CHITIN METABOLISM: NOT A TARGET OF AVERMECTIN/ MILBEMYCIN ACTIVITY IN INSECTS

Pamela M. Gordnier, J. Brezner and S. W. Tanenbaum

SUNY College of Environmental Science and Forestry, Syracuse, N.Y. 13210, U.S.A.

(Received for publication July 22, 1986)

The avermectins and milbemycins represent a new class of antibiotics with high insecticidal, acaricidal and anthelmintic activities[1,2]. Current evidence suggests that these compounds interfere with neurotransmission at the GABA receptor (refs 3, 4, and references therein). However, based on experiments using a methanol extract of *Streptomyces avermitilis* with brine shrimp and several microbial test organisms, it was reported[5] that chitin metabolism was the alternative major target of these drugs at low concentrations. We have examined this claim using commercially resolved avermectin B_{1a} (AVM) and milbemycin D (MBD) samples and crude chitinase and chitin synthetase preparations from a variety of insect species. In our experiments, neither compound had any apparent effect on chitin synthesis or on chitin hydrolysis. These findings parallel those recently published[6] in which it has been shown that pure AVM has no effect upon fungal chitin metabolism.

Tritiated chitin was prepared by the method of Molano *et al.*[7] using tritiated acetic anhydride (50 mCi/mmol) from New England Nuclear and chitosan from Pfanstiel Laboratories. The labeled polymer was characterized by selective hydrolysis at 40°C for 8 hours in 11 N HCl[8]. The excess mineral acid was removed by flash evaporation and the remaining syrup was taken up in distilled water. The hydrolysis products were analyzed by HPLC using an HPX 42A column (BioRad) held at 85°C with distilled water as the carrier phase (flow rate, 0.2 ml/minute). The bulk of the product was *N*-acetyl-D-glucosamine (NAG) with chitobiose and chitotriose present in very small amounts. Higher oligosaccharides were not detected but D-glucosamine was also present in significant amounts. This observation reflects either partial chitosan hydrolysis or its incomplete re-acetylation. The mixture of chitin oligosaccharides was purified by TLC on silica gel using *tert*-butyl alcohol - water - ammonia (70:30:1) as the developing phase[9]. The zone which contained NAG was scraped off and then eluted with water. The concentration of NAG was determined by a variation[10] of the Morgan-Elson assay. Its radioactivity was determined by scintillation counting in a xylene-based scintillation cocktail. From these procedures, the specific activity of the radioactive chitin was determined to be 14.2 dpm/nmol.

As test organisms for the chitinase and the chitin synthetase assays, fourth and fifth instar *Tenebrio molitor* larvae maintained on enriched whole wheat flour; *Acheta domestica* nymphs from a local pet store; and *Galleria mellonella* larvae from Carolina Biological Supply Company, were used. Insect chitinase extracts were essentially prepared by the method of Singh and Vardanis[11], except for the substitution of 50 mM citrate - phosphate buffer, pH 5.4. Purified *Streptomyces antibioticus* chitinase was obtained from Calbiochem. Chitin synthetase preparations were obtained following literature procedures[12~14]. Microsomes were prepared either by ultracentrifugation (for *Acheta* and *Galleria*) or by calcium chloride precipitation (*Tenebrio*). Avermectin B_{1a} (containing 6% B_{1b}) and milbemycin D (lot No. PM-41) donated by Merck, Sharp and Dohme and Ciba-Geigy, respectively, were dissolved in methanol for all assays. Polyoxin D (POD) from Calbiochem was dissolved in the assay buffer used for chitin synthetase controls. Protein assays were done either by the method of Markwell *et al.*[15] or by the Bradford assay[16].

The effects of AVM and MBD on chitinase preparations from *Streptomyces antibioticus*, *Tenebrio molitor*, *Acheta domestica* and *Galleria mellonella* were tested at drug concentrations between 1×10^{-5} and 1×10^{-6} M. At these relatively high levels, neither chemical inhibited chitinase by more than 10% for any of the test species (Table 1). Possible drug effects on insect chitin synthesis were next examined (Table 2). As a positive control, POD was also included in this series. *Tenebrio* chitin synthetase was inhibited less than 10% at AVM and MBD concentrations of 1×10^{-5} to $5\times$

Table 1. Effects of AVM or MBD or chitinases[a].

Compound	Concentration (M)	Control activity[b] (%)			
		Streptomyces	*Tenebrio*	*Acheta*	*Galleria*
AVM	1×10^{-5}	120	96	104	110
	1×10^{-6}	92	102	92	100
MBD	1×10^{-5}	94	91	98	105
	1×10^{-6}	96	94	100	107

[a] Assays were done according to the method of BODEN *et al.*[18], with the following changes; each assay contained 0.1 ml of the enzyme extract, 0.2 ml of tritiated chitin fines suspended in 50 mM citrate - phosphate buffer (pH 5.4) and 0.01 ml of methanol or test compounds dissolved in methanol. The assay mixtures were incubated at 36°C for 5 hours. A one hundred μl aliquots of the supernatent were taken for scintillation counting. Requisite Jack-bean *N*-acetyl-β-D-glucosaminidase was purchased from Sigma Chemical Company.

[b] Control (100%) activities were: *Tenebrio*; 1,745 dpm/mg protein; *Acheta*; 1,067 dpm/mg protein, *Streptomyces*; 7,490 dpm/mg protein, *Galleria*; 234 dpm/mg protein.

Table 2. Effects of AVM or MBD on chitin synthetases[a].

