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Characterization of Carboxylesterase from Malathion Degrading Bacterium: *Pseudomonas* sp. M-3

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Carboxylesterase, a group of relatively non specific enzymes, are widely distributed in nature and catalyze the hydrolysis of carboxylic acid esters to their respective acid anions and alcohols. Significant quantities of these enzymes have also been demonstrated in some microorganism capable of hydrolyzing malathion (Barik et al., 1984). The use of crude enzymes in waste water treatment for the removal of certain toxicants (e.g. parathion) has been proposed by several investigators (Klibanov, 1981;Munnecke et al.,1982). In order to explore the possibility of degrading high amounts of malathion in waste water an attempt has been made to study the characteristics of the enzymes found capable of degrading malathion in aquatic medium.

MATERIALS AND METHODS

Malathion (99.8%) was obtained from American Cyanamid Co. Princeton, N.J.. NAD^+ and Alcohol dehydrogenase of analytical grade were obtained from Sigma Chemical Company, St. Louis,Mo, U.S.A.

Pseudomonas sp. M-3, isolated from a papermill effluent was grown in mineral salts medium, pH 6.8, containing 100 µg of malathion (dissolved in 1% ethanol solution) per ml of sterile salts medium (Singh et al., 1986). The contents were incubated for 24 hours at 30 °C on a rotary shaker.

The bacterial cells were harvested by centrifugation (12,000 x g, 10 minutes) in late log phase of growth after 24 hours of incubation. The cells were resuspended in 250 ml of 0.1 M sodium phosphate buffer, pH 7.2 and centrifuged. The pellets were resuspended in 50 ml of 0.1 M sodium phosphate buffer, sonicated for 4 minutes

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using an Ultrasonic disintegrator P-2 (Vibronics, India) and centrifuged at 12,000 x g for 10 minutes to remove whole cells and cell debris. The resulting supernatant was stored at 4 °C for 24 hours and used for enzyme assay.

Carboxylesterase activity was determined following the method of Wolfgang Junge (1984)with certain modifications as detailed earlier(Singh, 1987) using malathion as substrate.

$$\text{Malathion} + H_2O \xrightarrow{\text{Carboxylesterase}} \text{Malathionmonoacid} + \text{Ethanol}$$

$$\text{Ethanol} + NAD^+ \xrightarrow{\text{Alcohol dehydrogenase}} \text{Acetaldehyde} + NADH + H^+$$

The rate of hydrolysis of malathion was monitored spectrophotometrically by allowing the product ethanol to react with NAD^+ in the presence of alcohol dehydrogenase. The increase in absorbance due to formation of NADH was measured at 340 nm using a Spectronic-21 spectrophotometer. The nmoles of NAD^+ reduced in a unit time was equivalent to nmoles of malathion hydrolysed.

The enzyme assay was carried out at 30 °C in a cuvette containing 2.5 ml of incubation mixture consisting of carboxylesterase enzyme preparation (100 µg protein), sodium phosphate buffer (0.1 M, pH 7.5), NAD^+ (1.8 mM) and alcohol dehydrogenase (25 units/ml). The reaction was initiated by adding 5 µl of 2 mM malathion dissolved in acetone into the cuvette at a final concentration of 4 µM. The increase in absorbance at 340 nm due to the formation of NADH was recorded using a Spectronic-21 spectrophotometer at 30 seconds interval for a period of 3 minutes. Protein concentration was determined using the method of Lowry et al (1951). The specific activity of the enzyme is expressed in nmoles malathion hydrolyzed/min /mg protein. Under the above assay conditions the amounts of NAD^+ and alcohol dehydrogenase were not rate limiting.

