Plant Physiol. (1984) 75, 675-678
0032-0889/84/75/0675/04/$01.00/0

Thermolysin Is a Suitable Protease for Probing the Surface of Intact Pea Chloroplasts[1]

Received for publication January 13, 1984 and in revised form March 8, 1984

KENNETH CLINE, MARGARET WERNER-WASHBURNE, JAEN ANDREWS[2], AND KENNETH KEEGSTRA*
Department of Botany, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT

Several proteases, *i.e.*, pronase, a mixture of trypsin and chymotrypsin, and thermolysin were screened as potential surface probes of isolated intact pea (*Pisum sativum* var Laxton's Progress No. 9) chloroplasts. Of these, only thermolysin met the criteria of a suitable probe. Thermolysin destroyed outer envelope polypeptides, but did not affect inner envelope polypeptides, envelope permeability properties or such chloroplast activities as metabolite transport and O_2 evolution.

Methods involving protease treatment have been extensively used for probing the surfaces of cells, organelles, and isolated membranes (5, 13). In general, the exogenously added protease is able to digest proteins exposed on the outer surface of such structures but is prevented from gaining access to proteins on the other side of the membrane barrier. Thus, protease treatment has been used for a variety of purposes including: localization of membrane proteins and enzymes (5, 13), determining the orientation of transbilayer polypeptides (5, 13), removing proteins adsorbed or bound to the surface of membrane-bounded structures, etc. (12).

The usefulness of protease treatment for such purposes relies on the inability of the protease to penetrate the bounding membrane. In many cases membrane rupture leading to protease penetration is not a problem because the debris from the ruptured material can be excluded from subsequent analysis by repurifying the remaining intact cells, organelles, etc. However, for double membrane-bounded organelles (chloroplasts, mitochondria), such a repurification procedure is not adequate since rupture of the outer membrane is not necessarily accompanied by rupture of the organelle. Consequently, special precautions must be undertaken to demonstrate that the exogenous proteases are limited in effect to the outer membrane.

Recently, several studies including one from our own laboratory have utilized proteases as a surface probe of chloroplasts (3, 4, 6, 8, 12). Due to the complexity of the chloroplast-bounding membrane, the envelope, and because of certain inconsistencies we found in our own prior experiments using protease treatment (4), we have examined several proteases to determine their usefulness as surface probes of pea chloroplasts. This communication documents the use of thermolysin, a relatively nonspecific metalloprotease, as a probe whose effects are limited to the outer envelope membrane of isolated, intact pea chloroplasts.

[1] Supported in part by a grant from the United States Department of Agriculture Competitive Research Grants Office. MWW and JA were supported by National Institutes of Health grant 5 T32 GM07215.

[2] Present address: ARCO Plant Cell Research Institute, Dublin, CA 94566.

MATERIALS AND METHODS

Proteases were obtained from Sigma; radiochemicals were obtained from New England Nuclear. All other chemicals were reagent grade.

Intact chloroplasts were purified from homogenates of 2- to 3-week-old pea seedlings (*Pisum sativum* var Laxton's Progress No. 9) by differential centrifugation followed by Percoll density gradient centrifugation as previously described (4). Chloroplast envelope membranes were isolated and subsequently subfractionated into inner and outer membrane fractions by the procedure described previously (4).

