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Plastid Protease Activity and Prolamellar Body Transformation during Greening[1]

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ABSTRACT

Two proteases active in and specific to oat etioplasts and up to 24-hour etiochloroplasts, only very slightly contaminated by other cellular compartments are described. The enzyme showed pH optima of 4.2 (acid) and 6.8 (neutral), temperature optima of 50 C and the highest level of enzyme activity was with prolamellar bodies (PLBs) as substrate. Both enzymes showed evidence of a sulfhydryl reagent requirement, particularly for the neutral enzyme. Levels of both proteases increased up to 4 hours of illumination of leaves, and then sharply decreased with the largest differences exhibited by the neutral protease. The pH values in the plastid stroma indicated that the neutral enzyme was likely to be the most important in PLB transformation. A comparison between plastid-associated and extra-plastidic protease activities showed similar properties, except the affinity toward PLBs, which was much higher for plastid proteases (K_m: 0.2 and 1.1 milligrams protein per milliliter, respectively).

PLBs[2] develop when the lack of light interrupts the formation of chloroplasts from proplastids, a process which leads to etioplasts instead. The PLBs consist of quasicrystalline arrays of branched tubular membranes, embedded in the etioplast stroma, which also penetrates into the structure between the tubules.

When exposed to light, this PLB structure quickly changes. An initial loss of crystallinity is followed by a progressive dispersal of PLB material into sheets of perforated lamellae, which become the primary thylakoids and the sites of photosynthetic electron transport (7, 19, 23).

Work on the chemical composition of PLBs (1, 2, 13, 14) indicates that *Avena* PLBs, in addition to lipids, consist of structural polypeptides. Because of the distinct change in structure immediately upon illumination of etiolated tissue, it is reasonable to suggest that the mobilization of these polypeptides could be an important primary event in the process of PLB transformation. As proteases are known to be involved in the mobilization of storage proteins in the course of developmental processes (20), we aimed to determine if protease activity is associated with developing plastids, and whether it catalyzes the mobilization of PLB proteins during the formation of thylakoid membranes.

MATERIALS AND METHODS

Isolation and Purification of Plastids. Seedlings of *Avena sativa* L. (var. Arnold) were grown in moist peat in a temperature controlled chamber at 25 C for 7 days in the dark, and thereafter illuminated up to 24 h with white light (Osram HQLS lamp, 400 w) providing 8.9 wm^{-2} at seedling level. About 50 g of laminae (uppermost 5 cm) were homogenized in 120 ml of a medium containing 0.35 M sorbitol, 50 mM Hepes-KOH (pH 7.6) and 0.2% (w/v) BSA. After filtration through four layers of a 35 μm aperture nylon cloth, plastids were collected by centrifugation (7 min, 600*g*) and resuspended as described earlier (8). For further purification, aliquots of the plastid suspension (200 μl, about 0.2 mg protein/ml) were pipetted into 450 μl microtubes (Beckman) containing 50 μl silicone oil (type AR 150, density 1.03; Wacker-Chemie, F.R.G.) on top of 30 μl of 0.6 M sucrose in 50 mM phosphate citrate buffer (pH 6.0) and 5 mM sodium metabisulphite. After centrifugation (45 s, 16.000*g*, 4 C; Beckman microfuge 152), the tips of the microtubes containing the pelleted plastids were cut, resuspended by vigorous shaking and used for enzyme assays.

Using this method, only plastids retaining their envelope membranes are pelleted (8, 11), which, in addition, show only very low contamination by other cell constituents.