Enzyme kinetic studies were carried out to determine the influence of pH, temperature and substrate concentration on the enzyme activity. The influence of selected salts and solvents on enzyme activity was determined by preincubating 100 µg enzyme protein for 5 minutes with 2.5 ml of 0.1 M sodium phosphate buffer, pH 7.5, containing salts and organic solvents at 5% level. The degradation product formed during the incubation of

malathion (50 ppm) with the cell free enzyme extract for half an hour were identified by TLC and infrared spectral analysis. The method used for extraction, separation and chromatography of malathion and its degradation products were same as described by Walker et al. (1974).

RESULTS AND DISCUSSION

Crude enzyme extracts obtained from Pseudomonas sp. M-3 capable of degrading malathion showed a specific activity of 900 nmoles malathion hydrolysed/min/mg protein. The Km and Vmax were 2.5 µM and 1240 nmoles malathion hydrolyzed/min/mg protein respectively (Figure 1a, 1b). The lower Km (2.5 µM) and higher Vmax (1240 nmoles malathion hydrolyzed/min/mg protein) value of the carboxylesterase of Pseudomonas sp. M-3 in comparison to some other bacteria suggest that the enzymes has higher affinity for malathion than that observed with the other bacterial enzymes (Walker and Stojanovic, 1974; Bourquin, 1977).

The enzyme was active between 20 to 45 ^{o}C with optimum activity occurring at 30 ^{o}C (Figure 2). The enzyme extract was active over a wide pH range (6-9) and the optimum enzyme activity occurred at pH 7.5 (Figure 3).

Cell free extract stored in aqueous solution at 4 ^{o}C for two weeks were found to retain 82% of the carboxylesterase activity indicating that the enzyme was stable in aqueous solution.

Effect of various salts and solvents up to a concentration of 5% was examined on the activity of carboxylesterase in view of the probability that they may be present in significant quantities in the industrial wastes. The enzyme activity was found to decrease by 15% in presence of sodium chloride (5%) and by 35% in presence of calcium chloride (5%) while the magnesium chloride (5%) had no significant effect on the activity of carboxylesterase. The lower concentrations of these salts had no significant effect on enzyme activity. Amongst the organic solvents examined, hexane and acetone were found to have no significant effect on the enzyme activity while xylene and chloroform inhibited the same by 30 and 18% respectively.

Carboxylesterase activity was assayed in Pseudomonas sp. M-3 grown on different energy sources in the presence or absence of malathion. The enzyme activity was found to be independent of the presence of malathion (Table 1) suggesting the constitutive nature of carboxylesterase enzyme of Pseudomonas sp. M-3.

