Acetylcholinesterase and Non-Specific Esterase Activities during the Regeneration of Planaria *Dugesia tigrina* (Girard)

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Biochemical modification of the acetylcholinesterase (AChE) and non-specific esterases of a planarian species [*Dugesia tigrina* (Girard)] were studied as a function of regeneration. The enzymatic activities were measured in regenerating planarians at regular time intervals from the beginning of the regeneration process to the complete restoration of the animal. Both enzymatic activities were substantially altered during the regeneration process of caudal and cephalic segments. Polyacrylamide gel zymograms for soluble α-naphthyl acetate hydrolytic activity revealed four bands of activities. These activities were fractioned into two major peaks on cation exchange high performance liquid chromatography, presented optimal pH between 6 and 7 and were inhibited by PMSF, DFP, BNPP and NaF but not by EDTA, TPCK, TLCK and *n*-ethyl maleimide.

Introduction

The regeneration is a complex physiological process which occurs in different organisms and consist in the replacement of lost structures. This phenomenon has been the subject of intense research and it was suggested that the formation of new tissues during the embryogenesis occurs at different command levels (Bronsted, 1969; Slack, 1987). At cellular level this process appears to comprise two distinct stages involving the programming of non-differentiated blastema cells followed by differentiation to determined cells which become part of missing structures (Spiegelman and Dudley, 1973). At molecular level it appears that a group of macromolecules including proteins (Smith and Wolpert, 1975; Steele and Lange, 1977) may play important inhibitory or stimulatory functions (Watanable and Child, 1935; Smith and Wolpert, 1975).

The morphological aspects of regeneration are well known. Some biochemical aspects of regeneration (Moraczewski *et al.*, 1987), myoblast differentiation (Hori, 1992), pattern of mitosis (Saló and Baguñà, 1984) and cellular activation (Martelly and Franquinet, 1984) have also been studied. Several drugs and chemicals have been shown to stimulate or inhibit regeneration in planarians, probably acting at cell proliferation (Franquinet, 1981; Baguñà *et al.*, 1989). Biochemical modification in the glycolytic metabolism of the planarians, *D. tigrina,* was studied as a function of regeneration. Significant differences in terms of metabolic parameters were observed between cephalic and caudal segments (Torres da Matta *et al.*, 1989). An investigation of the mechanism and metabolic regulation of the transformation which accompanies the regenerative process of planaria would require some prior understanding on the enzymatic action in the metabolism of amino acids and lipid. Recently, we have shown that the level of aminotransferases (Nery da Matta *et al.*, 1992), energetic substrates (Nery da Matta *et al.*, 1993) and lipid concentrations (Nery da

Matta *et al.,* 1994) are altered during the regeneration and that significant differences in terms of metabolic parameters were observed during the regeneration of *D. tigrina* planaria.

In the present report we studied the activities of esterases at regular time intervals from the beginning of regeneration, until the complete restoration of the animal. The assay was developed measuring the level of acetylcholinesterase (AChE, EC 3.1.1.7), and non-specific esterases with activity on short and long chain substrates. These studies constitute a necessary step towards a more detailed investigation on alterations of the enzymatic activities during the course of regenerative process of this animal.

Materials and Methods

Materials

Specimens of *D. tigrina* have been maintained in culture in our laboratory for ten years. This stock had not been observed undergoing sexual reproduction during this entire period and thus it was considered by the authors as being an asexual strain. The animals were kept at room temperature (20–25 °C) in plastic trays containing spring water (purity) and fed once a week with fresh chicken liver. Before experiments, the animals were fasted for one week. DTNB, acetylcholine iodine, DTT, NaF, octyl-glycoside, indoxyl acetate, PMSF, DFP, BNPP, TPCK, TLCK, MES, TES, DMSO and BSA were from Sigma Chemical Company, St. Louis, Mo., U.S.A. Acrylamide, bis-acrylamide, LGA and TEMED were from Bio-Rad Laboratories, U.K. All the other reagents were from Merck, Darmstadt, Germany.

Planarian culture

For regeneration studies, worms were cut transversely into sections by means of an incision behind the auricles. Cephalic and caudal sections were collected in two separate fresh water trays at room temperature. Samples were collected at 6 h intervals until 24 h and thereafter every 24 h until completion of the regeneration process (192 h).