For thermolysin treatment, purified intact chloroplasts were suspended to 1.5 mg of Chl/ml in wash buffer, 50 mM Hepes/NaOH, pH 7.5, containing 0.33 M sorbitol. Reactions were initiated by adding aliquots of a thermolysin stock solution which contained 1 to 5 mg of thermolysin/ml in wash buffer containing 10 mM $CaCl_2$. The final concentration of $CaCl_2$ in reaction mixtures was approximately 0.5 mM. After incubation at 4°C for 30 min, reactions were terminated by adjusting reaction mixtures to 5 mM EDTA above the amount required to bind the Ca^{2+} ions present. Intact chloroplasts were then repurified on Percoll density gradients as described (4) except that gradients contained 5 mM EDTA, or by centrifugation through 40% Percoll cushions for 4 min at $2500g_{max}$. The Percoll cushions were buffered with wash buffer, containing 5 mM EDTA and 0.1% BSA. Chloroplasts were then washed twice with wash buffer containing 5 mM EDTA. Each wash was accomplished by resuspension of chloroplasts followed by centrifugation at $1000g_{max}$ for 5 min. Control chloroplasts were subjected to the same treatment and repurification procedure with the exception that control chloroplasts did not receive thermolysin. For thermolysin treatment of isolated envelope vesicles, unfractionated envelope membranes obtained by freeze-thaw/flotation (4) were used without pretreatment. Reactions were initiated with thermolysin and terminated with EDTA as described above. Envelope membranes were then pelleted and washed with 10 mM Tricine/NaOH, pH 7.5, containing 2 mM EDTA before analysis.

Treatment of chloroplasts with trypsin/chymotrypsin was carried out in a similar manner by the procedure described (4). Treatment with pronase was carried out by the procedure used for trypsin/chymotrypsin except that an inhibitor was not used to terminate the reaction.

Chl was determined by the method of Arnon (1) and protein by the method of Markwell *et al.* (11) using BSA as standard. SDS-PAGE was carried out on 7.5 to 15% gradient gels as described (4). 3-Phosphoglyceric acid-dependent O_2 evolution was measured using the buffer and substrate conditions described by Barber and Thurman (2), except that 0.2 mM ATP was also included in the reaction mixture. The reaction was initiated by turning on a slide projector lamp which provided white light at 1500 $\mu E \cdot m^{-2} \cdot s^{-1}$. To determine the degree of intactness of chloroplast preparations, O_2 evolution of intact and hypoosmot-

ically-lysed chloroplasts was also measured in the presence of 1.0 mM potassium ferricyanide plus 2.5 mM NH_4Cl as described (10).

The permeability properties of chloroplasts were determined with [^{14}C]dextran, [^{14}C]sorbitol, and [^{3}H]H_2O using the silicone oil centrifugation procedure as described (7). Aspartate transport was measured using the silicone oil centrifugation procedure essentially according to Lehner and Heldt (9) except that a 120-μl chloroplast suspension aliquot (0.2 mg Chl/ml) was added to 120 μl of [^{14}C]Asp, mixed rapidly by pipetting, and 200 μl removed for assay. Rates were determined based on 15-s time points. Aspartate transport was measured under low light (20 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 4°C.

RESULTS AND DISCUSSION

We have screened several of the proteases which have been reported in the literature as probes of the surface of chloroplasts; pronase (6), a mixture of trypsin/chymotrypsin (12), and thermolysin (3, 8), a relatively nonspecific protease which requires divalent metal ions for activity. The basic protocol was to incubate intact pea chloroplasts with protease for 30 min; then, after termination of the reaction, repurify only the intact chloroplasts. These repurified, protease-treated chloroplasts, or envelope membranes isolated from them, were used for subsequent analysis. In the initial screen, we evaluated the usefulness of the various proteases using two criteria: (a) the effect of the protease treatment upon the intactness of the chloroplasts and (b) the ability of the proteases to penetrate the outer envelope membrane and digest inner membrane polypeptides.

None of the proteases tested seriously affected the intactness of the chloroplasts. In general, more than 70% of the chloroplasts survived a mild treatment. However, of the three different protease preparations, only thermolysin was unable to penetrate the outer envelope and digest inner envelope polypeptides. An SDS-PAGE analysis of envelopes isolated from chloroplasts treated with either pronase or trypsin/chymotrypsin showed that inner envelope membrane polypeptides had been destroyed. This problem was more serious with pronase (not shown), but was still significant with trypsin/chymotrypsin (4). Three of the approximately nine envelope polypeptides destroyed by the trypsin/chymotrypsin treatment are proteins associated with the purified inner envelope membrane (but not the outer). In a previous report, this observation led us to speculate that these three polypeptides were actually outer envelope polypeptides which copurify with the inner envelope membrane (4).

