid sequences share a high degree of identity with CP-MII (72%, 59%, and 53%, respectively) and to a lesser extent with CP-MI (37%, 38%, and 40%, respectively). Ser-CPs fall into two groups according to their primary structure: (*i*) the single-chain enzymes, such as CP-MIII, with ≈420 residues, and (*ii*) the two-chain enzymes, like CP-MI and CP-MII, composed of an A chain of around 260 residues and a B chain of about 160 residues. One extra disulfide bridge is present in the single-chain group (19). Doan and Fincher (11) showed that the two chains of CP-MI are encoded by a single mRNA. The A chain is encoded at the 5′ end of the mRNA and is in the newly synthesized precursor linked to the B chain by a 55-aa linker peptide. During enzyme maturation, the linker peptide is proteolytically excised. The deduced CP-MII.1, CP-MII.2, and CP-MII.3 precursors possess the characteristics of two-chain Ser-CPs. The aligned A- and B-chain protein sequences (Fig. 1) are separated by nonhomologous putative linker regions rich in proline and/or lysine and arginine residues. These sequences of 11–14 residues are, however, significantly shorter than the CP-MI linker peptide. Comparison of the aligned protein sequence of CP-MII.3 with the mature CP-MII protein reveals the presence of 77 extra N-terminal amino acids in CP-MII.3. The first 21 residues have the features of a typical eukaryotic signal peptide (20), whereas the following 56 residues are believed to make up a propeptide. Ser-CPs are normally synthesized as inactive preproenzymes with a propeptide of about 50–91 residues (9, 21).

Temporal and Spatial Expression of Ser-CPs in the Developing, Mature, and Germinating Barley Grain. The expression of the barley Ser-CP (*Cxp*) genes in various tissues of developing and germinating barley grains was analyzed with Northern blots using specific cDNA probes. Only *Cxp;2* mRNA was detected in developing tissues (Fig. 2), where it was present simultaneously in aleurone and endosperm between 20 and 30 days postanthesis and subsequently decreased over the following 10 days. *Cxp;1* and *Cxp;3* mRNAs were only detected in the germinating grain, where their levels were high (Fig. 2). The *Cxp;1* transcript was only detected in the scutellum and was most abundant after 1 day of germination, decreasing to a barely detectable level after 4 days. In contrast, *Cxp;3* mRNA was only expressed in the aleurone throughout the 5-day germination period with a maximum level at 3 days. The level of the *Cxp;2-1*, *Cxp;2-2*, and *Cxp;2-3* transcripts was below the detection limit by Northern blotting, and their quantitation required a more sensitive method. Each carboxypeptidase transcript was selectively amplified by RNA PCR and quantified with the appropriate cDNA probe (Fig. 3). The expression of the *Cxp;1* gene in the germinating embryo was confirmed; moreover, a lower level of expression was revealed in the aleurone, both during development and germination. *Cxp;2* transcripts were additionally detected in the germinating embryo. The *Cxp;3* gene was expressed only during germination, mainly in the aleurone, but also to a much lesser extent in the embryo. The *Cxp;2-1*, *Cxp;2-2*, and *Cxp;2-3* genes were expressed in the germinating embryo. Low levels of *Cxp;2-1* and *Cxp;2-2* mRNAs were also detected in the developing aleurone and embryo. All six mRNAs, with the exception of *Cxp;1* mRNA, were also expressed in the roots and to a lesser extent the shoots of the germinating seedling. The relative expression of *Cxp;2-1*, however, was greatest in the shoots.

