SUBSTRATE SPECIFICITY OF CARBOXYLESTERASE FROM *Vicia faba* ROOTS

Jana BARTHOVÁ[a], Hana LÖFELLMANNOVÁ[a] and Karel BENEŠ[b]

[a]*Department of Biochemistry, Charles University, 128 40 Prague 2*
[b]*Institute of Molecular Biology of Plants, Czechoslovak Academy of Sciences, 370 05 České Budějovice*

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Michaelis constants values and limiting rates of the reaction catalyzed by carboxylesterase isolated from the root tips of *Vicia faba* were determined. 1-Naphthyl acetate, 1-naphthyl butyrate, 1-naphthyl ester of N-acetylglycine, 1-naphthyl ester of L-leucine and 1-naphthyl ester of N-acetyl-L-leucine were used as substrates. The values for the enzyme from control plants were compared with the data for carboxylesterase from plants exposed to the effect of 2,4-dichlorophenoxyacetic acid. An electrophoretic analysis of enzymes from control plants and the tested plants was carried out.

Carboxylesterases constitute a numerous group of enzymes for which a broad substrate specificity and a pH optimum between 5 and 9 is characteristic. Carboxylester hydrolase (EC 3.1.1.1) is widely distributed, the enzyme from the liver of mammals has been investigated most thoroughly[1-3]. This enzyme is bound to the external membrane of the endoplasmatic reticula[4]. Its physiological role is not yet completely elucidated, it evidently takes part in the detoxification of xenobiotics of the ester and amide type[2,3,5]. In papers devoted to the study of plant enzymes, an especially high activity of carboxylesterase in the dividing meristematic cells of the root tips has been demonstrated[6,7]. Carboxylesterase is a very sensitive indicator of damage of plant cell[8] and therefore we turned our attention to its study.

EXPERIMENTAL

Material. Vicia faba L., var. Chlumecký (Oseva, Uherský Ostroh), acrylamide, 1-naphthyl acetate (NA) (Lachema, Brno), Fast Blue BB salt, 1-naphthyl butyrate (NB), N,N,N,'N'-tetramethylenediamine, Coomasie Brilliant Blue G 250, methylene-bis-acrylamide (Serva, Heidelberg), 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma, St. Louis), 1-naphthyl ester of N-acetylglycine (NAG), 1-naphthyl ester of L-leucine (NL) and 1-naphthyl ester of N-acetyl-L-leucine (NAL) (Institute of Organic Chemistry and Biochemistry, Prague). The remaining chemicals used were of analytical grade.

Cultivation of plants. The seeds of the bean were allowed to germinate for 4 days at 20 °C in a humid box in the dark. Between the fifth and the tenth day the control plants were cultivated hydroponically in Knop's nutritional solution[9] and the experimental plants in Knop's solution containing 2,4-D at a

concentration of 0.05 mmol l^{-1}. The tips of the main root, 15 to 20 mm long, were then worked up. The plant material was stored at −20 °C.

Extract ion of carboxylesterase. Frozen roots were ground with 0.1M citrate–phosphate buffer of pH 6.5 (1 part of the sample with 4 parts of the buffer) in the cold . The homogenate was centrifuged in the cold at 4 000 *g*, for 30 min. The extract of the enzyme was fractionated with ammonium sulfate. The active fraction precipitated between 40 to 70% saturation. The active fraction was dialyzed for 4 days in the cold against distilled water and eventually concentrated by freeze-drying.

Determination of the carboxylesterase activity. An amount of 0.1 ml of the enzyme solution was added to 3 ml of 1 mM solution of substrate in 0.1M citrate–phosphate buffer of pH 6, containing 3 mg of Fast Blue BB, temperated at 21 °C. After 30 min the reaction was stopped by addition of 5 ml of a mixture of ethyl acetate and isoamyl alcohol (4 : 1). A control experiment was carried out for each determination, in which the solution of the enzyme was added as the last, after addition of the organic solvent. The mixture was thoroughly shaken and absorbance of the organic phase of the sample was measured against the control experiment, at 560 nm. The activity of the enzyme is expressed in catals.

Analyses of proteins. The concentration of proteins was determined by the method of Bradford[10], using bovine albumin as a standard. The electrophoresis on polyacylamide gel was carried out according to Davies[11]. For detection of activity the medium for the determination of activities in solution was used.