Enzyme Assays. NADH: Cyt *c* reductase (antimycin A-sensitive), which showed the highest rates of activity compared to other commonly used mitochondrial markers, was used to measure the contamination of the plastid fraction by mitochondria according to the method of Lord *et al.* (12). Catalase, a microbody marker, was assayed by following the O_2 evolution in a Clark-type O_2 electrode. The reaction mixture contained in 50 mM Hepes-KOH (pH 7.6) 1.2 mM H_2O_2 in a total volume of 1 ml. NADP-GAPDH (plastids) was measured as described by Schulman and Gibbs (21). As a cytoplasm marker acid phosphatase was used, which was assayed with *p*-NPP as substrate at pH 5.6 for 5 and 10 min (3). Acid and neutral protease activities were routinely assayed using BSA (Sigma, type V), hemoglobin (Sigma), or purified PLBs from etioplasts as substrate essentially as given by Drivdahl and Thimann (5). If not otherwise stated, reactions were carried out at 50 C on a water bath for 90 min, and were terminated by the addition of 0.25 ml of 50% trichloroacetic acid to 1.75 ml assay volume. Blanks were prepared by adding trichloroacetic acid prior to the enzyme and α-amino nitrogen in the supernatant determined (16). Protein in the extracts was measured by the naphthalene blue-black procedure according to Bramhall *et al.* (4).

Isolation and Purification of Prolamellar Bodies. Etioplasts were pelleted and lysed in 20 ml of 25 mM Hepes/KOH (pH 7.6) for 25 min at 0 C, and thereafter centrifuged (3,000*g*, 10 min) to sediment the internal membrane fraction. This membrane fraction is known to contain PLBs with adhering prothylakoids (22, 23). After resuspension (20 ml of 25 mM Hepes as above), the preparations were treated with a loosely fitting TenBroeck homogenizer (about 10 strokes), or with ultrasound (twice 5 s, 20 kHz; [24]) to release a substantial proportion of the prothylakoids from the PLBs, and then were layered on top of a 0.9 M sucrose cushion. After centrifugation in a swing out rotor (Beckman L 50, SW 27: 1.5 h, 21,000 rpm, mean centrifugal force 60,000*g*) the prolamellar body enriched pellet was collected, resuspended in Hepes, and the

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[2] Abbreviations: PLB: prolamellar body; NADP-GAPDH: NADP-dependent glyceraldehyde-3-phosphate dehydrogenase; *p*-NPP: *p*-nitrophenyl phosphate; DMO: 5.5-dimethyloxazolidine-2,4-dione; BME: β-mercaptoethanol.

step gradient centrifugation repeated once more. The PLBs were finally resuspended in 2 mM Hepes-KOH (pH 7.0) to give a concentration of about 40 mg protein ml^{-1} (4%).

Measurement of Stroma pH. Stroma pH was measured and calculated from the distribution of the weak acid [^{14}C]DMO between plastid stroma and incubation medium as described by Heldt *et al.* (11). To obtain values as close as possible to those occurring *in vivo*, protoplasts were used to yield isolated plastids in less than 2 s. For this purpose etiolated or partially illuminated leaves were enzymically digested by incubation of leaf segments (about 1 mm in width [9]) in the following medium (2 h, 30 C): 0.6 M mannitol, 1 mM $CaCl_2$, 0.5% (w/v) BSA, 2% (w/v) Cellulysin (Calbiochem), and 5 mM Mes-KOH (pH 5.6). The released protoplasts were collected by filtration (200-μm aperture nylon net), sedimented (100 g, 5 min), and, after resuspension in 0.5 M sucrose containing 1 mM $CaCl_2$, were purified by a step gradient. For this purpose 5 ml of the protoplast suspension was overlayered by 2 ml of 0.5 M mannitol together with 1 mM $CaCl_2$ and 5 mM Mes-KOH (pH 6.0), and centrifuged for 2 min at 600*g*. The intact protoplasts, which banded at the interphase between the two layers, were collected by careful pipetting and stored on ice. Shortly before the experiment, aliquots were diluted with five times the volume of 0.5 M mannitol, the protoplasts pelleted (300*g*, 60 s), and resuspended in 0.35 M sorbitol, containing 20 mM Hepes-KOH (pH 7.6). Aliquots of the suspension (200 μl) were immediately mixed with [^{14}C]DMO (1 mM, 1.4 μCi/μmol) and pipetted into 450 μl microtubes, containing 30 μl 0.6 M sucrose, 50 μl silicone oil (AR 150), and a fixed nylon net (20 μm aperture; S. Robinson and W. Wirtz, personal communication) (Fig. 1). By a short run of the centrifuge (0.5 s, microfuge 152), protoplasts were forced through the nylon net and the plastids set free from

