Vol. 84, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 29, 1978 Pages 381-388

ACTIVATION OF OLD CUTICLE CHITIN AS A SUBSTRATE FOR CHITINASE IN THE MOLT OF MANDUCA

Maria L. Bade and Alfred Stinson, Dept. of Biology,
Boston College, Chestnut Hill, Mass. 02167

Received August 10, 1978

Summary. During the molt, chitin in the old cuticle of Manduca is digested by chitinase taken up from molting fluid, but the chitin in intact (= premolt) cuticle is not accessible to chitinase. As a prerequisite of digestion, old cuticle chitin is rendered competent to serve as chitinase substrate in a reaction attributable to trypsin-like proteolytic activity of molting fluid.

As an insect grows, it molts periodically, during which event the old exoskeleton is shed following synthesis of a new integument beneath it. Chief components of the exoskeleton are chitin and protein. Most of the material of old cuticle is broken down and recycled prior to shedding (1). Hydrolytic enzymes for digestion of old cuticle, i.e. chitinase (EC 3.2.1.14) and proteases, are present in the molting fluid (2). Activity reported in the literature for molting fluid chitinase, however, has consistently been several orders of magnitude too low to account for the quantity of chitin mobilized in the molt of large insects (1,3). Recently, it was demonstrated that old cuticle itself acquired the ability to degrade its constituent chitin at a high rate (4) and that this endogenous chitinase activity rose and fell with each molt (5). Evidence has now been obtained that old cuticle chitin takes up chitinase from molting fluid and that the chitin of intact (= premolt) cuticle is rendered competent to act as substrate through the activity of trypsin-like protease of molting fluid.

Methods. Larvae of the tobacco hornworm, Manduca sexta (Lepidoptera: Sphingidae) were reared and staged as previously described (5,6). Molting fluid was tapped from pharate pupae, mixed with an equal amount of cold 40% sucrose containing phenylthiourea, and frozen until use. Chitinase was assayed by measuring the amount of N-acetylglucosamine (7) liberated in 10 min at 37° by two 5 x 5 mm cuticle squares rinsed in cold 0.85% KCL and stored in cold 2% ascorbate (pH 5.5) until use. The reaction mixture contained, in 1.0 ml final volume, 50 mM phosphate-acetate buffer pH 7.0 and 1 mM $CaCl_2$. The cuticle squares served as source of both enzyme and endogenous substrate in determination of endogenous activity. Some assay mixtures

0006-291X/78/0842-0381$01.00/0

were supplemented with 0.2 mg chitobiase (EC 3.2.1.29) (8); later work showed this to be unnecessary. Weights were determined on blotted cuticle pieces prior to enzyme assay. Proteolytic activity in molting fluid was determined on 20 µl aliquots with HCl-denatured hemoglobin solutions in presence of 0.1 M Tris/cacodylate composite buffer. After 90 min at 37°, protein was precipitated with TCA and amino acids solubilized during the reaction determined with ninhydrin (9). Buffer-diluted molting fluid was pre-incubated for 1 h with 0.01 M inhibitor at 0°. Protein was measured by the method of Lowry (10) on the supernatant after boiling cuticle for 1 h in N/1 NaOH, and calculated with the aid of a standard curve constructed with bovine serum albumin. Tissue culture grade trypsin, hemoglobin, BSA, and inhibitors were purchased from Sigma Chemical Co., St. Louis, MO.

Results and Discussion

Cuticle prior to the molt lacks endogenous activity (5). Since the inner layers of old cuticle during much of the molt are bathed in molting fluid, intact cuticle obtained from actively feeding larvae was incubated with molting fluid buffered at pH 7.0 with phosphate-acetate buffer to see whether chitinase activity would subsequently be present in it. Following the pre-incubation, the cuticle squares were transferred with rinsing in ascorbate to fresh medium containing 0.2 mg chitobiase but no free chitin substrate, and endogenous chitinase activity was measured. Results are shown in Fig. 1. Appreciable endogenous chitinase activity was induced in intact cuticle by enzymatically active molting fluid (curve A) while no such activity was evident if the molting fluid was first boiled (curve B). If the cuticle was deproteinized by boiling with N/1 NaOH prior to incubation with active molting fluid, the remaining chitin skeleton acquired very high cuticle-bound chitinase activity at the most rapid rate (curve C). The only conclusion consistent with these results is that chitinase is present in molting fluid and moves into the cuticle in the course of the molt; the possibility that molting fluid activates a prechitinase present in cuticle (11,12) is decisively ruled out.

