**Histochemical Localization of Acetylcholinesterase in Leguminous Plant, *Siratro (Macroptilium atropurpureum)* **

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Received February 5, 1993

Abstract: Acetylcholine (ACh) and acetylcholinesterase (AChE) have previously been demonstrated to occur in leguminous plant, *Siratro (Macroptilium atropurpureum)*. In the present work, the localization of acetylcholinesterase (AChE) within selected organs of *Siratro* is described. Histochemical techniques have been employed to show the presence of acetylcholinesterase activity in petiole, stem, root and the primary and secondary pulvini. No histochemical evidence was observed in petiole, stem and root segments. The enzyme was only present in the primary and secondary pulvini. The AChE in tissues of the primary and secondary pulvini appeared to be in the endodermal cells around the vascular system. Particularly, after the cytochemical reaction dark spots of copper sulfide strongly stained the endodermal cells that circled around the vascular system in the primary pulvinus. The results confirmed a high ACh content and a high enzyme activity was present in the primary and secondary pulvini of *Siratro*. In addition, it is demonstrated that the primary pulvinus is associated with ACh function in *Siratro* plants.

Key words: Acetylcholine-hydrolyzing enzyme, Histochemistry, Pulvinus, *Siratro*.

マメ科植物サイラトロにおけるアセチルコリン分解酵素 (AChE) の組織化学的検出: 桃木芳枝・桃木節雄*（東京農業大学生物産業学部・九州農業試験場）

要旨: マメ科植物サイラトロ (*Macroptilium atropurpureum*) におけるアセチルコリン (ACh) およびアセチルコリン分解酵素 (AChE) の存在については、すでに報告した。本報では、サイラトロの葉柄、茎、根、そして第1葉柄、第2葉柄における AChE の局在性を組織化学的法によって確認した。AChE は、葉柄、茎および根には検出されず、第1および第2葉柄のみに検出された。葉柄での AChE の反応は、維管束の中心柱を囲む内皮細胞に現れ、ときに、第1葉柄における硫化鉄の暗褐色は強く呈色し、中心柱を囲む内皮細胞全体に認められた。一方、第2葉柄では、中心柱の周囲に存在する内皮細胞の一部に呈色反応を認めた。これらの結果は、第1および第2葉柄の特異部位に ACh および AChE の局在を明かにし、さらに、第1葉柄が主として ACh の作用にかかわっていることを裏付けたものである。

キーワード: アセチルコリン分解酵素、サイラトロ、組織化学的検出、葉柄。

In a previous study, changes in acetylcholine (ACh)-hydrolyzing activity and ACh content in tissues of leguminous plant, *Siratro (Macroptilium atropurpureum)* after heat stress were reported. Heat stress increased the ACh-hydrolyzing activity about 4 and 2 fold in the primary and secondary pulvini, respectively. The content of ACh in the primary and secondary pulvini also changed dramatically after heat stress. Further, Momonoki detected acetylcholinesterase (AChE) at the interface between stele and cortex of the mesocotyl of *Zea mays* by measuring the hydrolysis of acetyltiocoline and by the liberation of labeled acetate from [1-14C]ACh. In earlier work, Fluck and Jaffe found AChE to be localized in the area between the cell wall and cell membrane in *Phaseolus aureus* Roxb using cytochemical techniques. AChE activity in the mesocotyl of maize was also detected in a

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* This work was presented at the 195th meeting of the Crop Science Society of Japan held in April, 1993.
Fig. 2. Histochemical localization of ACh-hydrolyzing enzyme (AChE) in petiole, stem and root of Siratro (*Macropitium atropurpureum*).

A, Petiole (control, ×40); B, Petiole (treatment, ×40);
C, Stem (control, ×40); D, Stem (treatment, ×40);
E, Root (control, ×50); F, Root (treatment, ×50).
Cross sections were made 25–35μm thick using a Microslicer.
crude membrane fraction\textsuperscript{14}).

The present work will detect histochemical localization of AChE in various organs of Siratro plants.

**Materials and Methods**

**Plant materials**

The seeds of Siratro (Macroptilium atropurpureum) were germinated in petri dishes with two layers of wet filter paper, transferred to soil-filled paper pots, and placed in a greenhouse. Plants with a fully expanded first trifoliate leaf were used for the experiments. The primary pulvinus was defined as the joint-like thickening at the base of the petiole, and the secondary pulvini was defined also at the joint-like thickening at the base of the leaflet (Fig. 1).

**Histochemical demonstration of AChE**

Demonstration of AChE in primary and secondary pulvini, stem, petiole and root of Siratro plant was attempted. AChE was detected by a modification of the methods of Koelle\textsuperscript{8,9}) using cross sections of the organs made with a Microslicer (Dosaka, DTK-1000) \textasciitilde 25-30 \(\mu\)m in thickness. The following reagents were employed in preparing the substrate solution for the enzyme reaction: A) 3.75 g glycine, 2.5 g CuSO\textsubscript{4} 5H\textsubscript{2}O in 100 ml distilled water, B) 0.2 M maleate buffer, pH 6.0, C) 40\% Na\textsubscript{2} SO\textsubscript{4} (w/v), D) 9.52 g MgCl\textsubscript{2} in 100 ml distilled water, E) a little of copper thiocholine, F) 23 mg acetylthiocholine (ATHCh) iodide, as a substrate of AChE, dissolved in 1.2 ml distilled water plus 0.4 ml of 0.1 M CuSO\textsubscript{4}. Then, the reagents of A (0.6 ml), distilled water (2.1 ml), B (1.5 ml), C (9.0 ml), D (0.6 ml), E (trace), and F (1.2 ml) were mixed. The mixture was filtered after 15 min. The filtrate was used for the enzymic

![Fig. 3. Histochemical localization of AChE-hydrolyzing enzyme (AChE) in the primary pulvinus of Siratro (Macroptilium atropurpureum).](image)

A : The primary pulvinus (control, \(\times 100\)) ;
B : The primary pulvinus (control, \(\times 30\)) ;
C : The primary pulvinus (treatment, \(\times 80\)).