Preparation of crude extracts and obtention of soluble fraction

Washed segments of intact planarians were disrupted by homogenizing in a potter homogenizer using 1 ml of cold extraction solution (0.1 M potassium phosphate buffer, pH 8.0) for acetylcholinesterase assay and freeze-thawed four times in 10 mM MES, pH 6.4 containing 1% octyl-glycoside for other esterase assay. The homogenates were centrifuged at $105,000 \times g$ for 30 min at 4 °C and the supernatants assayed immediately for enzyme activities.

Acetylcholinesterase and α-naphthyl esterase activities assay

AChE activity was determined spectrophotometrically using acetylthiocholine as substrate (Ellman *et al.,* 1961). The activity was expressed as µmol of acetylthiocholine hydrolyzed $\times$ min^{-1} $\times$ mg protein^{-1}.

α-Naphthyl ester hydrolase activity was determined at 235 nm as previously described (Mastropaolo and Yourno, 1981). α-Naphthyl acetate (NA) and α-naphthyl myristate (NM) were used; 50 mM, solutions in ethylene glycol monomethyl ether and chloroform were prepared, respectively. For the assay 4 µl of substrate was added to 10 mM MES, pH 6.4 buffer maintained at 37 °C, with a final volume of 1.0 ml, in a semimicro quartz cuvette. Enzyme samples (10–15 µl) were added and the rate of increase in absorbance was measured at 235 nm. One esterase unit was defined as the amount of enzyme which hydrolysis 1.0 µmol of α-naphthyl ester in 1.0 min at 37 °C.

Polyacrylamide gel electrophoresis and detection of esterase activity

Electrophoresis was carried out on mixed agarose-polyacrylamide gels (Benoist and Schwencke, 1990). A 4% (pH 6.7) stacking gel and 7% (pH 8.3) running gel containing, respectively, 0.2% and 0.4% of low gelling point agarose (LGA) were used. The polymerization of acrylamide was conducted at room temperature (25 °C) and the gelification of the agarose at 4 °C. Pre-electrophoresis of 15 min (two times) was conducted at 40 mA (4 °C) and the electrophoresis at 25 mA in running buffer (50 µM Tris–0.38 mM glycine, pH 8.3) containing 5 mM MgCl$_2$.

Hydrolytic activity to NA and NM was detected by coupling them with the dye Fast Garnet GBC (Yourno and Mastropaolo, 1981). Hydrolytic activity to 3-indoxyl acetate was revealed as de-

scribed (Benoist and Schwencke, 1990). Briefly, the gels were first equilibrated for 15 min in 100 ml of 100 mM Hepes-KOH, pH 6.8, containing 100 mM NaCl and 5 mM MgCl (E 1 buffer). Then with 20 ml of 50 mM Mes-KOH, 50 mM Tes-KOH, pH 6.8, containing 100 mM NaCl and 5 mM $MgCl_2$ (E 2 buffer). The revealing mixture contained 1 mM indoxyl acetate. As control was used carboxyl esterase and the reactivity was visible in 5–10 min.

Denaturing gel electrophoresis was performed in SDS (Laemmli, 1970) and protein detected by staining with silver (Niellsen and Brown, 1984).

Effect of inhibitors

Studies of the effect of inhibitors on hydrolase activity in gels were carried out by incubating segments cut from gel runs in parallel in baths of E 1 and E 2 buffer containing one of the following inhibitors at the indicated concentration: PMSF (2.5 mM), TPCK (0.5 mM), TLCK (0.5 mM), EDTA (0.1 mM) and NaF (40 mM). The gels were then stained for esterase activity as described before.

For the spectrophotometric assay the different inhibitors were added 10 min before the substrate and the α-naphthyl ester hydrolase activity measured as described (Mastropaolo and Yourno, 1981).

High performance cation exchange chromatography (HPCEC)

HPCEC was performed on an automatic Shimadzu 6A System using a polyacrylate (PA)-CM (10 μm; Shimadzu, Kyoto, Japan) column (8 mm× 10 cm) maintained at room temperature (25 °C). The samples were prepared in 25 mM acetate buffer, pH 5.0, and separated using a linear gradient from 0 to 0.3 M KCl-acetate buffer in 35 min and a flux of 0.8 ml min^{-1}. Eluates were monitored at 280 nm using a SPD-6AV detector controlled by a SCL-6B microcomputer. Data were recorded on a C-R6A Chromatopac computing integrator. Protein was determined using bovine serum albumin as standard (Lowry *et al.*, 1951).