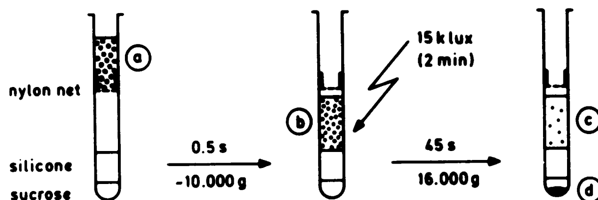

FIG. 1. Scheme for the measurement of the stroma pH of plastids derived from protoplasts. A protoplast suspension (a) was centrifuged through a 20 μm aperture nylon net, resulting in a protoplast homogenate (b). This was incubated with the weak acid [^{14}C]DMO in the light or in the dark, and the uptake of [^{14}C]DMO by plastids terminated by another centrifugation over silicone oil (AR 150). This resulted in a purified plastid pellet (d) and a supernatant (c) which contained some broken plastids and most of all other cellular compartments. Temperature: 8 C.

protoplasts by this procedure were incubated (still within the protoplast homogenate in the layer above the silicone oil) for 2 min at 8 C in the light (15 klux on microtube surface) or in the dark, leaving the tubes in the uppermost position of the centrifuge; after this time the uptake of [^{14}C]DMO was stopped by an additional centrifugation of 45 s at 16,000*g*. The stroma pH was calculated from the specific activity within the incubation medium, the amount of DMO taken up into the stroma space of the plastid pellet (tritiated water space minus sorbitol permeable space [8]), and the pK value of DMO (6.64) according to Heldt *et al.* (11). It was assumed that all DMO anions in the sorbitol impermeable [^{3}H]H_2O space are located in the stroma. The error introduced by this assumption is low with the thylakoid space of developed chloroplasts (11), and should be even lower with less developed plastids as these contain only few thylakoid membranes.

Statistics. The experiments were carried out in duplicates with at least two replicates. The values given are means of at least four different measurements. In some cases standard deviations are given to indicate significantly differing results.

RESULTS

Purity of Plastid Preparations. The levels of activity of catalase, NADP-GAPDH, Cyt *c* reductase (antimycin A-sensitive), and phosphatase associated with different fractions obtained from etiolated and up to 24-h-greened leaves of oats (Table I) show a pronounced increase in purity of plastids, as indicated by the reduced specific activities of catalase (microbodies), phosphatase (cytoplasm/vacuoles) and Cyt *c* reductase (mitochondria), which were obtained after silicone oil filtration in comparison to unfiltered plastid suspensions. In parallel, the specific activity of the plastid marker NADP-GAPDH increased with purification. On average (0-, 4-, and 24-h stages) the percentages of the activities within the purified plastid fraction with respect to the homogenate were 6-12 (catalase), 171-183 (NADP-GAPDH), 0.5-0.9 (Cyt *c* reductase), and 0.2-2.2 (phosphatase). This indicates that the plastid preparations used for the experiments described herein were only slightly contaminated by other cellular compartments.

Properties of Proteases of Purified Plastids: Effects of Sulfhydryl Reagents. Sulfhydryl reagents like BME, cysteine, glutathione, or dithioerythritol (DTE) (Table II) increased protease activities (acid and neutral) with a concentration optimum of 5 mM, and the uncharged sulfhydryl reagent BME being the most effective (acid protease: 144%; neutral protease: 270% of control).

Temperature. Both enzymes evince surprisingly high temperature optima, (50 C) (Fig. 2), showing a reaction rate that is linear for at least 90 min at this temperature.

pH Optimum. The levels of activity of proteases from 4-h

Table I. *Levels of Specific Activities of Marker Enzymes*

Values given represent specific activities found in the homogenate, in unfiltered plastids, and in plastid fractions obtained after silicone oil centrifugal filtration. The leaf material used was etiolated or additionally illuminated for 4 and 24 h. H = homogenate; P = plastids (600*g*, 7 min) without silicone filtration; P + Si = plastids after silicone oil filtration (16,000*g*, 45 s). Numbers in parentheses: per cent of activity in the homogenate.