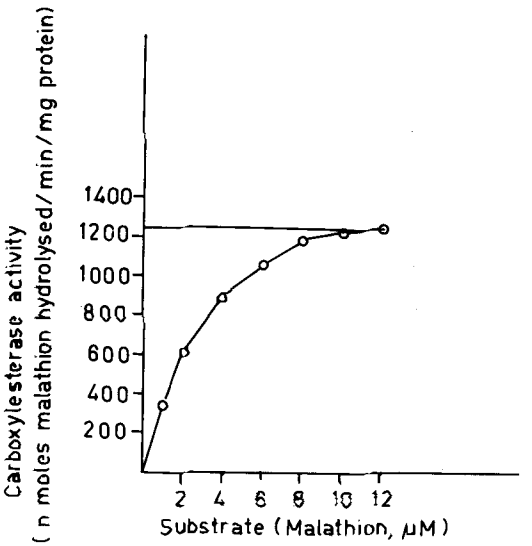

Figure 1a. Carboxylesterase Activity as a Function of Substrate Concentration

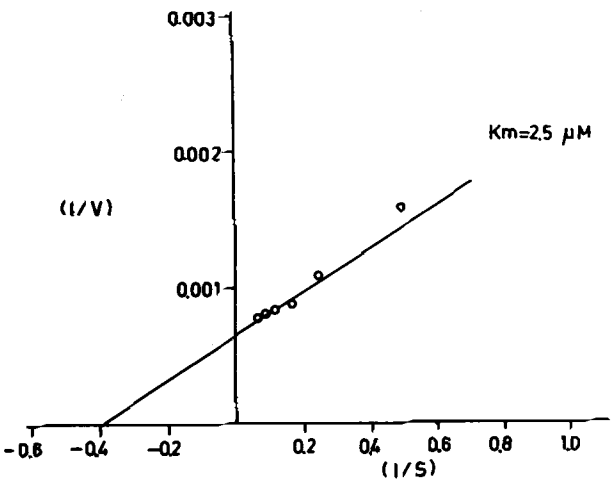

Figure 1b. Line Weaver-Burke Plot Representation of Figure 1a

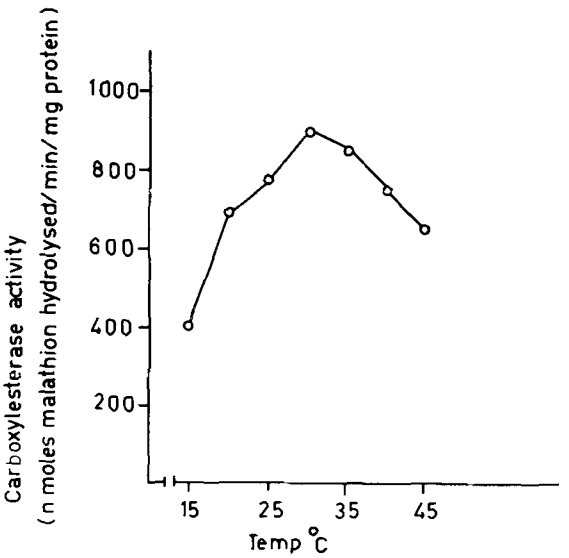

Figure 2. Carboxylesterase Activity as a Function of Temperature

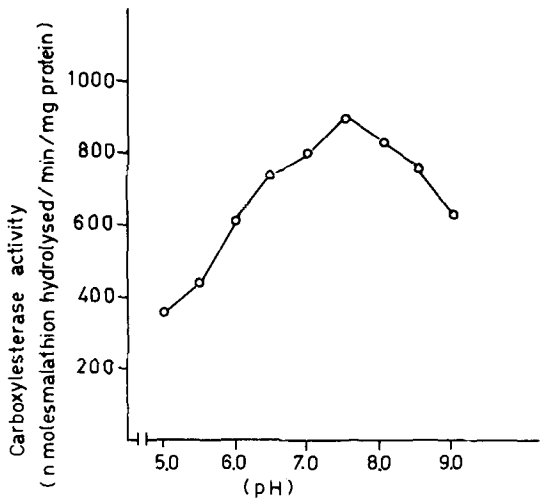

Figure 3. Carboxylesterase Activity as a Function of pH

Table 1. Carboxylesterase activity in cell free extracts of Pseudomonas sp. M-3 grown on various energy sources in the presence or absence of malathion

Energy sources used for the growth of Pseudomonas sp. M-3 from where the cell free extract was made	Specific activity (nmoles malathion hydrolysed/min/mg protein)
Malathion (100 ppm)	No growth No activity
Ethanol 1% (v/v)	890 ± 32
Ethanol 1% (v/v) + Malathion (100 ppm)	900 ± 36
Glucose 0.2% (w/v)	896 ± 35
Glucose 0.2% (w/v) + Malathion (100 ppm)	912 ± 32
Succinate 0.2% (w/v)	865 ± 35
Succinate 0.2% (w/v) + Malathion (100 ppm)	870 ± 40

Pseudomonas sp. M-3 was grown on different carbon sources, e.g. ethanol (1%), glucose (0.2%) and succinate (0.2%) in the presence and absence of 100 µg/ml malathion. The cells were harvested, sonicated and carboxylesterase activity assayed in 12,000 x g supernatant using 4 µM malathion as described in materials and methods.

Data represents mean ± S.E. of three values.