Results

Fig. 1 shows the enzymatic activity of AChE during the head and tail regeneration of planarian.
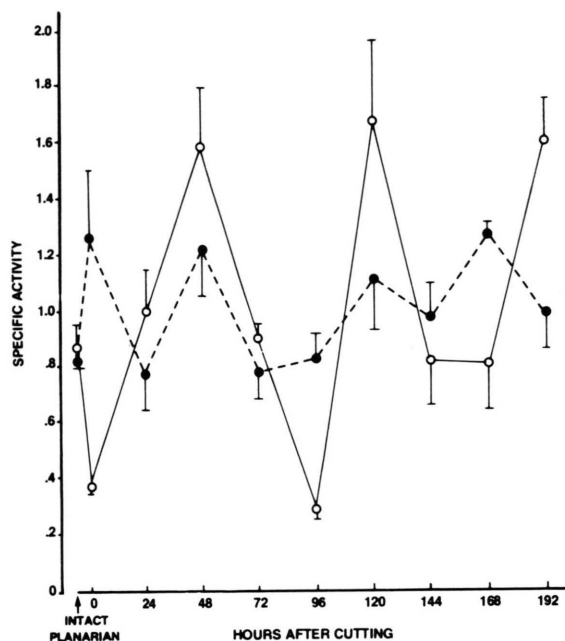
Fig. 1. Profiles of AChE activity during the regeneration of tail (filled symbols) and head (open symbols) of *D. tigrina* planarian. The enzymatic activity was expressed in μmol of acetylthiocholine hydrolyzed × min^{-1} × mg protein^{-1}. The level of the intact planarian was included for comparative purpose. The results represent the media ± S.D. of three experiments.

Fig. 2. Electrophoretic detection of esterase enzyme activity using the 3-indoxyl acetate as coupling dye. Intact *D. tigrina* planarian were disrupted by three cycles of freezing-thawing in 10 mM Mes, pH 6.4 and the 105,000×*g* soluble (a) and insoluble (b) fractions were analyzed by agarose-polyacrylamide gel electrophoresis. Each lane contained 50–70 μg of protein.

The results revealed that in the cephalic region the AChE activity is about 2.5- to 3-fold lesser than in the tail at time zero of the regeneration. After this period an intense oscillation is observed during the cephalic regeneration until the complete restoration of the animals. This activity was further detected in the $105,000 \times g$ insoluble fraction. Only one band of activity was observed on polyacrylamide gel electrophoresis using the 3-indoxyl acetate as substrate (Fig. 2b). No activity band was identified on the soluble extract (Fig. 2a).

Soluble enzyme preparations were prepared simultaneously from intact, head and tail regenerating planarian segments, and analyzed by LGA-PAGE. With α-NA (C2) as substrate a total of 4 electrophoretically distinguishable esterase bands were detected from intact planarians (Fig. 3). The enzyme pattern obtained with the same fraction from head and tail regenerating segments were quantitatively different during the regeneration with only traces of the four bands observed at 24, 48, 96 and 120 h of regenerating heads and at 72, 120 and 192 h of regenerating tails (Fig. 3). Essentially the same pattern of enzyme bands was obtained with regenerating head and tail, although

the total activity was higher at 96 h and 192 h of the regenerating tail and head, respectively. Quantitative estimation of esterase activities, as determined by spectrophotometric assay, typically revealed a 1.8- to 3.1-fold increase in esterolytic activity in the regenerating tail (Fig. 4).

With α-naphthyl myristate (C18) as substrate, no enzyme or enzyme activity was observed or detected by LGA-PAGE and spectrophotometric assay, respectively. Thus, suggesting that the soluble homogenates of *D. tigrina* planarians did not possess hydrolytic activity to longer chain esters.

The optimum pH at soluble esterase activities from adult planarians on α-NA, was obtained between pH 6.0 and 7.0 (Fig. 5). Thus, the effect of a series of specific and non-specific serine hydrolase

Fig. 3. Electrophoretic patterns of soluble esterases during the regeneration of head (H) and tail (T) of *D. tigrina* planarian. The numbers on the left side of the figure represent the identified bands. Each lane contained 50–70 µg of protein.