Period of Illumination (h)	0			4			24		
Fraction	H	P	P + Si	H	P	P + Si	H	P	P + Si
Enzyme									
Catalase μmol O_2 h^{-1} mg $protein^{-1}$	2,888	519	350 (12)	4,686	546	303 (6)	2,799	381	161 (5.7)
NADP-GAPDH μmol NADPH h^{-1} mg $protein^{-1}$	1.2	1.6	2.2 (183)	5.1	6.4	8.7 (171)	7.2	9.8	13.1 (182)
Cyt c reductase μmol Cyt *c* h^{-1} mg $protein^{-1}$	13.6	0.46	0.08 (0.6)	56.4	1.74	0.27 (0.5)	12.5	0.56	0.11 (0.9)
Phosphatase μmol *p*-NPP h^{-1} mg $protein^{-1}$	124.6	7.7	2.8 (2.2)	234.7	7.2	2.1 (0.9)	67.6	3.7	0.16 (0.2)

Table II. *Effect of Sulfhydryl Reagents*

Substances were added directly from pH-adjusted stock solutions to suspensions of purified plastids (P + Si; Table I) and the protease assay was carried out at 50 C. Period of illumination: 4 h; substrate: hemoglobin.

Sulfhydryl Reagent Concentration	Enzyme Activity							
	Acid (pH 4.2)				Neutral (pH 6.8)			
	BME	Cysteine	Glutathione	DTE	BME	Cysteine	Glutathione	DTE
mM	*μmol α-NH₂ mg protein⁻¹ h⁻¹*							
0	1.53	1.53	1.55	1.52	1.28	1.29	1.30	1.27
1	1.86	1.77	1.60	1.59	2.35	1.75	1.60	1.45
2	2.05	1.83	1.68	1.63	2.85	2.39	2.15	1.70
5	2.21	1.90	1.75	1.70	3.46	2.83	2.72	2.05
10	2.15	1.85	1.70	1.65	3.38	2.69	2.60	1.83
15	1.55	1.73	1.63	1.53	2.83	2.40	2.20	1.66
20	0.98	1.20	1.45	1.40	1.76	1.89	1.75	1.49
25	0.48	0.90	1.23	1.45	0.95	1.68	1.48	1.26