Various enzymes were tested for their ability to substitute for molting fluid in its ability to engender in cuticle the capacity to break down its own constituent chitin, a multi-step process (6) which will without prejudice as to its nature be referred to as "inducing endogenous activity." Each

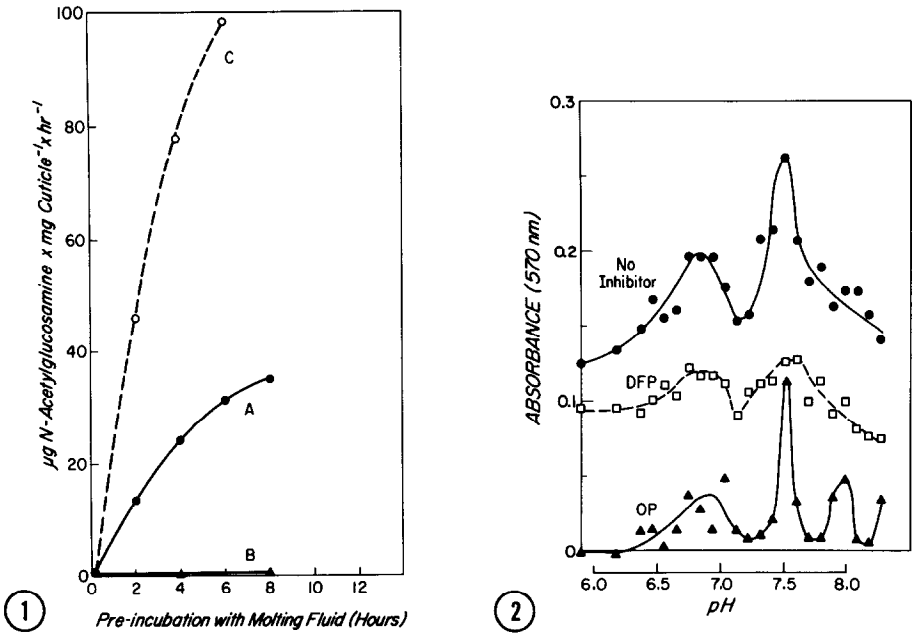

Fig. 1 Cuticle chitinase activity developed in vitro in Manduca cuticle.

Cuticle squares were incubated with inhibitor-treated molting fluid (40 mM phosphate-acetate buffer pH 7.0, 0.1 ml molting fluid 1:1 with 40% sucrose plus phenylthiourea, 2 cuticle squares, small crystal of thymol, 0.4 ml final volume, 25°). Following incubation, they were rinsed, weighed, and assayed for endogenous chitinase activity developed (40 mM phosphate-acetate buffer pH 7.0, 0.2 mg chitobiase, 1.0 ml final volume, 10 min at 37°). Each point average of 2-6 determinations.

Curve A: intact cuticle, active molting fluid
Curve B: intact cuticle, boiled molting fluid
Curve C: deproteinized cuticle, active molting fluid

Fig. 2. Differential inhibition of molting fluid proteases with suitable inhibitors.

Final concentrations were 0.25 M cacodylate (pH 7.0), 0.2% hemoglobin in 0.06 N HCl, 0.01 M inhibitor, 1 mM $CaCl_2$ + 20 µl molting fluid 1:1 with 40% sucrose containing PTU. Molting fluid was pre-incubated for 1 h at 0° with all components except substrate; the solutions were then brought to 37°C, substrate was added and a zero time aliquot withdrawn immediately. A second sample was withdrawn after 90 min. Protein in samples was precipitated with TCA and ninhydrin in the supernatant determined according to Spies (9).

DFP: diisopropylfluorophosphate
(trypsin inhibitor)

OP: ortho-phenanthroline
(inhibitor of neutral protease)

Table 1. Cuticle chitinase activity developed by enzyme treatments.

For each determination, 2 cuticle squares were treated 3 h at 25°C under optimum buffer and pH conditions for each enzyme (13). Cuticle pieces were rinsed and either activity without further added substrate determined immediately or second 3 h treatment given at 25° as noted, after which endogenous activity was determined. 2 to 4 determinations per value shown ± range.