Compound	Concentration (M)	Control activity[b] (%)		
		Tenebrio	*Acheta*	*Galleria*
AVM	1×10^{-5}	92	97	108
	5×10^{-7}	94	—	—
MBD	1×10^{-5}	107	105	104
	5×10^{-7}	86	—	—
POD	5×10^{-5}	10	72	52
	5×10^{-7}	47	—	—

[a] Assays were carried out by the method of COHEN and CASSIDA[12,13]. Tritiated UDP-*N*-acetylglucosamine was obtained from New England Nuclear.

[b] Controls (100%) of radioactivity incorporated into chitin were: *Tenebrio*; 131~284 dpm/mg protein (range), *Acheta*; 87 dpm/mg protein, *Galleria*; 234 dpm/mg protein.

10^{-7} M. However, with POD in this range, a dose-dependent inhibition of the enzyme complex was observed. Neither AVM nor MBD showed any effect on chitin synthetase from *Acheta* or *Galleria* at 1×10^{-5} M (Table 2). *Acheta* chitin synthetase activity was reduced to 72% of the control in the presence of 5×10^{-5} M POD, while *Galleria* chitin synthetase activity was reduced to 52% control. We attribute the differences in POD activity to interspecial sensitivities in their chitin synthetase enzymes[17].

Because the avermectins and the milbemycins are active in low concentrations against nematodes and arthropods, it is of considerable interest to pinpoint their biochemical mode of action. While all preliminary evidence points toward their blockage of neurotransmission[3,4], other targets for these novel compounds may be involved at the molecular level. In this vein, evidence was presented by CALCOTT and FATIG[5] that AVM interferes with chitin synthesis and turnover in susceptible organisms. Their inferences as regards drug inhibition of chitin synthesis and turnover were recently contested by ONISHI and MILLER[6], who found that avermectin B_{1a} had no effect on chitin synthesis or turnover in several fungi. It was further indicated[6] that the previously observed effects[5] were most likely produced by trace amounts of oligomycin in the unfractionated methanol extracts. Here, we have studied drug effects on the activity of chitinase and chitin synthetase from a variety of insects at concentrations that were greater than or equal to those used by CALCOTT and FATIG (1×10^{-7} to 1×10^{-8} M) and have been unable to detect any significant inhibitions. These results suggest, for insects, that it is unlikely that AVM and MBD interact directly with critical chitinase or chitin synthetase enzymes. Accordingly, it would appear that the next logical step to elucidate the mechanism of action of these remarkable natural products

would be to seek their specific neuroreceptors.

Acknowledgments

This investigation was supported by the U.S. Department of Agriculture Cooperative Research Service (McIntire-Stennis Program).

References

1) CAMPBELL, W. C.; R. W. BURG, M. H. FISHER & R. A. DYBAS: The discovery of ivermectin and other avermectins. *In* Pesticide Synthesis Through Rational Approaches. ACS Symposium Series No. 255. *Ed.*, P. S. MAGEE, pp. 5~20, 1984
2) MISHIMA, H.: Milbemycin: A family of macrolide antibiotics with insecticidal activity. *In* IUPAC Pesticide Chemistry 2. *Eds.*, J. MIYAMOTO & P. C. KEARNEY, pp. 129~134, Pergamon Press, Oxford, 1983
3) ABALIS, I. M.; A. T. ELDEFRAWI & M. E. ELDEFRAWI: Actions of avermectin B1a on the γ-aminobutyric acid receptor and chloride channels in rat brain. J. Biochem. Tox. 1: 69~82, 1986
4) TANAKA, K. & F. MATSUMURA: Action of avermectin B1a on the leg and the nervous system of the american cockroach. Pestic. Biochem. Physiol. 24: 124~135, 1985
5) CALCOTT, P. H. & R. O. FATIG, III: Inhibition of chitin metabolism by avermectin in susceptible organisms. J. Antibiotics 37: 253~259, 1984
6) ONISHI, J. C. & T. W. MILLER: The lack of antifungal activity by avermectin B1a. J. Antibiotics 38: 1568~1572, 1985
7) MOLANO, J.; A. DURAN & E. CABIB: A rapid and sensitive assay for chitinase using tritiated chitin. Anal. Biochem. 83: 648~656, 1977
8) RUPLY, J. A.: The hydrolysis of chitin by concentrated hydrochloric acid, and the preparation of low-molecular-weight substrates for lysozyme. Biochim. Biophys. Acta 83: 245~255, 1964
9) POWNING, R. F. & H. IRZYKIEWICZ: Separation of chitin oligosaccharides by thin layer chromatography. J. Chromatogr. 29: 115~119, 1967
10) ENGHOFFER, E. & H. KRESS: An evaluation of the Morgan-Elson assay for 2-amino-2-deoxy sugars. Carbohydr. Res. 76: 233~238, 1979
11) SINGH, G. & A. VARDANIS: Chitinases in the house fly, *Musca domestica* pattern of activity in the life cycle and preliminary characterization. Insect Biochem. 14: 215~218, 1984
12) COHEN, E. & J. E. CASIDA: Properties of *Tribolium* gut chitin synthase. Pestic. Biochem. Physiol. 13: 121~128, 1980
13) COHEN, E. & J. E. CASIDA: Inhibition of *Tribolium* gut chitin synthase. Pestic. Biochem. Physiol. 13: 129~136, 1980
14) COHEN, E.: Chitin synthetase activity and inhibition in different insect microsomal preparations. Experientia 41: 470~472, 1985
15) MARKWELL, M. K.; S. M. HAAS, L. L. BIEBER & N. E. TOLBERT: A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 87: 206~210, 1978
16) BRADFORD, M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72: 248~254, 1976
17) STODDART, R. W. (*Ed.*): The Biosynthesis of Polysaccharides. pp. 254~258, Macmillan Publishing Co., New York, 1984
18) BODEN, N.; U. SOMMER & K. D. SPINDLER: Demonstration and characterization of chitinases from the drosophila K cell line. Insect Biochem. 15: 19~23, 1985