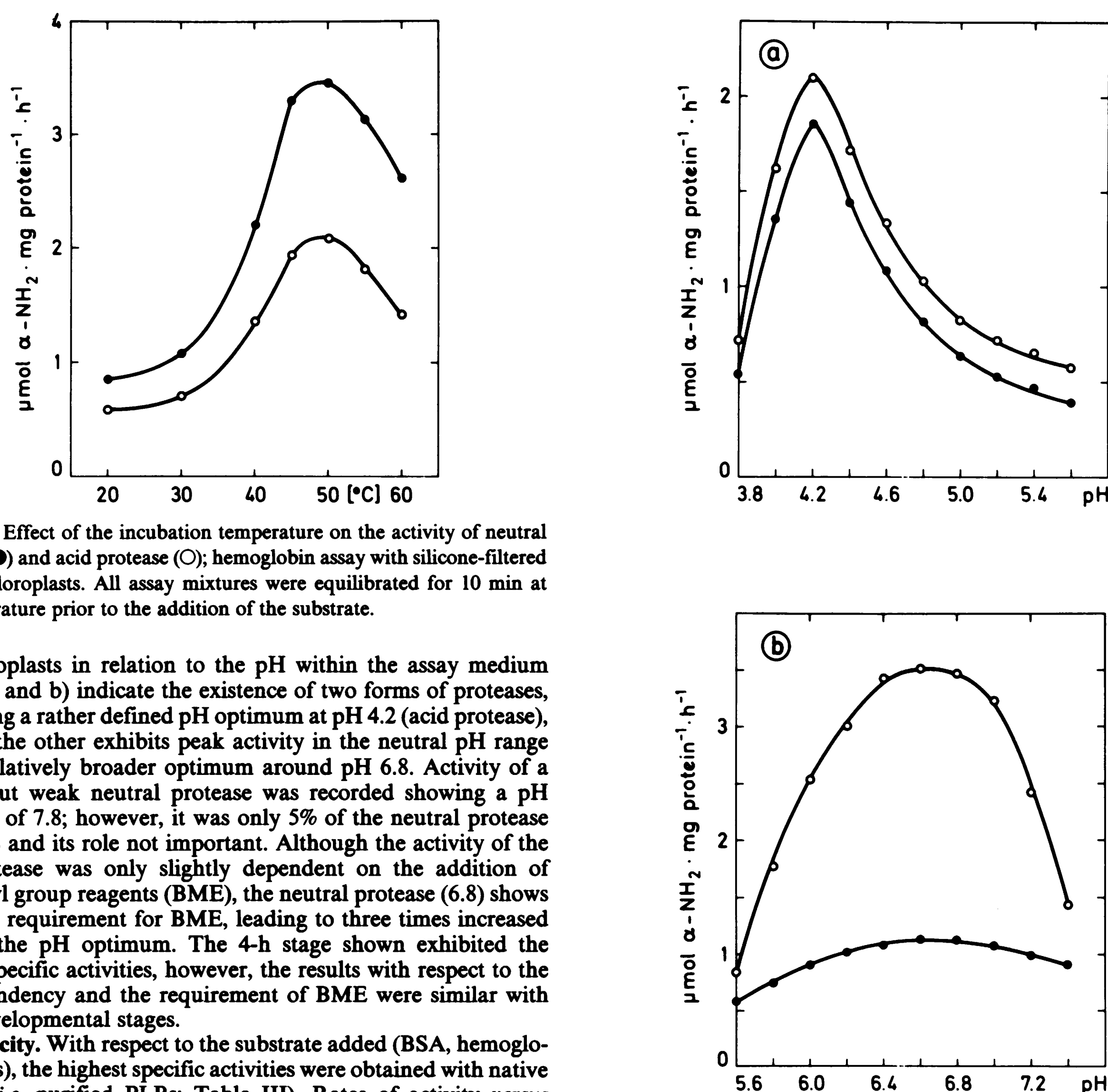

FIG. 2. Effect of the incubation temperature on the activity of neutral protease (●) and acid protease (○); hemoglobin assay with silicone-filtered 4-h etiochloroplasts. All assay mixtures were equilibrated for 10 min at the temperature prior to the addition of the substrate.

FIG. 3. Effect of the medium pH on the activity of (a) acid protease; (b) neutral protease. Hemoglobin assay with silicone-filtered 4-h etiochloroplasts using Na-phosphate buffers (b) or Na-acetate buffers (a): 50 C; (○) + 5 mM BME; (●) without BME.

etiochloroplasts in relation to the pH within the assay medium (Fig. 3, a and b) indicate the existence of two forms of proteases, one having a rather defined pH optimum at pH 4.2 (acid protease), whereas the other exhibits peak activity in the neutral pH range with a relatively broader optimum around pH 6.8. Activity of a second but weak neutral protease was recorded showing a pH optimum of 7.8; however, it was only 5% of the neutral protease at pH 6.8 and its role not important. Although the activity of the acid protease was only slightly dependent on the addition of sulfhydryl group reagents (BME), the neutral protease (6.8) shows a distinct requirement for BME, leading to three times increased rates at the pH optimum. The 4-h stage shown exhibited the highest specific activities, however, the results with respect to the pH dependency and the requirement of BME were similar with other developmental stages.

Specificity. With respect to the substrate added (BSA, hemoglobin, PLBs), the highest specific activities were obtained with native protein (*i.e.* purified PLBs; Table III). Rates of activity *versus* substrate concentration are given as double reciprocal plots for both acid and neutral proteases (Fig. 4, a and b). To overcome difficulties at low substrate concentrations from proteins introduced as impurities, amounts of hydrolysis occurring in the absence of added substrate were measured as blanks and subtracted

Table III. *Hydrolysis of Different Protein Substrates*

Assay was performed with suspensions of purified plastids (P + Si; Table I) at 50 C and a substrate concentration of 4%. Period of illumination: 4 h.