Preincubation Enzyme	Activity (nmol N-acetylglucosamine per mg cuticle per hour)
Molting fluid	28.6±8.1
Doubly inhibited* molting fluid	2.6±0.8
Trypsin	0
Trypsin, followed by doubly inhibited* molting fluid	107.6±4.0
Trypsin, followed by molting fluid	88.1±12.5
Buffer, followed by doubly inhibited* molting fluid	7.6±1.0

*Molting fluid pretreated for 15 min at 25° with 0.01 M diisopropylfluorophosphate (trypsin inhibitor) and 0.01 M 8-hydroxyquinoline (neutral protease inhibitor).

enzyme was incubated with intact cuticle squares for 3 h at 25° under optimal conditions for the particular enzyme (13) and the cuticle squares were tested for endogenous chitinase activity induced. The results are given in Table 1. Neither chitinase in molting fluid with inhibited proteolytic activity or trypsin alone was able to substitute for molting fluid. Absence of any effect of chitinase on intact cuticle shows that the chitin of intact cuticle is masked from attack by chitinase and must be rendered competent to serve as substrate by some molting fluid activity distinct from chitinase. Suitable controls with colloidal chitin established that the observed effect was not on molting fluid chitinase itself, but rather on the treated cuticle.

It seemed probable that some of the proteolytic activity of molting fluid might play a role in rendering cuticle chitin susceptible to chitinase. Bade and Shoukimas (14) demonstrated that two distinct kinds of protease are present in Manduca molting fluid, one trypsin-like with alkaline pH optimum and subject to DFP inhibition, the other with pH optimum slightly below neutrality, activated by calcium and inhibited by metal chelators. It is possible by choice of appropriate inhibitors to inhibit each of these activities differentially without materially affecting proteolysis by the other. This is shown in Fig. 2. (A separate series of experiments established that the slight overall depression in proteolytic activity in presence of DFP is due to the presence of the small amount of isopropyl alcohol in which the DFP is dissolved). Experiments were designed to test for the involvement of the known molting fluid proteases in the activation of cuticle chitin. Intact cuticle was treated for 3 h under various conditions and then incubated for 3 h more with molting fluid in which both types of proteolytic activity, but not chitinase, had been inhibited. The cuticle was then tested for endogenous activity developed. The results are also shown in Table 1. It is apparent that trypsin treatment followed by exposure to molting fluid chitinase leads to very high chitinase uptake by initially intact cuticle. As expected, doubly inhibited molting fluid alone is unable to produce significant chitinase activity in intact cuticle. Buffer extracts a few, but not the major part, of masking molecules.

Since molting fluid proteases can be differentially inhibited, we tested the ability of various inhibitors to interfere with induction by molting fluid of endogenous chitinase activity in intact cuticle. Cuticle squares were incubated at 25° for 3 h in buffer-diluted molting fluid which had been pretreated for 15 min with 0.01 M inhibitor; at the end of the incubation, the cuticle squares were rinsed and assayed for endogenous chitinase activity as usual. The results are shown in Table 2. It is evident that all trypsin inhibitors tested interfered with the ability of

Table 2. Effect of inhibitors on induction of cuticle chitinase activity by molting fluid.

Buffer-diluted molting fluid was pretreated with 0.01 M inhibitor for 15 min, after which cuticle squares were incubated with the molting fluid for 3 h. (80 mM phosphate-acetate buffer pH 7.0, 0.1 ml molting fluid 1:1 with 40% sucrose plus phenylthiourea, 0.5 ml total volume, 25°). Cuticle squares were then rinsed, weighed, and assayed for endogenous chitinase activity; activity per mg cuticle weight developed was compared to uninhibited control. 4-6 determinations per value shown ± range. TPCK = L-1-tosylamide-2-phenylethylchloromethyl ketone (chymotrypsin inhibitor); TLCK = N-α-p-tosyl-L-lysine chloromethyl ketone (trypsin inhibitor).