Kinetic measurements. The initial rate of the reaction catalyzed by carboxylesterase was always determined for at least 6 values of substrate concentrations, from 1.5 to 4.0 mmol l^{-1}. For each concentration of the substrate a control experiment was carried out. The value of the Michaelis constant (K_m) and the limiting (maximum) reaction rate (V) were determined according to Wilkinson[12] using a computer.

RESULTS

The values of K_m and V were determined for five substrates of carboxylesterase, i.e. 1-naphthyl acetate (NA), 1-naphthyl butyrate (NB), 1-naphthyl N-acetylglycinate (NAG), 1-naphthyl L-leucinate (NL) and 1-naphthyl N-acetyl-L-leucinate (NAL). For each of them five independent determinations were carried out, calculating the average value and the standard deviation. The results are summarized in Table I.

TABLE I

Effect of 2,4-D on the activity of carboxylesterase in the root tips of *Vicia faba*. The values represent the mean of 5 determinations; no error of a determination exceeded 10%. For details see Experimental

Substrate	Control plants			Experimental plants		
	K_m mmol l^{-1}	V μCat	V/K_m ml s^{-1}	K_m mmol l^{-1}	V μCat	V/K_m ml s^{-1}
NA	0.78	7.99	10.2	2.00	13.30	6.7
NB	0.41	2.72	6.6	0.37	10.37	28.0
NAG	3.40	1.70	0.5	1.60	1.58	1.0
NL	0.55	0.70	1.3	2.00	0.87	0.4
NAL	2.00	0.36	0.18	2.20	0.29	0.13

The value of V decreases rapidly with increasing molecular weight of the substrate, both for the carboxylesterase from control plants and for the enzyme of the experimental plants. It decreased twenty times in the case of the control enzyme and even more than forty times in the case of the experimental one when the mass (weight) of the substrate is increased about twice. The value *of* K_m is lowest for NB in the case of the enzyme both from control and from experimental plants. In the case of the control material the values of K_m for N-acetylated substrates are distinctly higher than for the substrates with a free amino group. In the case of the enzyme from experimental plants the differences in K_m values are much less distinct.

When comparing the specific activity (expressed by the V/K_m ratio) from control and experimental plants cultivated in a 2,4-D solution, we observed an up to fourfold increase of the value for NB. A double specific activity of the enzyme from experimental plants was still found for NAG. For NA the value V did increase twice, but the value K_m increased more than twice simultaneously and therefore its specific activity is comparable with that of the control. For NL K_m is four times higher than for the control enzyme and therefore the specific activity of the enzyme from experimental plants with this substrate is also distinctly lower. In the case of NAL the kinetic parameters of both enzymes are comparable.

We further carried out an electrophoretic analysis of the enzyme preparation from control and experimental plants. For detection we used NA, NAG, NB and NL. From the densitograms of the electrophoreograms it is evident that under the effect of 2,4-D the pattern of the carboxylesterase activities has changed (Fig. 1). The number of carboxylesterase zones was reduced in experimental plants, for NA and NAG as substrates, to a single one, in contrast to three for control plants. The substrates NB and NAL displayed a single zone of enzyme activity both for control and the experimental plants.

DISCUSSION

The localization and the activity of the enzymes represent important indicators of the growth and the differentiation of cells. In this connection carboxylesterase[6,7] seemed an especially suitable labelling enzyme. For the study of the properties of this enzyme we selected five esters, differing in the acyl part of the molecule, i.e. NA, NB, NAG, NL and NAL. From the results it is evident that the value V of the reaction catalyzed with carboxylesterase decreases with the size of the substrate molecule, i.e. with the extension of the acyl residue. Its extension by two methylene units brings about a decrease of the V value by 3 to 5 times. Since the V value depends in fact on the rate of decomposition of the enzyme–substrate complex, it means that the larger the acyl part of the substrate, the less reactive is the acyl derivative of the enzyme. The reaction catalyzed with esterase has the character of the so-called "covalent catalysis", meaning that in the course of the catalysis a reactive chemically modified derivative of the enzy-

me molecule is formed. In the case of carboxylesterase this is the acyl derivative of the enzyme[13-15]. From our measurement it follows that the acetyl derivative of the enzyme is the most labile one, the enzyme with the attached residue of butyric acid is hydrolyzed more slowly, while the one which has the residue of acetylleucine in its active centre is hydrolyzed the slowest.