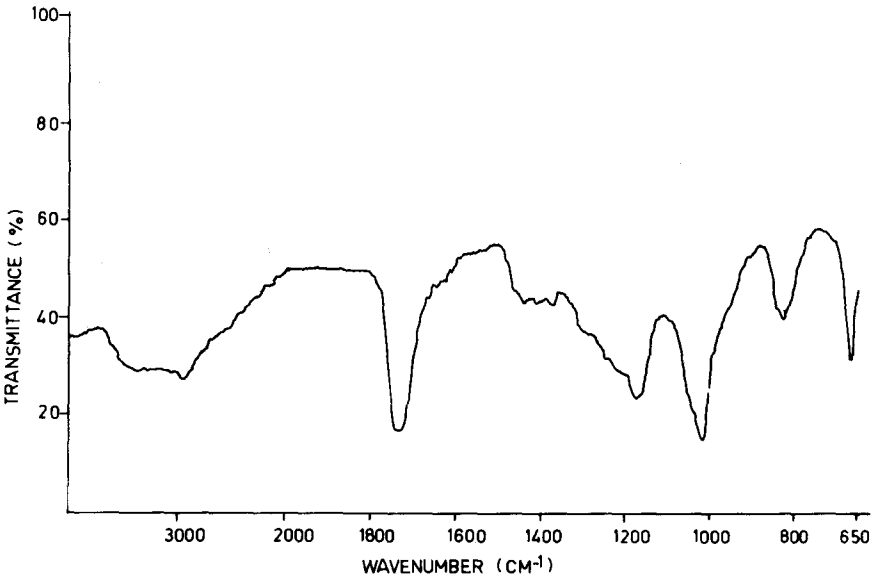

Figure 4. Infrared Spectral Tracing of the Metabolic Product of Malathion

The infrared absorption spectrum of the single metabolite isolated using TLC showed major bands at 655, 1,380, 1,730 and reduced adsorption at 1,100 and 1,170 cm^{-1} (Figure 4). These characteristics were found to be identical with those of malathion monocarboxylic acid (Walker and Stojanovic,1974). Accordingly, the metabolite was identified as malathion monocarboxylic acid. Malathion monoacid was the only product formed when cell free enzyme extracts were used as compared to enzyme extracts obtained from some microorganisms isolated from soil where both malathion mono and dicarboxylic acid were formed (Bourquin,1977).

Lack of any significant effect of high salts and solvent concentrations on the enzyme activity and the ability of the enzyme to actively carry out the hydrolysis of the pesticide over a broad range of temperature and pH, suggests a great potential for its use to treat the industrial effluent. However further studies are needed to confirm this.

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REFERENCES

Barik S, Munnecke DM, Fletcher JS (1982) Enzymatic hydrolysis of malathion and other dithioate pesticides. Biotechnology Letters 4:795-798.

Bourquin AW (1977) Degradation of malathion by saltmarsh microorganism. Appl Environ Microbiol 33:356-362.

Junge W (1984) Carboxylesterase. In: Bergemeyer HU (ed) Methods of Enzymatic Analysis, Vol 4. Verlag Chemie Weinheim, Deerfield Beach, Florida, Basel. p 2.

Klibanov AM (1981) 3rd Symposium Biotechnol in Energy Production and Conservation. Gatlinburg, TN. May.

Lowry OH, Rosenbrough Nj, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265-275.

Munnecke DM, Johnson LM, Talbot HW, Barik S (1982) Microbial metabolism and enzymology of selected pesticides. In:Chakrabarty AM (ed) Biodegradation and Detoxification of Environmental Pollutants. CRC Press, Inc, Florida, p 1.

Singh AK, Shanker R, Seth PK (1986) Isolation and characterization of a ethanol utilizing bacterium. Ind J Microbiol 26:257-260.

Singh AK (1987) Microbial degradation of some pesticides. Ph.D. Thesis, Banaras Hindu University, Varanasi, India.

Walker WW, Stojanovic BJ (1974) Malathion degradation by an Arthrobacter sp. J Environ Quality 3:4-10.

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