Analysis of envelopes derived from thermolysin-treated chloroplasts indicated that only outer membrane proteins were digested. Figure 1 depicts an SDS-PAGE analysis of unfractionated envelope membranes obtained from chloroplasts treated with 0, 5, 50, and 200 μg/ml thermolysin. From this gel it appears that seven polypeptides are destroyed or greatly reduced in quantity by the thermolysin treatment. A comparison with polypeptide profiles of purified outer and inner envelopes from untreated chloroplasts demonstrates that all of the digested polypeptides are outer envelope membrane polypeptides. This specificity for outer envelope polypeptides is not the result of a general insensitivity of the inner membrane proteins to thermolysin. Treatment of isolated envelope vesicles (see "Materials and Methods") results in the destruction of a large number of inner membrane polypeptides (not shown). This demonstrates that these inner membrane proteins would have been destroyed if they had been exposed on intact chloroplasts.

Inner and outer envelope membranes were purified from the thermolysin-treated chloroplasts in an effort to further characterize the protease treatment and to assure that the disappearance of the outer membrane polypeptides from the SDS gel was not simply due to a protease-induced loss (stripping) of the outer membrane from the chloroplasts. Comparable amounts of outer and inner membrane were recovered from protease-treated as from control chloroplasts (Table I). SDS-PAGE analysis of the purified outer membranes reveals in more detail the pattern of digested proteins (Fig. 2). Ten of the approximately 20 outer membrane polypeptides were destroyed or greatly reduced in quantity by the treatment (see arrows Fig. 2). In addition, various membrane-bound degradation products appear in these samples (asterisks, Fig. 2). Examination of purified inner membranes shows that inner membrane polypeptides are unaffected. Although some bands of the polypeptide profile do disappear as a result of the treatment, these are outer membrane polypeptides present in these preparations as a result of outer membrane contamination.

Of special interest in this analysis is the fate of the 86-kD polypeptide. One-dimensional SDS gels show the presence of a major band at 86 kD in both purified inner and outer membrane subfractions (compare inner and outer envelope membrane reference samples in Fig. 1). In an effort to determine if the 86-kD band in inner and outer membranes was one or two polypeptides,