The affinity of the enzyme to the substrate may be evaluated on the basis of K_m values. From our results it follows that the longer the acyl residue of the substrate, the higher is its affinity to the enzyme, meaning that the K_m value is lower. However, this is valid only for an aliphatic and unbranched chain. From a comparison of K_m values for NB and NAG, or NL and NAL, it follows that the enzyme preferentially binds hydrophobic substrates. In the case of NL it should be considered that its affinity to the enzyme is affected positively by the hydrophobic part of the molecule, and negatively by the free amino group. The specific activity of the substrate to the enzyme represents a further kinetic parameter, evaluated by the V/K_m ratio. It may be stated unambiguously that the substrates with aliphatic chains in the acyl part of the molecule (NA and NB) have a much higher specific activity than the more polar or branched substra-

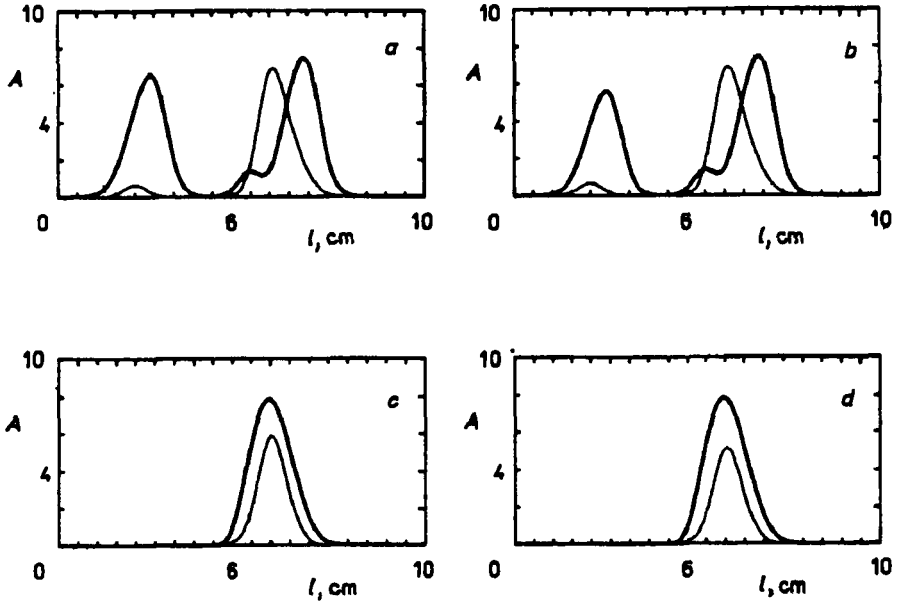

FIG. 1

Densitograms of the electrophoreograms of carboxylesterase from root tips of *Vicia faba*. Thick line control plants, thin line experimental plants; *a* substrate NA, *b* substrate NAG, *c* substrate NB, *d* substrate NAL. For details see Experimental

tes. The plant carboxylesterase, similarly as the animal enzyme[16], binds preferentially non-polar groups into the acyl site of the active centre and the affinity of the substrate to enzyme increases with increasing length of its acyl part. However, in contrast to the animal enzyme, in the case of plant carboxylesterase its acetyl derivative, but not acyl derivatives with longer chains[16], was the most reactive one.

Individual substrates were used for detection of electrophoreograms of the enzyme preparation. For NA and NAG three zones of activity were determined, while when using NB and NAL as substrate only one zone was detected. Only one of these forms of the enzyme cleaves all the substrates studied. The others probably represent the residues of other esterases or are a form of carboxylesterase which underwent some posttranslation modifications. Sialylation or various aggregations of the enzyme molecules[17] may come into consideration.

For modelling the situation of the negative effect of the medium on the plant cell we chose the effect of the herbicide 2,4-D. From histochemical analysis we know[8] that carboxylesterase is a suitable indicator of the damage to the plant cell. From these analyses it follows that a four days effect of 2,4-D at 0.05 mmol l^{-1} concentration elicits distinct changes both in the concentration of soluble proteins and in the activity and localization of carboxylesterase in tissue slices of root tips of the plant. From the mentioned results it follows that the character of the enzyme is also changed with respect to its kinetic properties. These changes are most distinct if NA or NB are used as substrate. In the case of other substrates the specific activity of the enzyme from experimental plants is lower by an order of magnitude and it is difficult to interpret the results. The kinetic properties of carboxylesterase, especially if NA or still better NB are used, should be utilized – in our opinion, for the evaluation of the functional state of the plant cell.

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