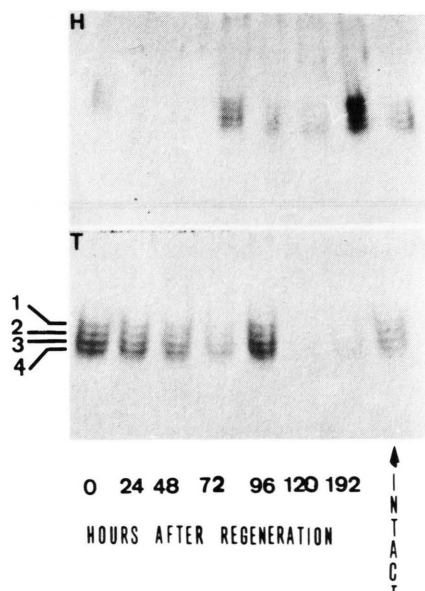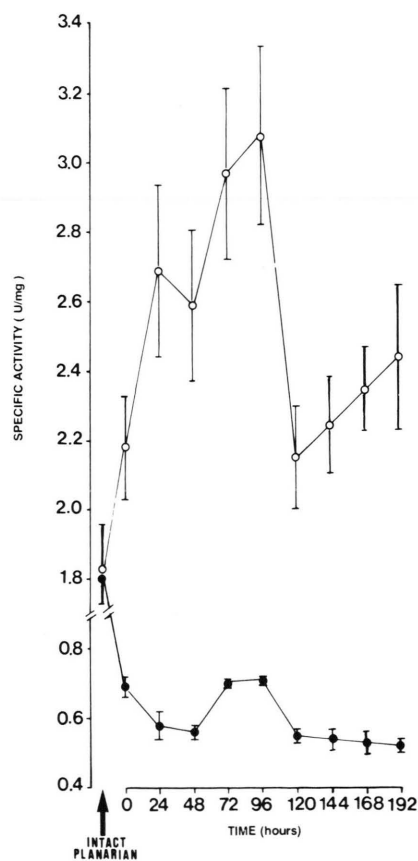Fig. 4. Activity level of esterase during the regeneration of the tail (open symbols) and head (filled symbols) of *D. tigrina* planarian using as substrate α-naphthyl acetate. The enzymatic activity was expressed in µmol of α-NA hydrolyzed × min^{-1} × mg protein^{-1}. The results represent the media ± S.D. of three experiments.
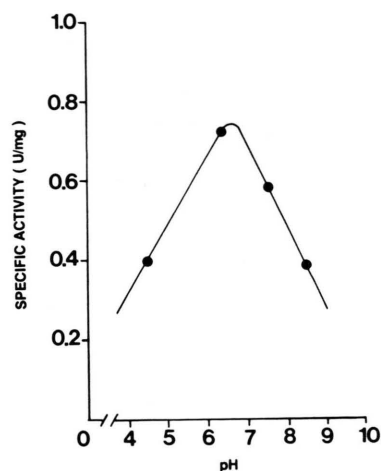
Fig. 5. Determination of the optimum pH of the soluble esterase activity on α-naphthyl acetate. Reaction conditions were described in Materials and Methods. Maximum activity was (69.56 nmol of naphthol) 0.77 U/mg of protein at pH 6.4.

Table II. Effects of inhibitors on the α-naphthyl ester hydrolase activity. 105,000×*g* soluble extract was tested for inhibition against a variety of inhibitors. Data are mean values from two experiments and showed a variation not greater than 5%.

Reagents	Concentration [mM]	Activity [%]
Control		100
PMSF	1.0	0
DFP	5.0	4
BNPP	0.2	68
N-Ethyl maleimide	1.0	100
EDTA	1.0	100
TPCK	0.1	100
TLCK	0.1	100

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropylfluorophosphate; BNPP, bis(*p*-nitrophenyl)-phosphate; EDTA, ethylenediaminotetraacetic acid; TPCK, ʟ-1-tosylamido-2-phenylethylchloromethyl ketone; TLCK, 1-chloro-3-tosylamido-7-amino-ʟ-2-hepatone hydrochloride.

inhibitors on the activity of individual esterase enzyme bands were determined at pH 6.8 and the results are showed in Table I. With α-NA as substrate all four bands were PMSF and fluoride-sensitive, but EDTA, TPCK and TLCK resistant. The enzymatic activity to α-NA was reduced in 100%, 96% and 32% in presence of PMSF, DFP and BNPP, respectively (Table II).