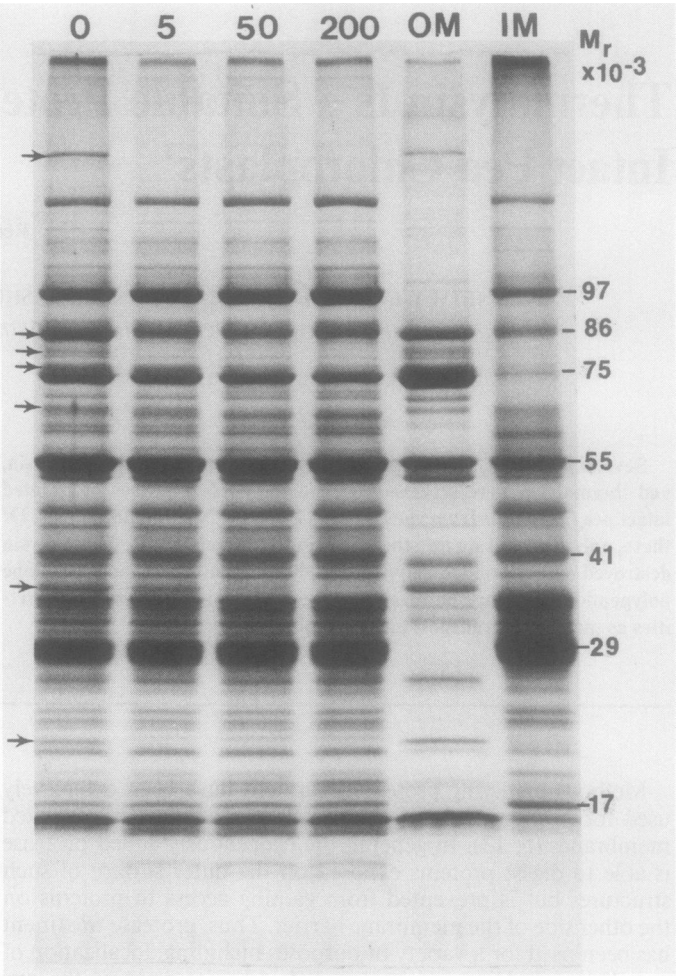

FIG. 1. Effect of thermolysin treatment of intact pea chloroplasts on polypeptides of the envelope membranes. Chloroplasts were treated with thermolysin and then repurified as described in "Materials and Methods". Envelope membranes (unfractionated) were isolated from the treated chloroplasts and analyzed by SDS-PAGE. Each lane contains 30 μg of protein. Arrows designate polypeptides which were destroyed by the treatment. Lane designations 0, 5, 50, and 200 are envelope membranes isolated from chloroplasts treated with 0, 5, 50, and 200 μg thermolysin/ml, respectively; OM, an outer envelope membrane sample from untreated chloroplasts; IM an inner envelope membrane sample from untreated chloroplasts.

Table I. *Properties of Pea Chloroplasts Treated with Thermolysin*

Properties	Thermolysin		
	0	25	200
		μg/ml	
Chloroplasts remaining intact (% of total)[a]	94	91	90
3-PGA-dependent O_2 evolution (μmoles O_2/h·mg Chl)[b]	23.3	24.0	23.5
Transport of 25 μM aspartate (nmoles/h·mg Chl)[c]	530 ± 50	720 ± 80	770 ± 30
Dextran volume (μl/mg Chl)[c]	19 ± 0.9	20 ± 1.1	18 ± 0.9
Sorbitol volume (μl/mg Chl)[c]	24 ± 1.5	21 ± 0.9	22 ± 3.5
Water volume (μl/mg Chl)[c]	50 ± 1.7	50 ± 1.2	51 ± 1.8
Recovered outer envelope membrane (μg/mg Chl)[d]	8	8.2	7.4
Recovered inner envelope membrane (μg/mg Chl)[d]	10.8	8.7	8.5

[a] These values represent the percentage of chloroplasts which migrated as intact chloroplasts in the Percoll gradients following the protease treatment. Their intactness was verified to be greater than 95% by the ferricyanide-dependant O_2 evolution assay described in "Materials and Methods".

[b] Each value is the average of duplicate measurements.

[c] Each value represents the average of three measurements ± standard deviation.

[d] These preparations are the enriched inner membrane (pool 1) and highly purified outer envelope membrane (pool 4) from isopycnic sucrose gradients described in (4).

we have previously analyzed the envelopes by two dimensional gel electrophoresis (14). The results indicated that there are two different 86-kD polypeptides, one in the inner membrane and one in the outer. The present results derived from thermolysin treatment of chloroplasts support that conclusion. As can be seen in Figure 2, the outer membrane 86-kD polypeptide is destroyed by the protease treatment whereas the inner membrane 86-kD polypeptide is unaffected.