Inhibitor	% Endogenous activity developed
None	100±16
Diisopropylfluorophosphate†	20±3
1,10-Phenanthroline‡	106±20
8-Hydroxyquinoline‡	111±23
TPCK	100±3
TLCK†	17±9
p-Chloromercuric benzoate	101±6
Soybean trypsin inhibitor†	23±12
Ovomucoid trypsin inhibitor†	68±7

†trypsin inhibitor
‡inhibitor of neutral protease

molting fluid to induce chitinase activity. None of the other inhibitors tested did so. Several compounds that strongly inhibit neutral proteases (14,15) failed to inhibit induction of endogenous chitinase in intact cuticle.

The results given are consistent with the conclusion that cuticle chitin is unmasked during the molt by a relatively specific proteolytic reaction mediated by trypsin-like molting fluid protease. The proteolytic activity probably makes the chitin accessible to chitinase rather than affecting its structure more directly, but elucidation of the molecular mechanism of unmasking must await more detailed knowledge of the manner in which chitin and protein are associated in intact cuticle.

A number of cases are now known where proteins are converted into final (active) form by specific proteases. In the cases explored, which range from blood coagulation and tissue repair to assembly of structural proteins and activation of hormones, the timing and rate of physiological processes such as development and defense reactions are regulated through partial proteolysis of precursor proteins (16). The data on the interplay of protease and chitinase in insect molting here presented permit the interference that insect molting is regulated not only on the hormonal level, where its regulation has been intensively studied, but also on the level of breakdown of the macromolecules that comprise the exoskeleton. In the molt, which is an indispensable step in insect development, trypsin-like molting fluid protease renders cuticle chitin susceptible to degradation by molting fluid chitinase. This may be viewed as a novel type of activation through proteolysis by which a macromolecule, not itself a protein, is enabled to participate as substrate in subsequent hydrolytic reactions.

Acknowledgements. This work was supported by the United States Public Health Service (Grant ES00650) and in part by the National Science Foundation. We thank Helen Di Muzio and Michael Dewey for faithful service as Manduca nurses, and P.W. Robbins and H. Lipke for critical reading of the manuscript.

REFERENCES

1. Bade, M.L. and Wyatt, G.R. (1962) Biochem. J. 83, 470-478.
2. Passonneau, J.R. and Williams, C.M. (1953) J. Exper. Biol. 30, 545-560; Jeuniaux, C. and Amanieu, M. (1955) Arch. Internat. Physiol. Biochim. 63, 94-103; Katzenellenbogen, B.S. and Kafatos, F.C. (1970) J. Insect Physiol. 16, 2241-2256.
3. Jeuniaux, C. (1963) Chitine et Chitinolyse. These d'aggregation Liege, Belgium. 149 pp.; Kimura, S. (1973) J. Insect Physiol. 19, 115-123; Spindler, K.-D. (1976) Insect Biochem. 6, 663-667.
4. Bade, M.L. (1974) Biochim. Biophys. Acta 372, 474-477.
5. Bade, M.L. (1975) FEBS Letters 51, 161-163.
6. Bade, M.L. and Stinson, A. Insect Biochem. (in press).
7. Reissig, J.L., Strominger, J.L., and Leloir, L.F. (1955) J. Biol. Chem. 217, 959-966.
8. Jeuniaux, C. (1966) in Complex Carbohydrates (Neufeld, E.F. and Ginsburg, V., Eds.) p. 644-650 Vol. VIII of Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., Eds.); Acad. Press, New York.
9. Spies, J.R. (1957) in Methods in Enzymology Vol. III (Colowick, S.P. and Kaplan, N.O., Eds.) p. 468-471; Academic Press, New York.
10. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
11. Bade, M.L. and Stinson, A. (1976) The 8th Miami Winter Symposia: Proteolysis and Physiological Regulation.

12. Bade, M.L. (1976) Xth Internat. Biochem. Congress, Hamburg, Germany.
13. Anon. (1967, 1972) Worthington Enzyme Manual, Worthington Biochemical Corp., Freehold, N.J.
14. Bade, M.L. and Shoukimas, J.J. (1974) J. Insect Physiol. 20, 281-290.
15. Matsubara, H. and Feder, J. (1971) in The Enzymes (Boyer, P.D., Ed.) 3rd ed., p. 721-795 Vol. 3; Acad. Press, New York.
16. Neurath, H. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D.B. and Shaw, E. Eds.) Cold Spring Harbor Laboratories, p. 51-64.