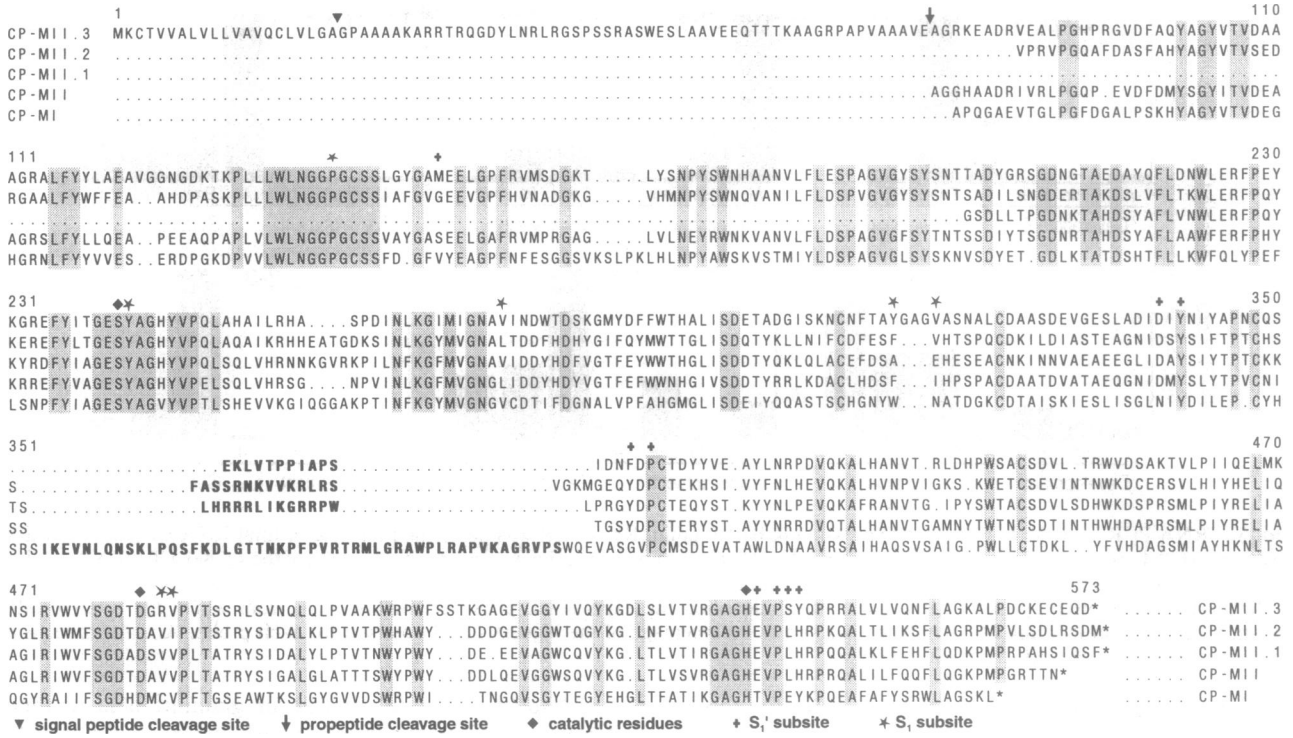

FIG. 1. Sequence comparison of barley two-chain Ser-CPs. CP-MII.1, CP-MII.2, and CP-MII.3 are deduced precursor protein sequences from the respective cDNA clones. CP-MI and CP-MII are the determined sequences of the mature proteins (12). The deduced sequence of the CP-MI linker peptide (11) is presented in boldface type. Residues of the catalytic site and of the S_1 and S_1' sites of Ser-CPs were determined from analogy with yeast carboxypeptidase Y and from the three-dimensional structure of CP-WII (17, 18). The C termini were determined by protein (CP-MI and CP-MII) or cDNA (CP-MII.1–.3) sequencing.

The accumulation and secretion of CP-MI, CP-MII, and CP-MIII proteins were investigated on Western immune blots of protein extracts from developing, mature, and germinating tissues (Fig. 4). During seed maturation, CP-MI is present at a low level in its mature processed form in the aleurone and in the endosperm at an early stage. The enzyme is subsequently degraded, since it is absent from the mature grain. CP-MI is synthesized *de novo* early in germination in the scutellum, increasing considerably from day 1 to day 2, and is stable in this tissue. Low levels were detected in the endosperm, indicating secretion from the embryo, since the endosperm loses vitality at seed maturation. Although CP-MI transcripts can be found in the aleurone during germination, this does not lead to the accumulation of detectable levels of CP-MI. CP-MII appeared simultaneously in the developing aleurone and endosperm between 14 and 20 days postanthesis and accumulated in both tissues, resulting in high levels in the mature grain. During germination, CP-MII accumulated in the embryo between days 1 and 5. The amount of CP-MII in the aleurone during germination was stable, which reflects the absence of CP-MII transcripts. The abundance of CP-MII in the endosperm after 1 day of germination was notably higher than that in the mature grain, which indicates that CP-MII was secreted from the embryo. In the developing and mature grain, CP-MII was present in its mature form and was active (data not shown). CP-MIII appeared specifically during germination in the aleurone. It was, in addition, barely detectable in the embryo, reflecting the level of mRNA in these two tissues. CP-MIII was most abundant in the endosperm, indicating an efficient secretion from the aleurone and/or the embryo.