To further document the structural integrity of the envelopes on thermolysin-treated chloroplasts, several other properties were examined (Table I). The ability to carry out 3-phosphoglyceric acid-dependent O_2 evolution and the ability to transport aspartate both require functional translocators on the inner envelope membrane. Both of these functions were unaffected by the protease treatment of chloroplasts (Table I). This demonstrates that the treatment does not affect inner membrane translocators, nor does it affect the O_2 evolving apparatus within the chloroplasts. Finally we have measured the permeability properties of the treated chloroplasts. The permeability properties of chloroplasts were determined by the silicone oil centrifugation procedure to determine access of dextran, sorbitol, and water to various compartments of the chloroplasts. In an untreated chloroplast dextran is excluded from both membranes of the envelope, sorbitol can pass through the outer but not the inner envelope, and water can enter all of the chloroplast compartments. As can be seen in Table I, the dextran, sorbitol, and water volumes of protease-treated chloroplasts are essentially the same as control chloroplasts, demonstrating that the protease treatment has not caused major changes in the permeability of the two envelope membranes.

Our results with pea chloroplasts can be compared with recent reports of protease treatment of spinach chloroplasts (3, 8). Joyard *et al.* (8) tested several different proteases and concluded that thermolysin is a suitable probe of the surface of spinach

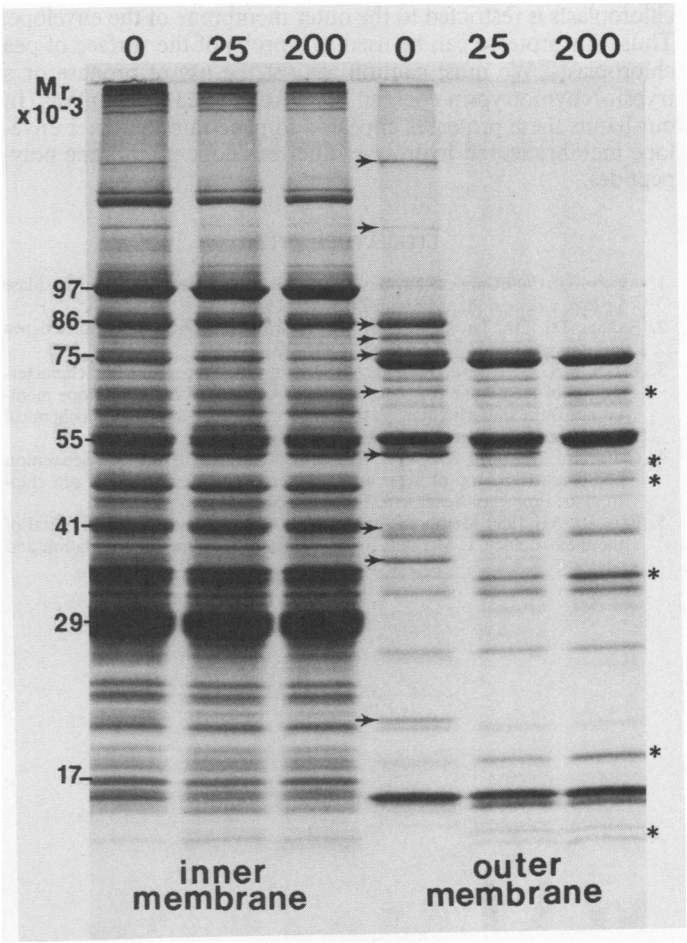

FIG. 2. SDS-PAGE analysis of outer and inner envelope membrane subfractions purified from thermolysin-treated chloroplasts. Chloroplasts were treated with varying amounts of thermolysin as described in "Materials and Methods". After termination of the reaction, intact chloroplasts were repurified and used to prepare inner and outer envelope membrane fractions. Inner envelope lanes contain 30 μg of protein; outer envelope lanes contain 20 μg of protein. Arrows designate polypeptides which disappear as a result of the protease treatment; asterisks designate degradation products. Lane designations 0, 25 and 200 are envelope membranes isolated from chloroplasts treated with 0, 25, and 200 μg thermolysin per ml respectively.

