Vol. 87, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1979 Pages 349-353

MOLTING FLUID CHITINASE: A HOMOTROPIC ALLOSTERIC ENZYME

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Received February 2,1979

Summary. The molting fluid of the tobacco hornworm has chitinase activity which shows allosteric behavior with chitin. A parabolic curve is obtained on a double reciprocal plot of $\frac{1}{v}$ vs. $\frac{1}{[S]}$, and a sigmoid curve results when v (N-acetylglucosamine produced) is plotted against [S] (chitin concentration in terms of N-acetylglucosamine concentration). The Hill coefficient with insect chitin is 1.95 ± 0.08. Allostery of the chitinase enables the insect to exert additional control over the integrity of structure of its cuticle.

During the molt, insects break down and recycle up to 90% of old cuticle material (1). Since this material is chiefly chitin and protein, both chitinases and proteases from molting fluid are involved in breaking down old cuticle (e.g. 2,3). We now bring evidence that molting fluid chitinase shows positive cooperativity when good quality colloidal insect chitin (4) is used as substrate in the assay of molting fluid chitinase activity.

Methods. Larvae of Manduca sexta, the tobacco hornworm, were reared and staged as previously described (4,5,6) from eggs kindly supplied by Dr. John Reinecke, USDA Experiment Station, Fargo, North Dakota. Preparation of chitin substrate from insect larval cuticles and the assay method have been described in detail (4) and will therefore be given only briefly: Manduca larval cuticles are obtained by stripping them from fifth instar larvae; they are rinsed in ascorbate (2%, pH 5.5), deproteinized by boiling in dilute NaOH (1M, 5 changes), and dissolved under controlled conditions in aqueous sulfuric acid. The colloidal chitin is precipitated in ice-cold 50% ethanol and washed with deionized water to about pH 5. The stock solution contains about 6 mg/ml chitin in 0.1 M phosphate-acetate buffer (pH 6.5) and is diluted if necessary.

The assay consists of measuring the rate of evolution of N-acetylglucosamine by 10 µl molting fluid from colloidal chitin, prepared as given above, during a short interval at 37°C; the assay mixture contains, in 1.0 ml final volume, 50 mM phosphate-acetate buffer (pH 6.5), 1 mM $CaCl_2$, 20 µl diluted centrifuged molting fluid (see below), and the desired quantity of colloidal chitin. A 10-minute assay period is used routinely; this is extended to 20 minutes if low product yield is expected. N-acetylglucosamine is assessed quantitatively at the beginning and end of the assay period by a modification of the assay developed by Reissig et al. (7). Molting fluid chitinase under these conditions produces N-acetylglucosamine at a linear rate for 20 minutes (4).

Whole molting fluid is used as the enzyme source. Molting fluid is tapped from pharate pupae and quick-frozen after dissolving in it a few small crystals of phenylthiourea to suppress tyrosinase activity. Before use, the cold thawed molting fluid is diluted 1:1 with 0.1 M phosphate-acetate buffer (pH 6.5 and 0.01 M in 8-hydroxyquinoline 5-sulfonate to suppress neutral protease (4)) and

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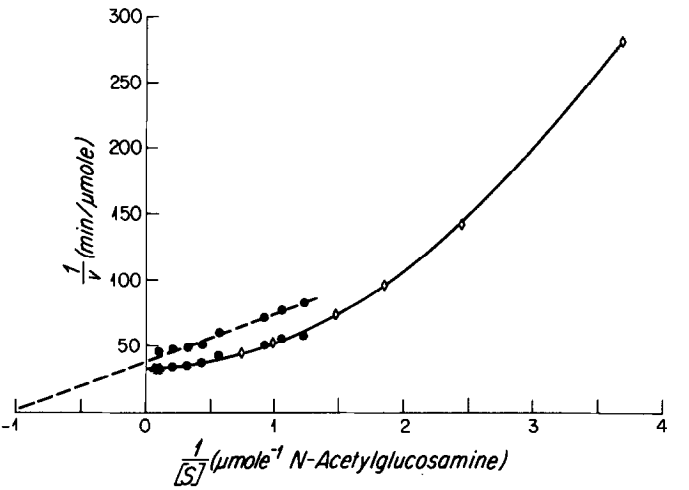

Figure 1. Double Reciprocal Plot of Chitinase Activity vs. Chitin Concentration.

Tubes were set up containing, in 1.0 ml, 50 mM phosphate-acetate buffer pH 6.5, insect colloidal chitin, 1 mM $CaCl_2$, and 20 µl of diluted molting fluid (see text), and stirred at 37°C. 0.1 ml samples were removed after 5 and 25 minutes and N-acetylglucosamine determined (4). Each point represents the average of 4 to 6 separate determinations.

----- straight line drawn through one set of points (4)
_____ results from three separate runs normalized to same molting fluid activity

centrifuged for 10 minutes at 5000xg. 20 µl aliquots of the diluted centrifuged molting fluid serve as enzyme source in the chitinase assay.

Results and Discussion.

A straight line can be drawn through a limited number of points in a double reciprocal plot of rate of N-acetylglucosamine production vs. chitin concentration (4, and dashed line Fig. 1). If, however, rate determinations are extended to lower chitin concentrations, the points on the double reciprocal plot are seen to fall on a parabolic curve typical of an allosteric enzyme. Since only the chitin concentration is changing, chitinase must react cooperatively with its substrate (or possibly with an intermediate produced in the course of breakdown of chitin from a macromolecule to N-acetylglucosamine).