Cross sections were made \textasciitilde 25-33\(\mu\)m thick using a Microslicer.

The dark residues show AChE-positive spots of copper sulfide after cytochemical reaction in tissues.
reaction. The ATCh employed as substrate is hydrolyzed at a more rapid rate than ACh by AChE because of the weaker linkage of the 
\[ \text{O} \quad \text{O} \]
\[ \text{C} \quad \text{S} \quad \text{C} \quad \text{O} \]
Di-isopropylfluorophosphate (DFP) was used as an inhibitor of nonspecific esterases since ACh is hydrolyzed by both specific cholinesterase (AChE) and nonspecific esterases. As a control, sections were immersed in 24% (w/v) sodium sulfate without DFP for pretreatment and incubated in a medium, without ATCh, to which was added distilled water instead of F solution.

To detect AChE, sections were immersed in 24% (w/v) sodium sulfate containing 100 nM DFP at 38°C for 30 min as a pretreatment. Then, sections were incubated in a medium containing reagents of A, B, C, D, E and F at room temperature for 30 min. After incubation, sections were washed sequentially in 20% (w/v) and 10% (w/v) aqueous sodium sulfate saturated with copper thiocelione, for 5 min and 1 min at room temperature, respectively. Sections were then washed in distilled water saturated with copper thiocelione for 1 min at room temperature. Finally, sections were immersed in ammonium sulfide saturated with copper thiocelione for 20 min at room temperature. The cytochemical reaction was as follows: 1) ATCh was hydrolyzed by the AChE in the tissue, 2) the produced thiocholine, was changed to a white precipitate of copper thiocelione by copper ions, 3) the white precipitate was converted to dark residues of copper sulfide by a reducing action of ammonium sulfide.

Results

In these histochemical studies, localization of AChE in tissues of Siratro plant was examined by determining the position of the end product, dark residues of copper sulfide of a cytochemical reaction. We have examined tissue from five regions of the plant, that is, stem, petiole, root and the primary and secondary pulvini. The histochemical evidence in stem, petiole and root is shown in Fig. 2. No dark residues of copper sulfide after the cytochemical reaction were observed in experimental preparations of stem, petiole and root segments. There were no differences between experimental preparations and control preparations in these tissues. Figures 3 and 4 show AChE-positive residues in the primary and secondary pulvini. When experimental preparations were compared to the control preparation, a difference in the intensity of dark residues of copper sulfide in both of the pulvini was apparent after a 30 min incubation period at room temperature.

The AChE activity in tissues of the primary (Fig. 3-C) and secondary pulvini (Fig. 4-B, C) appeared around the vascular system, especially in endodermal cells, as if AChE sticks to, or around, the cell wall (Fig. 4-C). An especially heavy concentration of AChE was observed in the primary pulvinus (Fig. 3-C).

Discussion

Acetylcholine is a chemical transmitter serving to propagate an electrical stimulus across the synaptic junctions of animals. Both ACh and AChE have been reported to occur in a number of higher plants. Also, ACh and AChE have previously been demonstrated to occur in Siratro plant. The objective of this research was to examine the histochemical localization of AChE activity in tissues of Siratro plant. The enzyme reaction was only found in cells around the vascular system of the primary and secondary pulvini. The dark-brown residues of copper sulfide were especially evident in endodermal cells which circled around the vascular system in the primary pulvinus. Also, there was staining in the secondary pulvinus but in a smaller area as compared to that in the primary pulvinus. In both areas, localization of AChE seems to be sticking to the cell wall or around the cell wall. Fluck and Jaffe suggested that AChE was located in the area between the cell and cell membrane which makes the AChE system a candidate for a regulator of stele to cortex transport. In previous work of Momoniki, a working hypothesis, the voltage-gating theory, has been proposed. Acetylcholine occurs at the interface between the stele and cortex in plants. The stele cells on the inside of the endodermal cell wall would act like a presynaptic cell in the animal system and release Ca²⁺ by a bioelectric change, i.e., an action potential. The released Ca²⁺ would trigger release of ACh. The released ACh
would diffuse to the junction between stele and cortex on the outside of the endodermis, and there bind to an ACh receptor. The propagated action potential would open damper-like gates in the plasmodesmatal channel. Thus, the histochemical evidence of AChE activity in the tissues suggests one of these functions are likely. Because AChE cannot move, an altered transport of ACh or altered AChE activity would probably occur.

Acknowledgments

The authors wish to thank Dr. Juanita Ladyman for helpful discussions and critical review of the manuscript and Miss M. Suzuki and Mr. N. Gushiken for skillful technical assistance.

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* Translated from Japanese by the author.