Biochemical Characterization of an Acetylcholine-hydrolyzing Enzyme from Bean Seedlings

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ABSTRACT

An acetylcholine hydrolyzing enzyme was prepared and purified (40 times) from dwarf bean hypocotyl hooks. The purity of the enzyme was proved by polyacrylamide gel electrophoresis. The molecular weight of the enzyme was determined to be 65,000 daltons. Enzyme activity was the highest at pH 8.0 and between 30 and 36°C. The enzyme had an apparent affinity constant (K_a) for acetylcholine of 460/micromolar. The affinity for substrate analogs increased from butryrylthiocholine to propionylthiocholine to acetylthiocholine. The enzyme activity was inhibited by choline, neostigmine, physostigmine, manganesate, and calcium. Magnesium had no influence on the enzyme activity. We conclude that the enzyme from dwarf beans is an acetylcholinesterase (EC 3.1.1.7).

Acetylcholine is one of the several naturally occurring esters of choline in animals and plants. Although its only well understood function is that of a neurotransmitter (25) it is found in nonnervous tissues of multicellular animals, protozoa, bacteria, and all plants so far investigated (11, 17). The physiological function of acetylcholine in plants and other nonnervous organisms remains unclear and controversial. Besides the transmitter function of acetylcholine in the nervous systems of animals there are many other reactions of acetylcholine in nervous or nonnervous tissues of many organisms which are not understood. To overcome this ignorance it is necessary to investigate the pathways of synthesis and degradation, especially in plant tissues. Rivo and Jaffe (27) isolated the enzyme cholinesterase (EC 3.1.1.8) from mung bean roots (Phaseolus aureus, Roxb.), Kasturi and Vasanatharan (21) identified it in peas (Pisum sativum L.), and Wettlaufer (29) isolated it from etiolated hypocotyl hooks of dwarf beans (Phaseolus vulgaris L.). We report data which characterize an acetylcholine-hydrolyzing enzyme from green hypocotyl hooks of dwarf beans.

MATERIALS AND METHODS

Dwarf beans (P. vulgaris, var. St. Andreas, erste Ernte, grünhülsig) were grown in a constant temperature room at 20°C ± 1°C, in plastic tanks covered with a glass plate. The substrate (moist Vermiculite) was sterilized and the light conditions were 16 h red light (Philips fluorescent tubes TL 40 W/15 with Flexiglas filter, Röhm No. 501 red, 4.8 w m⁻²) and 8 h dark. The dwarf bean seedlings were harvested after 7 days and the hook part excised above the cotyledons.

The hooks were homogenized in 10 mM K-phosphate (buffer A, pH 7.0) using 4 ml/g fresh weight, with an Ultra-Turrax homogenizer. After stirring for 30 min the homogenate was filtered through two layers of a 55-μm mesh nylon net. The residue was resuspended in 2 volumes (w/v) of 10 mM K-phosphate and 4% ammonium sulfate (buffer B, pH 7.0) and stirred for 1 h. It was then filtered again through a 55-μm mesh nylon net. The filtrate, which contained the solubilized enzyme, was centrifuged for 30 min at 40,000g. Ammonium sulfate was added to the supernatant fluid to 80% saturation and the mixture was stored overnight at 4°C. The precipitated proteins were centrifuged for 30 min at 40,000g and then dissolved in 5 ml of buffer A. This protein solution (max 70 mg/ml) was put on a column of Sepharose 6B which had previously been equilibrated with buffer A, and elution was done with buffer A. The flow rate was 18 ml/h and fractions of 3 ml were collected. To avoid overlapping with the other protein bands, we did not use all of the fractions which showed acetylcholine hydrolyzing activity (only 10 fractions, no 32-42, were used). Ammonium sulfate was added to 80% saturation to the collected fractions and they were stored overnight at 4°C. The precipitated proteins were then centrifuged for 30 min at 40,000g, the pellet redissolved in 3–5 ml buffer (depending on the protein concentration) and dialyzed for 24 h against buffer A. The purification factor obtained by this procedure was about 40 ×. The enzyme could be stored at 4°C for several weeks without changes in activity after the solution had been saturated to 40% with glycerol.