Substrate	Enzyme Activity	
	Acid (pH 4.2)	Neutral (pH 6.8)
	μmol α-NH₂ mg protein⁻¹ h⁻¹	
Prolamellar bodies	3.26 ± 0.31	4.19 ± 0.25
Hemoglobin	2.45 ± 0.27	3.25 ± 0.26
BSA	1.05 ± 0.23	1.16 ± 0.22

from all other values. For hemoglobin (Fig. 4a) as substrate, the concentration needed to cause half-maximal activity (K_m) is 1.17 mg protein/ml with V_{max} being 4 (acid protease) and 5.4 (neutral protease) μmol α-NH_2 formed mg protein^{-1} h^{-1}. In contrast, with isolated and purified PLBs as substrate, K_m is 0.2 mg protein/ml, and V_{max} is 5.4 (acid protease) and 6.9 (neutral protease) μmol α-NH_2 formed mg protein^{-1} h^{-1} respectively. While on a protein basis V_{max} is only slightly higher with PLBs, the affinity of both proteases towards PLB protein is more than five times that towards hemoglobin. Acid and neutral proteases differ only in the maximum velocity, but exhibit identical substrate affinities.

In addition, experiments were performed, in which protoplasts were used as a source for plastid isolation (see under "Materials and Methods"), and protease specificities determined within the plastid pellet after silicone oil filtration, and the fraction above the silicone layer (protoplast homogenate with less than 5% contamination by plastids according to the distribution of the plastid marker NADP-GAPDH).

Proteases associated with plastids obtained in this way showed the same characteristics (K_m, V_{max}) as described above. In contrast, proteases contained within the supernatant fraction differed in their affinities toward PLBs (K_m: 1.1 mg protein/ml; V_{max} 3.8 (acid) and 5.1 (neutral) μmol α-NH_2 formed mg protein^{-1} h^{-1}), whereas the values for K_m and V_{max} with hemoglobin as a substrate were identical to those found for plastid proteases.

A comparison of specific activities of proteases within protoplast homogenates (unfiltered) and plastids obtained from these homogenates by silicone filtration, showed an increase from 1.09 (acid) and 1.30 (neutral) to 2.10 and 2.75 (μmol α-NH_2 formed mg protein^{-1} h^{-1}) respectively within filtered plastids (protoplasts from 4 h illuminated primary leaves; hemoglobin as substrate).

These findings suggest that the levels of protease activity found to be associated with the purified plastid pellets are specific to plastids, and not due to impurities stemming from extraplastidic sites and taken through the silicone oil layer.

Levels of Activity of Proteases of Purified Etioplasts and 1 to 24-h Etiochloroplasts. The developmental course of protease activities within purified plastids, isolated from etiolated and up to 24-h-greened primary leaves of oats, is given in Fig. 5, a and b. Under all conditions (acid or neutral protease; hemoglobin or PLB protein as substrate) the levels of enzyme activity were highest within 4-h etiochloroplasts, with the increase being most pronounced for the neutral enzyme. Times of illumination longer than 4 h under all conditions resulted in a marked decrease in rates of α-NH_2 production. After 24 h of illumination values were close to or lower than those of the etiolated stage.

Stroma pH of Purified Etioplasts and 1- to 24-h Etiochloroplasts. As both proteases have more or less defined pH optima (Fig. 2, a and b), exhibiting only limited levels of activity above pH 7.2, it seemed important to measure the stroma pH during the period of greening investigated. To measure a stroma pH value as close as possible to that occurring under *in vivo* conditions, protoplasts enzymically isolated from the respective stages of greening leaves were used to get plastids in less than 2 s, which in addition to the rather low permeability of the plastid envelope towards protons (25) should be nearly unaffected by any external pH other than that of the cytoplasm. The results achieved by this method and using the weak acid DMO as a pH probe (Fig. 6) indicate a significant pH drop, shortly after the start of illumination, showing a minimum around pH 6.7 within 2-h etiochloroplasts, compared to a pH of about 7.3 in the etioplast stroma. Times of greening longer than 2 h again are coupled to an increase of the stromal pH, reaching values of above 7.5 at the 24-h stage, with significant differences between light and dark treatment from 6 h of greening and onwards.

Compared to the pH-dependent levels of activity of the neutral protease (Fig. 2b), these results indicate optimum conditions for this enzyme within the plastid stroma during a developmental period (1 to 4 h of greening) where it also shows highest specific activities *in vitro* (Fig. 4, a and b).