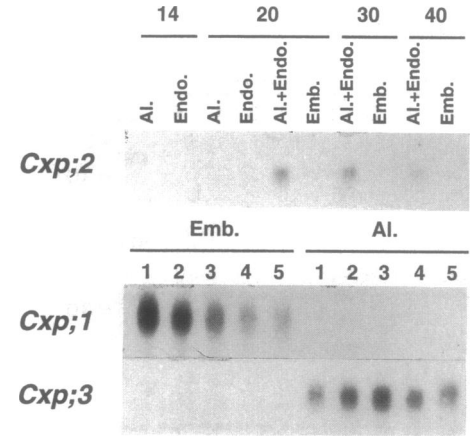

FIG. 2. Northern analysis of *Cxp* gene expression in barley grain. Total RNA extracted from developing (14–40 days postanthesis) and germinating (1–5 days) seeds (20 μg from each tissue but 10 μg from aleurone of germinating grains) was separated on 6% formaldehyde gels, blotted, and hybridized with probes specific for each of the six Ser-CP mRNAs. Signals were observed only in the autoradiograms shown in the figure. Al., aleurone; Endo., endosperm; Emb., embryo.

DISCUSSION

The three additional cDNA clones provide unequivocal evidence for the existence of more than three Ser-CPs in the barley grain. Furthermore, our three clones represent further examples of two-chain Ser-CPs that are synthesized as single-chain protein precursors with a central linker peptide, previously only demonstrated for CP-MI (11). As in CP-MI, the three linkers are rich in arginine, lysine, and/or proline residues, but they are significantly shorter. As they are largely hydrophilic, they probably form a loop on the surface of the molecule and may carry sequence or structural signals necessary for the correct folding and transport of the cognate enzyme. Considering the high degree of identity between CP-WII (wheat homolog of CP-MII, 95% sequence identity), CP-MII, CP-MII.1, CP-MII.2, and CP-MII.3, one can as-

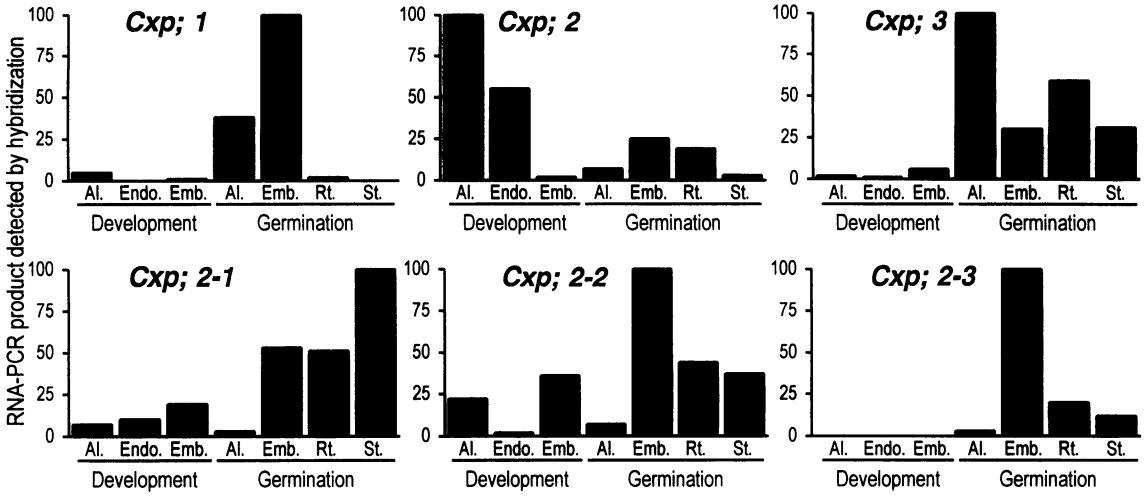

FIG. 3. RNA PCR analysis of *Cxp* gene expression in barley grain. RNA PCR was performed on 1 μg of total RNA extracts from tissues of developing (20 days postanthesis) and germinating (3 days) barley grain, using primer pairs specific for each of the six Ser-CP mRNAs. Aliquots withdrawn during PCR amplification were separated on 0.7% agarose gels, blotted, and hybridized with the corresponding probe. The quantified hybridization signal plotted against PCR cycle number identified the exponential phase of amplification, from which the relative mRNA abundance was determined. mRNA abundance is given as a percentage of the highest signal obtained for each probe. Al., aleurone; Endo., endosperm; Emb., embryo; Rt., root; St., shoot.

