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DE NOVO SYNTHESIS OF ACETYLCHOLINESTERASE IN ROOTS OF *PISUM SATIVUM*

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Key Word Index—*Pisum sativum*; Leguminosae; pea; acetylcholinesterase activity; enzyme synthesis.

Abstract—*Pisum sativum* seeds contain a conserved acetylcholinesterase (AChE) which is active during the early stages of germination. The enzyme activity soon disappears and reappears after 72 hr of germination. A protein devoid of catalytic ability, but exhibiting similar chromatographic and electrophoretic properties as the active AChE, could be detected after 24 hr of germination. The pattern of incorporation of labelled amino acids into AChE and the influence of cycloheximide revealed that the AChE found in the roots from 72 hr onwards was entirely new. During this period of growth, the AChE protein accounts for 4–10% of the total proteins in the root tissue.

INTRODUCTION

Some of the enzyme proteins in the developing seeds may be converted into inactive forms and conserved in the seeds. These inactive proteins are activated during germination, and this does not involve *de novo* synthesis of proteins [1–6]. Apart from the conserved enzymes, there are many enzymes which are synthesised entirely *de novo* [7–13]. It has been shown that in the case of *Pisum sativum* that AChE activity varies with the age of the seedlings [14]. Investigations have been carried out to determine the reasons for such variation in AChE activity during early stages of growth.

RESULTS AND DISCUSSION

Acetylcholinesterase activity during germination and early stages of growth

Soon after imbibition, a considerable amount of AChE activity was detected in the seeds, but this activity was soon lost and by 48 hr the ability to hydrolyse acetylthiocholine had completely disappeared (Fig. 1). Interestingly, an inactive protein chromatographically and electrophoretically similar to active AChE was obtained both from the entire seedlings and also from excised roots. The enzyme activity reappeared 72 hr from imbibition in the roots. The reappearance of activity in the roots may be due to either reactivation of the inactive AChE or new synthesis of enzyme. Three different approaches were used to resolve these possibilities. They are physical and enzymatic methods of activation of the inactive AChE; incorporation of labelled amino acids into AChE; and influence of protein synthesis inhibitor.

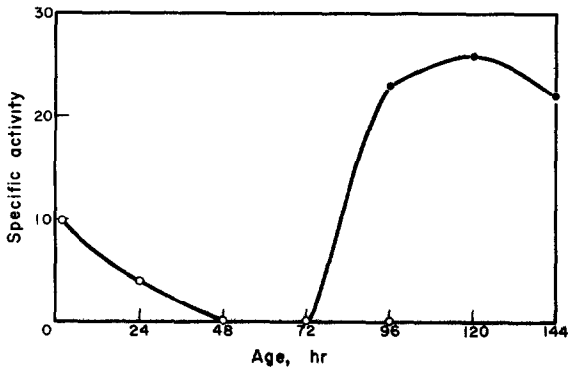

Fig. 1. AChE activity at different times after germination of pea seeds. O—O whole seedling ●—● excised roots.

Physical and enzymatic methods of activation of inactive AChE

It is known that some of the proteins get activated on repeated freezing and thawing [15]. However, this method had no influence on the inactive AChE from pea roots. Mild proteolysis with trypsin or chymotrypsin has been reported to activate enzymes [16]. Even this method failed to activate the inactive protein.

Radioactive tracer studies

1. *Incorporation of labelled amino acids from* Chlorella *protein hydrolysate-[^{14}C] into AChE at different times of germination and growth.* The pattern of incorporation of labelled amino acids from *Chlorella* protein hydrolysate-[^{14}C] into AChE protein is depicted in Fig. 2. It is evident that there is no synthesis of AChE during first 72 hr of germination. The *de novo* synthesis of AChE in root tissue starts soon after 72 hr, as there was a high incorporation of the label into the AChE of root tissue, after 72 hr of growth. Thus, all the catalytic activity that was observed during early stages of germination was due to conserved protein in the seed.

2. *Double labelling studies.* To confirm the results a double labelling experiment was performed and the results are presented in Table 1. When the seeds were grown for the first 72 hr in the presence of [^{3}H]- or [^{14}C]-labelled amino acid and then shifted to non-labelled medium, very little radioactivity was present in the AChE purified from roots after 120 hr of growth, but when allowed to grow in the presence of [^{3}H]- or [^{14}C]-labelled amino acid for up to 120 hr, very high radioactivity could be