DISCUSSION

To our knowledge, proteases have mainly been investigated with germinating seeds or senescing tissues. No attempts have been made to localize protease activity in compartments other than vacuoles (15, 20). The results presented here demonstrate that there are also distinct levels of protease activities associated with plastid fractions that are only slightly contaminated by other cellular compartments.

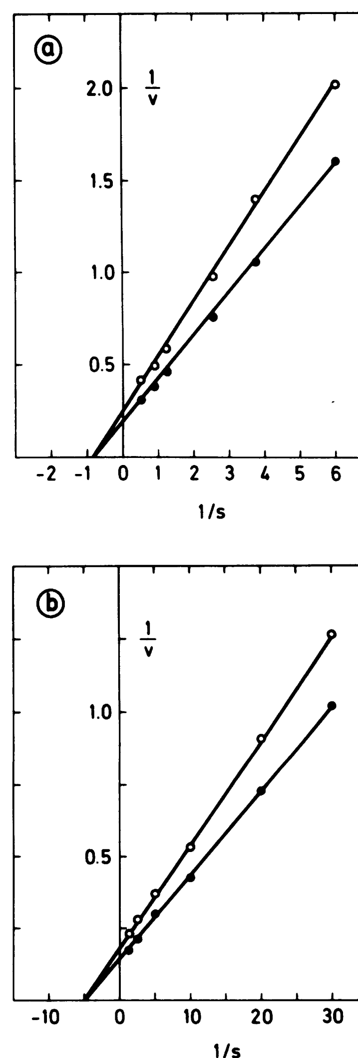

FIG. 4. Lineweaver-Burk plots showing the relationship between substrate (a: hemoglobin; b: PLBs) concentration and the rate of hydrolysis of proteases (○: acid; ●: neutral) from silicone-filtered 4-h etiochloroplasts.

The high temperature optima (50 C) for both neutral and acid proteases, as well as the extended period of linear reaction rates (90 min), suggest that the enzymes are relatively stable, at least in the presence of saturating substrate concentrations and sulfhydryl reagents. Similar high temperature optima have already been reported for proteases from maize endosperm (10) and from senescing oat leaves (5), indicating considerable stability of these enzymes.

The pH optima shown seem to be characteristic for oat tissue. Under comparable conditions Drivdahl and Thimann (5) obtained identical results with purified proteases from senescing oat leaves.

The enzymes from oat plastids degraded several proteins, but at different rates. It has been suggested (17) that such differences in rates are due to the existence of susceptible peptide bonds in exposed regions of protein molecules, which facilitate the binding to the active center of the protease. Although the plastic proteases exhibited rather broad specificities in that all proteins tested were degraded, preparations of PLBs as substrate yielded the highest rates of activity, with more than a five times higher affinity compared to hemoglobin on a protein basis. Harvey and Oaks (10) also achieved the highest rates of hydrolysis by an acid protease from maize endosperm with a natural storage protein (gliadin) as substrate. It seems likely that, *in vivo*, for plastid protease the prolamellar body of etioplasts could be the substrate, as far as the polypeptides stored therein are concerned. This view is supported by the finding that proteases from extra-plastidic sites (protoplast homogenate without plastids) show a much lower affinity towards PLBs.