sume that these enzymes have a similar fold. In the three-dimensional structure of CP-WII (17, 18), the C terminus of the A chain and the N terminus of the B chain lie in close proximity to each other and to the active site of the enzyme. While a linker of 20 or more amino acids may serve to cover the active site and inhibit the enzyme, this role may be structurally impossible for the shorter linkers of CP-MII.1–3.

The three-dimensional structure of CP-WII has allowed the identification of the residues crucial for the substrate specificity of the two-chain Ser-CPs (17, 18, 22). These residues, which make up the binding subsite for the C-terminal substrate residue (S_1' site) and for the penultimate substrate residue (S_1 site), are shown in Fig. 1. In the CP-MII.2 sequence, these residues are similar to those of CP-MII. However, while CP-MII.1 and CP-MII.3 have residues similar to those of CP-MII in their S_1' subsite, they differ from CP-MII in the residues making up their S_1 subsite and may therefore exhibit new substrate specificities. In particular, the presence of Arg-485 may render CP-MII.3 efficient toward substrates with aspartic or glutamic acid at their C terminus, which are normally poorly hydrolyzed by Ser-CPs.

The maturation of Ser-CPs requires the cleavage of the propeptide and, for the two-chain Ser-CPs, the linker peptide. Since Ser-CPs have no endoproteolytic activity, their maturation involves other enzymes. Processing of precursor polypeptides in the plant cell is still obscure and can occur at a wide variety of sites in the sequences. However, a barley aspartic protease (23–25) has been described that shares sequence and specificity similarities with yeast proteinase A, a protease participating in the processing of the yeast Ser-CP, carboxypeptidase Y (26). The barley enzyme can process 2S albumin precursors *in vitro* (27), suggesting a regulatory function for this protease and a possible role in processing of propeptides and linkers in barley Ser-CPs. Such a processing mechanism would require the concerted action of endoproteases and exoproteases to yield the correct N and C termini. All five barley two-chain Ser-CPs have serine residues at the actual or putative C terminus of their A chain and of their