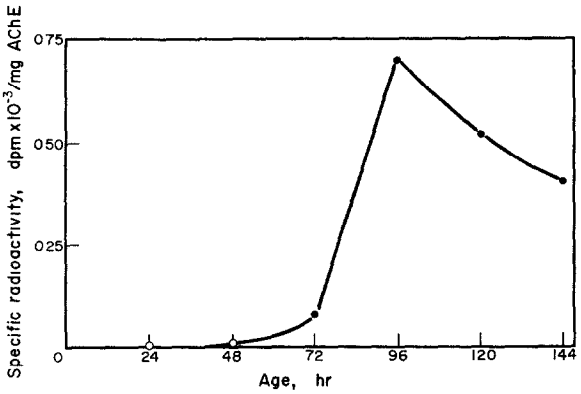

Fig. 2. Incorporation of amino acids from *Chlorella* protein hydrolysate-[^{14}C] into AChE at different times after germination of pea seeds. O—O whole seedling ●—● excised roots.

detected in AChE, indicating that incorporation of labelled amino acid into AChE occurs after 72 hr. The same results were obtained when the seedlings were transferred from non-labelled medium to labelled medium, after 72 hr of growth.

When seedlings were shifted from [^{3}H]- to [^{14}C]-medium after 72 hr of growth, all the radioactivity could be accounted for in [^{14}C]-isotope only. Similarly when the seedlings were transferred from [^{14}C]- to [^{3}H]-medium, no radioactivity due to [^{14}C]-isotope was detected. That is, only the label present after 72 hr alone is incorporated. From these results it is clear that most efficient and rapid synthesis of AChE occurs after 72 hr of growth.

Inhibitor studies

To demonstrate that active AChE synthesis occurs after 72 hr of growth, seedlings were treated with cycloheximide after 72 hr growth and the pattern of incorporation of labelled amino acids from *Chlorella* protein hydrolysate-[^{14}C] was followed (Table 2). The addition of cycloheximide to the seedlings almost completely prevented the incorporation of radioactive amino acids into the AChE. In the absence of the drug, there was an active incorporation of the isotopic amino acids into the AChE, indicating that new synthesis of AChE occurs after 72 hr of growth.

Table 1. AChE synthesis using histidine-[^{3}H] and phenylalanine-[^{14}C]

Label during 0–72 hr	Label during 72–120 hr: nil		histidine-[^{3}H]		phenylalanine-[^{14}C]	
	Activity in channel*					
	[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]
Nil	—	—	1 83	—	—	0 81
Histidine-[^{3}H]	0 01	—	1 33	—	0.30†	0 76
Phenylalanine-[^{14}C]	—	0 04	1 53	—‡	—	0.94

* Specific radioactivity in AChE (dpm × 10^{-3}/mg AChE). † Spillage due to [^{14}C] isotope in the [^{3}H] channel. ‡ The very small contribution of [^{3}H] to [^{14}C] channel is ignored. Seeds which were allowed to germinate in Petri plates were divided into 3 subsets To one subset from each group phenyl- (1 μCi/ml) was added. To the second group histidine-[^{3}H] (1 μCi/ml) was added. The third group was left as control. Seeds were germinated under this condition up to 72 hr. After 72 hr the seedlings were washed in cold sterile H_2O and transferred to fresh plates. Each of the above groups were further divided into 3 subsets. To one subset from each group phenylalanine-[^{14}C], to the second histidine-[^{3}H] and to the third sterile H_2O was added. The seedlings were allowed to grow for 48 hr more, i.e. up to 120 hr. Then the roots of the seedlings were excised and AChE was purified from roots.

Table 2. Effect of cycloheximide on the incorporation of amino acids from *Chlorella* protein hydrolysate-[^{14}C] into AChE*

Duration of treatments in hr	Specific radioactivity (dpm × 10^{-3}/mg AChE) with cycloheximide	without cycloheximide
24	0.03	0.7
30	0.04	0.6

* Seeds were germinated and after 72 hr, cycloheximide (10 μg/ml) was added. After stated time intervals chlorella protein hydrolysate-[^{14}C] was added (1 μCi/ml). After 5 hr, the roots were excised and AChE was extracted and purified. The radioactivity in the purified protein was determined.

Rate of AChE synthesis

The rate of synthesis of the enzyme after 72 hr of growth was followed with time, and the results are shown in Fig. 3. AChE activity, AChE protein and the rate of incorporation of labelled amino acids into AChE increased with time. Hence, from these studies it is evident that pea seeds contain a conserved AChE which is active during the early stages of growth and later becomes inactivated. The modulation of AChE activity is still a challenging one and it was not possible to locate the factor involved in the inactivation of AChE in pea. However, judging from the fact that external acetylcholine as well as organophosphorus pesticides inhibit lateral root formation in the plant (unpublished data), it is likely that the enzyme plays a role in the regulation of initiation of lateral roots.