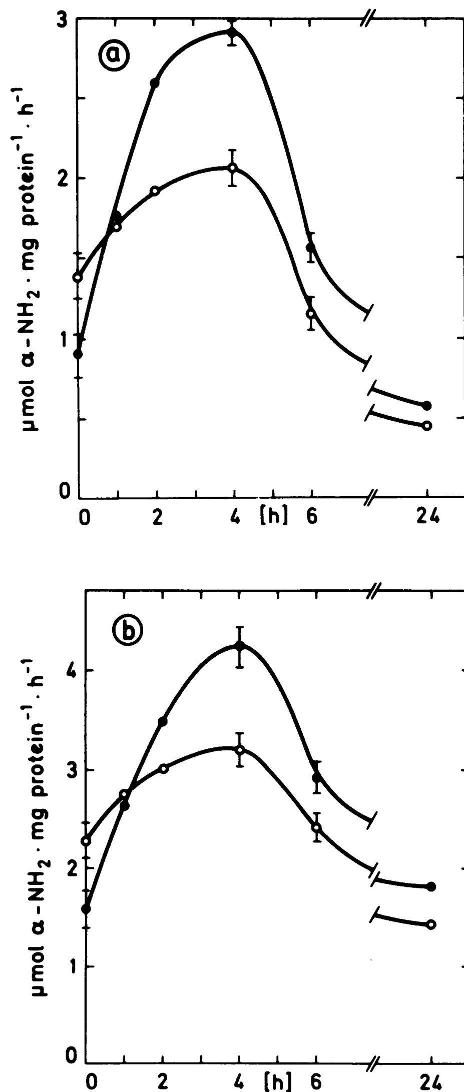

FIG. 5. Time course of activity levels of proteases (○: acid; ●: neutral) from etioplasts (0 h) and 1- to 24-h etiochloroplasts after silicone oil filtration (a) hemoglobin assay; (b) PLB assay. Temperature: 50 C. I = SD (n = 4).

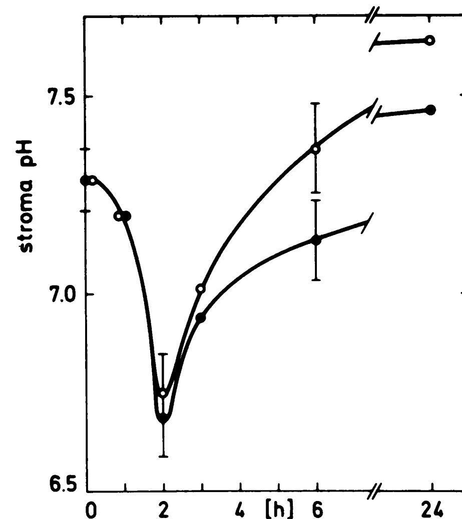

FIG. 6. Stroma pH of etioplasts and 1- to 24-h etiochloroplasts as calculated from the uptake of [^{14}C]DMO into the sorbitol-impermeable space of the organelles (see Fig. 1). Incubation with [^{14}C]DMO was for 2 min (8 C) in the dark (●) or in the light (○, 15 klux). I = SD (n = 4).

During the course of early degradation of PLB protein, the pH of the plastid stroma is lowered. This should favor the activity of the neutral protease, which showed the most pronounced increase upon illumination. The decreased stroma pH could be a consequence of the release of free amino acids by the protease action or/and of the uptake of organic acids from the cytoplasm as discussed earlier for this developmental stage (8). The lowest pH value measured at about 2 h of illumination (6.7 ± 0.13) is very close to the pH optimum of the neutral protease (Fig. 3b).

During development the PLB disappears (within 4–6 h of illumination [24]), being the substrate for prothylakoids and finally grana and stroma membranes. With the formation of these membranes, possibly in part accompanied by the resynthesis of membrane proteins from amino acids set free during the protease action, photosynthetic electron transport reactions occur, exhibiting first signs of water splitting at 4 h of illumination (24). This is well demonstrated by the significant increase of the stroma pH on illumination over that of plastids kept in the dark (Fig. 6; 6 h). As a consequence of the formation of thylakoid membranes, the amount of organic acids needed for the synthesis of membrane constituents decreases, resulting in slightly alkaline stroma pH values (about 7.4) even in the dark. This creates an environment, which inhibits the activity of the still existing protease in a developmental stage (4–6 h of greening) where PLBs have mainly disappeared and no more protease activity for PLB degradation is necessary.

A comparison of the neutral protease located in and specific to plastids with that extracted from 5-day-old oat shoots (18) exhibits differences with respect to the effects of sulfhydryl reagents. While the enzyme from oat shoots is inhibited up to 85% by 5 mM glutathione, the plastid enzyme shows a 100% stimulation at the same concentration. In addition, BME enhances plastid protease activity up to a concentration of 20 mM only, whereas the enzyme extracted from shoots is stimulated even at 100 mM BME. The function of the acid protease, the activity level of which also transiently increases during greening, is not clear and has still to be investigated.

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