chloroplasts. Joyard *et al.* report that about 20 envelope polypeptides were affected by the thermolysin treatment. Because these analyses were carried out on a mixture of inner and outer envelope membranes, a direct comparison with our results is difficult. In a subsequent publication, Block *et al.* (3) presented the polypeptide profiles of outer envelope membranes prepared from control and from thermolysin-treated spinach chloroplasts. A visual comparison of these data with our results shows that the polypeptide profiles of the outer envelope membranes from pea and spinach are quite similar. Many of the polypeptides in the pea chloroplast outer envelope appear to have counterparts in spinach chloroplast outer membrane. In addition, the pattern of outer envelope polypeptides digested by thermolysin is similar for pea and spinach. A notable exception to this similarity is that the outer membrane of spinach chloroplasts contains protease-sensitive polypeptides at 10 kD and 12 kD. Although our SDS-PAGE system is capable of resolving polypeptides in this region (Fig. 1), we do not observe comparable polypeptides in the outer envelope membranes of pea chloroplasts.

In conclusion, our results strongly suggest that under the described conditions the effect of thermolysin treatment of intact

chloroplasts is restricted to the outer membrane of the envelope. Thus, this protease can be used as a probe of the surface of pea chloroplasts. We must caution against the use of pronase or a trypsin/chymotrypsin cocktail as probes of pea chloroplasts. In our hands these proteases appeared to penetrate the outer envelope membrane and hydrolyze inner envelope membrane polypeptides.

LITERATURE CITED

1. ARNON DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15
2. BARBER DJ, DA THURMAN 1978 Transport of glutamine into isolated pea chloroplasts Plant, Cell Environ 1: 297–303
3. BLOCK MA, A-J DORNE, J JOYARD, R DOUCE 1983 Preparation and characterization of membrane fractions enriched in outer and inner envelope membranes from spinach chloroplasts. I. Electrophoretic and immunochemical analyses. J Biol Chem 258: 13273–13280
4. CLINE K, J ANDREWS, B MERSEY, EH NEWCOMB, K KEEGSTRA 1981 Separation and characterization of inner and outer envelope membranes of pea chloroplasts. Proc Natl Acad Sci USA 78: 3595–3599
5. ETEMADI AH 1980 Membrane asymmetry. A survey and critical appraisal of the methodology. I. Methods for assessing the asymmetric orientation and distribution of proteins. Biochim Biophys Acta 604: 347–422
6. FISH L, VR FRANCESCHI, R STOCKING 1979 Effects of pronase on isolated chloroplasts. Plant Physiol 64: 1012–1014
7. HELDT HW 1980 Measurement of metabolite movement across the envelope and of the pH in the stroma and the thylakoid space of intact chloroplasts. Methods Enzymol 69: 604–613
8. JOYARD J, A BILLECOCQ, SG BARTLETT, MA BLOCK, N-H CHUA, R DOUCE 1983 Localization of polypeptides to the cytosolic side of the outer envelope membrane of spinach chloroplasts. J Biol Chem 258: 10000–10006
9. LEHNER K, HW HELDT 1978 Dicarboxylate transport across the inner membrane of the chloroplast envelope. Biochim Biophys Acta 501: 531–544
10. LILLEY RMcC, MP FITZGERALD, KG RIENTIS, DA WALKER 1975 Criteria of intactness and the photosynthetic activity of spinach chloroplast preparations. New Phytol 75: 1–10
11. MARKWELL MAK, SM HAAS, LL BIEBER, NE TOLBERT 1978 A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 87: 206–210
12. MULLET JE, N-H CHUA 1983 *In vitro* reconstitution of synthesis, uptake, and assembly of cytoplasmically synthesized chloroplast proteins. Methods Enzymol 97: 502–509
13. STECK TL 1974 The organization of proteins in the human red blood cell membranes. A review. J Cell Biol 62: 1–19
14. WERNER-WASHBURNE M, K CLINE, K KEEGSTRA 1983 Analysis of pea chloroplast inner and outer envelope membrane proteins by two-dimensional gel electrophoresis and their comparison with stromal proteins. Plant Physiol 73: 569–575