Some preliminary purification of the enzymes on HPLC was performed. HPLC-ion exchange chromatography of separate homogenate of intact planaria resulted in partial purification of the enzyme activities to α-NA ester substrate. Two peaks with retention time of 4.85 min (peak 1) and 7.21 min (peak 3) presented activity to α-NA (Fig. 6). The end peak 2 and 4 shows a substantial

Table I. Sensitivity of electrophoretically distinguishable soluble esterases to inhibitors.

| Esterase enzyme No. | Effect of hydrolase inhibitors | | | | |
	PMSF	TPCK	TLCK	NaF	EDTA
1	S	R	R	S	R
2	S	R	R	S	R
3	S	R	R	S	R
4	S	R	R	S	R

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; TPCK, ʟ-1-tosylamido-2-phenylethylchloromethyl ketone; TLCK, 1-chloro-3-tosylamido-7-amino-ʟ-2-hepatone hydrochloride; S, sensitive; R, resistant.

Fig. 6. Cation exchange HPLC of *D. tigrina* soluble extract (105,000×*g* supernatant) on PA-CM. 1.0 mg of protein (capable of producing 19 μmol of α-naphthol × min^{-1}) were applied to the column, and this was eluted as described under m.m. Absorbance at 280 nm was monitored and 1 ml fractions were collected. They were pooled, concentrated by ultrafiltration and then analyzed for esterase activity. Arrows indicate peaks with NA esterase activity.

amount of 280 nm absorbing material, but no hydrolytic activity was detected (data not shown).

Discussion

The principal biological role of acetylcholinesterase is termination of impulse transmission of cholinergic synapses by rapid hydrolysis of neurotransmitter acetylcholine. Histochemical studies on planarian *Procotyla fluviatilis*, show that AChE activity is localized in elements of the nervous system and is more intense in the cells and fibers comprising the submuscular and subepidermal nervous plexuses (Lentz, 1968). The enzyme was localized less intensively in the cephalic ganglia while large nerve bundles and epidermal sensory cells were unreactive. By the analysis of Fig. 1 we can see that during the morphological stages of blastema development and definitive differentiation of *D. tigrina*, the AChE activity increased and decreased substantially in head and tail regeneration. The fractionation of disrupted planarians at $105,000 \times g$ showed that this activity is distinct from the activity to α-NA found in the soluble fraction.

In addition, this study also demonstrated that *D. tigrina* planarian contains multiple molecular forms of esterase acting on α-NA. Separation of at least two of the forms was achieved using HPCEC. Different from the other invertebrates which present two and three bands on electrophoresis, the planaria produce four distinct bands. All four bands were inhibited by the same inhibitors suggesting to represent the same molecular entity. Electrophoretic surveys of α-NA esterases in invertebrates almost always describe heterozygotes as having a two-banded phenotype, consistent with a monomeric structure, although a few surveys have identified such esterases active in both monomeric and trimeric forms (Inkerman *et al.*, 1975; Searle, 1986).

Several neutral proteases in mammalian tissues are serine hydrolases and all serine hydrolases that have been investigated exhibit some degree of non-specific esterase activity (Benoist and Schwencke, 1990). PMSF has been reported to inhibit serine hydrolases much more effectively than it inhibits acetylcholinesterase and BNPP inhibits non-specific carboxylesterases but not acetylcholinesterase (Fahrney and Gold, 1963). The inhibition at a very high grade of all of the esterase enzymes by PMSF and DFP suggests that the detected bands are serine hydrolases. The observation that these isoenzymes that are inhibited by PMSF are also inhibited by BNPP would suggests that those esterases have substantially different specificities. However, the inhibition of the enzymatic activity by BNPP may be due to its non-specific inhibition ability (Fahrney and Gold, 1963). The failure of TLCK and TPCK to inhibit any of the esterase isoenzymes indicates that none of them represent the non-specific action of trypsin-like or chymotrypsin-like enzymes.

Since the metabolic function of the non-specific esterase is not known in most cases, it is difficult to speculate on the significance of the esterase enzymes level modification in cells when the animals are regenerating. Notwithstanding, an intense esterase activity could be observed during the regeneration of the tail in relation to the head (Fig. 4). The observed quantitative alteration in enzyme patterns could result from either selective repression of synthesis or enhancement of degradation of some enzymes in response to one or more factors that are technically difficult to distinguish between nutrient deprivation, reabsorption of tissues or accumulation of toxic metabolites. The activity obtained only on short chain substrate suggests that the non-specific esterases detected in this work are probably not involved in various aspects of lipid turnover in membranes. Irregardless of the validity of this proposal, the observation that esterase levels are altered in response to cut shock is consistent with the suggestion that these enzymes may play an important, as yet unelucidated, role in the intracellular protein turnover and consequently in the regeneration of planarians.

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