A possible explanation for these observations is that chitinase exists in two forms, in equilibrium with each other and their substrate, and that one of

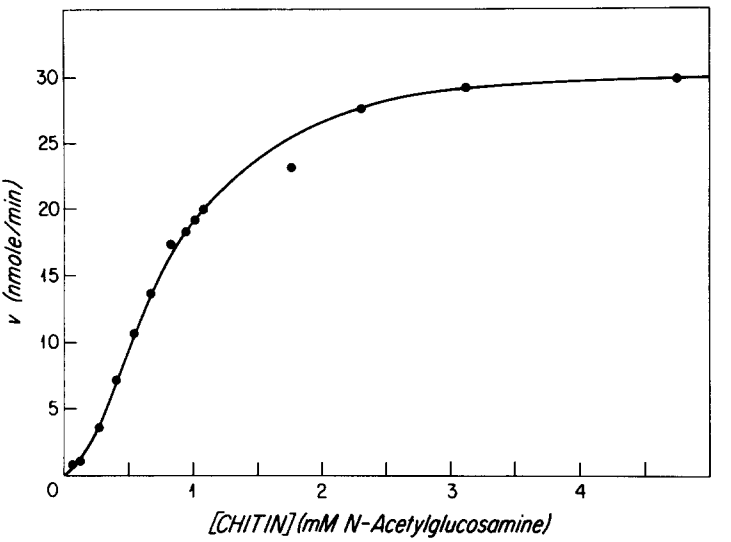

Figure 2. Chitinase Activity vs. Chitin Concentration.

v vs. [S] plot of values taken from Fig. 1, plus additional values obtained from tubes with very low chitin concentrations.

these conformers has much lower affinity for its substrate than the other (8). At low substrate concentrations, relatively more of the enzyme would exist in the low-affinity form than at higher concentrations, and thus sigmoidal kinetics would be expected in a plot of rate vs. substrate concentration. Fig. 2 shows that a S-shaped curve indeed results if the data shown on the solid line in Fig. 1 are plotted in this manner (note that Fig. 2 includes several points at very high substrate dilutions which the scale of the ordinate of Fig. 1 does not permit to be plotted). This is consistent with the idea that chitinase has more than one binding site for chitin, and that in the absence of chitin it consists of a relatively inactive form with low affinity for chitin and a much more active form with higher affinity for the substrate. As chitin concentration increases, more chitin is bound to the high affinity form of chitinase, more inactive chitinase converts to the active form, and chitinase activity therefore rises rapidly during an intermediate concentration interval of chitin.

The data shown in Figs. 1 and 2 were used to construct a Hill plot of log $\frac{v}{V_{max}-v}$ vs. log [S]; V_{max} was estimated from the intercept of the ordinate of Fig. 1. A value of 1.95 ± 0.08 was obtained for the Hill coefficient n_H by a least squares fit to the linear part of the curve (a total of 3 points at very low and very high substrate concentrations markedly departed from linearity; these points were omitted in the calculation of n_H ± R.M.S. error (8)). Least squares polynomial fits to $\frac{1}{v}$ as a function of $\frac{1}{S}$ were also compared. The R.M.S. error was a little over 6 for the second and third degree fits and 1.78 for the fourth degree fit. It appears probable that molting fluid chitinase binds at least 2 substrate molecules per molecule of enzyme.

The present instance may well be the first case reported of an extracellular hydrolase that is under allosteric control. No other case is known to us; as a general rule, hydrolases are relatively simple, stable, low molecular weight enzymes (10). Molting fluid chitinase has a molecular weight greater than 100,000 Daltons and appears to consist of several subunits of about 34,000 molecular weight (Bade and Stinson, unpublished).

While explanations other than cooperativity are possible for the sigmoidicity observed for chitinase, the idea of an allosteric molting chitinase is very attractive. Possession of such an enzyme would confer considerable selective advantage on an insect. The structure of cuticle is in some respects analogous to the structure of vertebrate skin: A fibrous material, chitin in insects but collagen in vertebrates, is embedded in a matrix that consists of protein in insects and glycosaminoglycans in vertebrates (9). Insects are absolutely dependent on the state of their cuticle for survival, and the state of the cuticle must be fitted to the demands of the moment. Prior to the molt, the integrity of the cuticle must not be breached, while during the molt, old cuticle must break down at maximal rates. If chitin were to break down prematurely, the insect could not survive because the tensile strength of the cuticle would drop rapidly, but failure of old cuticle to break down during the

molt also dooms it, since it then cannot shed the old cuticle so as to resume feeding and grow into the new cuticle.

Recently, we reported (3) that cuticle chitin must be "activated" by trypsin-like protease before it can be attacked by chitinase; this activation may consist in an unmasking of chitin to free it of masking protein(s). We have shown elsewhere (6) that chitinase present in intact cuticle can act only on exogenous chitin; thus the effective concentration of chitin which can serve as chitinase substrate approaches zero in intact cuticle prior to the molt. Possession of a homotropic allosteric molting fluid chitinase permits additional fine control of the breakdown of cuticular macromolecules: Prior to the molt, it prevents breakdown of chitin molecules which might accidentally become unmasked, while during the molt it promotes rapid breakdown of naked chitin.

Acknowledgments. This work was supported by Grant ES00650 from the Institute of Environmental Health Sciences, NIH. Our thanks are due to Dr. J. M. Yos for the least squares curve fitting, Michael Dewey and Judith Hanley for faithful caterpillar nursing, and Drs. Arlene Wyman and Evan Kantrowitz for critical reading of the manuscript.

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