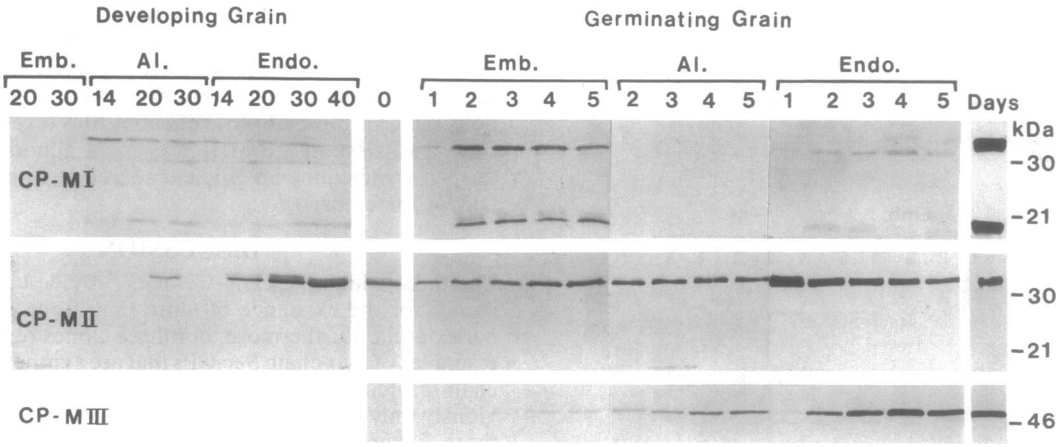

FIG. 4. Accumulation and secretion of Ser-CPs CP-MI, CP-MII, and CP-MIII in barley grain. Western blots of protein extracts from developing (14–40 days postanthesis), mature (0 days), or germinating (1–5 days) barley grains were incubated with CP-MI, CP-MII, or CP-MIII antiserum as indicated. Between 40 days postanthesis and 1 day of germination, the aleurone and endosperm could not be separated; therefore, the lanes Endo.40 (of the developing grain) and Endo.1 (of the germinating grain) represent both tissues. The lane on the far right represents controls with pure CP-MI–III. For each tissue, an equivalent proportion of the protein extracts was loaded in each lane. The 32- and 28-kDa proteins seen in the CP-MI immune blot were recognized by antibodies present in the serum prior to immunization with CP-MI and were not related to CP-MI. The CP-MII antiserum only recognized the A chain of CP-MII. For abbreviations see Fig. 2.

known linkers, with the exception of CP-MII.1 (see Fig. 1). This suggests that excision of the linker peptide by cleavage of the two peptide bonds after the serine residues flanking the linker could alternatively be achieved by a single endoprotease, other than the above-mentioned aspartic endoprotease, with the required specificity. Peptide hormones and the yeast mating factor are synthesized as large precursors containing several repeats of the mature peptide separated by sequences rich in basic amino acids. Maturation of such peptides is achieved by the joint action of specific endoproteases, cleaving after dibasic sites, and of exopeptidases, trimming the peptide to its mature form (28). Although well characterized in animals and yeast, no such endoprotease has been described in plants. The abundance of basic residues in the linkers of two-chain Ser-CPs suggests a role for such processing endoproteases and represents a third hypothesis for the maturation process of Ser-CPs.

The use of monospecific antibodies has shown that during grain maturation CP-MII, in its mature active form, is the only Ser-CP to accumulate in significant amounts in the endosperm. It is also found in its active form in the mature grain. As judged by transcript analysis, germination is accompanied by *de novo* synthesis of all six Ser-CPs, either in the embryo or in the aleurone, and their partial secretion into the endosperm, in accordance with previous reports (7, 10). The abundance of *de novo*-synthesized Ser-CPs during germination and their presence in the endosperm support the thesis that these enzymes participate, in concert with cysteine endoproteases, in the breakdown of storage protein to supply nutrients for the growing seedling (29, 30). Small peptides of 2–5 residues are abundant hydrolytic products found in the endosperm and are subsequently transported into the embryo via a specialized uptake system in the scutellar epithelium, where they are degraded into free amino acids (31, 32). The presence of CP-MI and CP-MII in the embryo suggests a role in the degradation of those peptides, and furthermore CP-MI and CP-MII may be involved in the degradation of storage proteins (33) in the embryo.

Gliadins, the major storage proteins of wheat, are hydrolyzed by GA_3-dependent cysteine endoproteases during germination. Dunaevsky *et al.* (29) observed that the rate of hydrolysis *in vitro* was dramatically enhanced when the gliadin was first incubated with an aspartic protease and a carboxypeptidase, purified from mature wheat seeds. This indicates that proteases present in the mature seed are required for the rapid and efficient mobilization of reserve protein during germination. Indeed, partially hydrolyzed hordein has also been detected in the mature barley grain by immune blotting, suggesting a limited degradation of these storage proteins even before germination (30), perhaps involving CP-MII.

C-terminal processing has been shown for a form of barley β-amylase and for a barley peroxidase, both expressed in the endosperm of the developing grain. In both cases, this processing is likely to be effected by Ser-CPs, since it generates a C-terminal heterogeneity (34) or a C-terminal sequence unfavorable for further Ser-CP-catalyzed digestion (35). Furthermore, CP-MII has been shown to C-terminally process a barley α-amylase precursor *in vitro* and to be responsible, possibly in cooperation with other Ser-CPs, for the maturation of barley α-amylase *in vivo* (36). Thus, the Ser-CPs present in the developing and germinating grain or in the growing seedling could have a regulatory function. The physiological significance of such C-terminal processing events remains, however, to be elucidated.

In conclusion, the presence of Ser-CPs exhibiting new specificities is relevant for the concerted action of Ser-CPs during the breakdown of storage proteins. The complex pattern of expression of Ser-CPs in the barley grain and the participation of Ser-CPs in the maturation of proteins suggest that Ser-CPs play additional roles besides their participation in degradation of storage proteins.

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