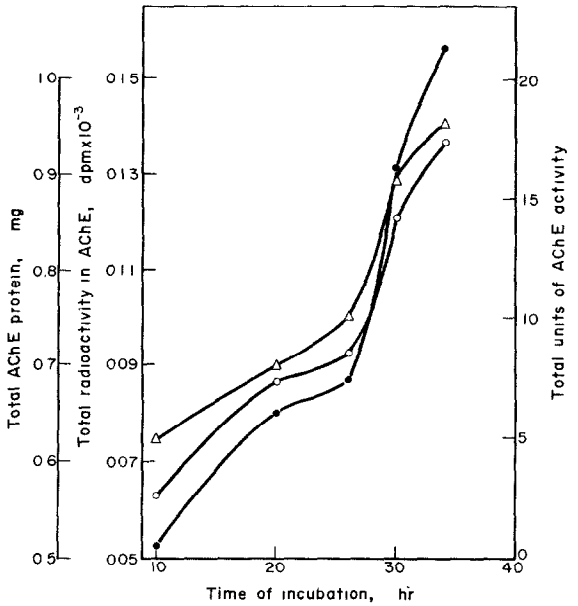

Fig. 3. Rate of AChE synthesis in pea roots. O—O total AChE protein ●—● total AChE activity △—△ total radioactivity in AChE.

EXPERIMENTAL

Pea seeds (*P. sativum* cv Bonovilla) were soaked in running H_2O for 4 hr and surface sterilized with 0.1% $HgCl_2$ for 10 min, then washed well with sterile H_2O and germinated in sterile Petri plates (10–15 seeds/plate) on sterile Whatman No. 1 filter paper. The same moisture level was maintained in all cases (4 ml sterile H_2O/plate). The seeds were germinated under 18 hr light and 6 hr dark cycle.

AChE activity in pea at different times of growth. AChE was purified from soaked seeds, sprouts as well as roots of pea seedlings, at different times of growth, as in ref. [14]. The enzyme activity was determined as in ref. [17].

Influence of trypsin and chymotrypsin treatment on inactive AChE. The purified inactive AChE was incubated with trypsin or chymotrypsin for 10, 20 and 30 min in 50 mM KPi buffer pH 8 at 37°. The ratio of AChE to trypsin or chymotrypsin used was 60:1 (3:0.05 mg). After incubation AChE activity was determined [17].

Incorporation of labelled amino acids from Chlorella *protein hydrolysate-[^{14}C] into AChE at different ages of seedlings. Chlorella* protein hydrolysate-[^{14}C] was added to seeds at different time intervals of germination. After 5 hr of incubation in the label, the seeds (or seedlings) were removed and washed thoroughly with cold sterile H_2O. Whole seedlings or roots of seedlings were taken for the isolation and purification of AChE. The radioactivity in the purified AChE was determined.

Double labelling studies. Seeds were divided into 3 groups. To one phenylalanine-[^{14}C] (1 μCi/ml) was added. To the second histidine-[^{3}H] (1 μCi/ml) was added, the third group was left as control. Seeds were allowed to grow under these conditions for up to 72 hr, after which seedlings were washed in cold sterile H_2O and transferred to fresh Petri plates containing sterile filter paper. Each of the above groups were divided into 3 subsets to which either phenylalanine-[^{14}C], histidine-[^{3}H] or sterile H_2O was added. The seedlings were then allowed to grow for 48 hr more, i.e. up to 120 hr, when they were collected for AChE purification.

Influence of cycloheximide on AChE synthesis. To 72 hr old seedlings cycloheximide (10 μg/ml) was added. After a further period of 24 or 30 hr, *Chlorella* protein hydrolysate-[^{14}C] (1 μCi/ml) was added and incubated for 5 hr. After 5 hr, the seedlings were washed with cold sterile H_2O. The roots were excised and AChE was purified. The required controls without antibiotic treatment were retained.

Rate of AChE synthesis. After 70 hr germination, seedlings were grown in the presence of *Chlorella* protein hydrolysate-[^{14}C] (0.4 μCi/ml). After 10, 22, 26 and 34 hr of incubation in the presence of label, 20 seedlings were removed and washed with cold sterile H_2O. The roots were excised and used for isolation of AChE. The total AChE protein, total AChE activity and total radioactivity of purified AChE were determined.

Purification of AChE. In all the radioactive expts, AChE was purified by the method of ref. [14], but omitting the preliminary buffer extraction step.

Enzyme activity was assayed by the method of ref. [17]. One unit of activity is the hydrolysis of 1 nmol of actylthiocholine/min at 30°. Sp. act. is in units of enzyme activity/mg protein.

Radioactivity was determined by spotting a known amount of sample on Whatman No. 3 filter paper square (2 × 2 cm). The air dried filter paper squares were transferred into scintillation fluid containing PPO and POPOP and radioactivity was measured in a liquid scintillation counter in the appropriate channels.

Protein concns were determined according to the method of ref. [18].

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