The enzyme activity of the acetylcholinesterase was determined by the method of Ellman et al. (9) with acetylthiocholine chloride as a substrate and DTNB² as a reagent. The substrate, substrate analogs, inhibitors, and effectors were dissolved in 0.5 mM K-phosphate (pH 8.0). Before the substrate was added the solution was preincubated at 34°C.

The reaction was measured by the change in light absorbance at 436 nm against a reference sample without enzyme. The protein contents of the preparations were determined by the method of Bradford (4). Polyacrylamide gel electrophoresis was used to prove the purity of the preparations according to Ornstein (26) and Davis (7). The separations were carried out in 5% gels with a current of 5 mamp/gel; about 100 μg of protein was added to each gel in Tris-glycine (pH 8.8). Tracking dye was bromophenol blue. The gels were stained for 15 min with Coomassie blue G-250 and destained with a 13% methanolic acetic acid solution. The gels were then scanned by a gel scanner at 579 nm.

The identification of the acetylcholinesterase activity in the gel was performed with the Ellman test and a single deep yellow band could be observed after the incubation of the gels in the substrate. The localization was identical with the Coomassie blue-stained band. The mol wt of the enzyme was determined by TLC on Sephadex G-200 super fine. Chymotrypsinogen, albumin (egg), albumin (bovine), aldolase, and catalase were used for calibration. Cyt C served as an indicator protein. In the case of TLC the R,

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² Abbreviations: DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid); kat: enzyme activity.
values of the calibration proteins were calculated relative to those of Cyt c. The R_{cyt} values were plotted against the logarithm of the mol wt of the protein. In the case of column chromatography the calibration proteins and the unknown protein were eluted separately with Cyt c as an indicator protein. The total elution volumes of the single proteins were determined in relation to the elution volume of Cyt c. The values were plotted as described in method 1.

Figure 1 shows a typical elution profile of one of the enzyme preparations. The other figures represent mean values from three of four determinations from the same preparations. All experiments were repeated at least four times and showed the same tendency but with variations in the absolute values dependent on the batch of beans used. Acetylthiocholine chloride, butryrylthiocholine chloride, DTNB, physostigmine sulfate, neostigmine sulfate, and choline chloride were purchased from Sigma Chemicals (München); Sepharose 6B and Sephadex G-200 were from Pharmacia Fine Chemicals (Freiburg). Calibration proteins and Cyt c were from Boehringer Mannheim.

RESULTS

Figure 1 shows the elution profile of a separation of the protein preparation using Sepharose 6B. Hydrolyzing activity was found in peak 2. Electrophoresis of this peak on a polyacrylamide gel gave a single band with a relative mobility of 0.37 (Fig. 1). In peak 1, which occurs in the void volume, as well as in peak 3, no acetylcholine hydrolyzing activity could be demonstrated. The mol wt of the enzyme was found to be 65,000 daltons using two methods (Fig. 2, A and B). Its specific catalytic activity was 2.1 µkat g^{-1}; Figure 3 shows a typical substrate hydrolysis of the prepared enzyme. K_m was determined using acetylthiocholine concentrations of 10 mM-100 µM; a Lineweaver-Burk plot showed a K_m of 460 µM. Optimum enzyme activity was found at pH 8.0 and between 30 and 36°C (Fig. 4, A and B).

Figure 5A shows the rates of hydrolysis of three esters of thiocholine by the enzyme, and the following sequence of hydrolysis velocities was found: acetylthiocholine-propionylthiocholine- butryrylthiocholine. The effect of choline on the enzyme activity was tested using concentrations of between 10 mM and 0.1 µM with a constant substrate concentration of 1 mM; complex interactions were found. Concentrations higher than 1 mM stimulated the enzyme activity whereas concentrations lower than 1 mM inhibited the enzyme activity. The highest inhibition was found with 20 µM choline (Fig. 5B). Figure 3 shows that there is no substrate inhibition by acetylcholine; we have tested concentrations up to 10 mM without any inhibitory effect. The enzyme activity was also affected by bivalent cations. To test this a Tris buffer was used instead of a phosphate buffer to avoid phosphate precipitations. Concentrations of cations were varied between 10 and 1 mM while the substrate concentration was 1 mM. Mg had no significant influence on the enzyme activity but Mn and Ca inhibited the enzyme activity (Fig. 6). The enzyme activity was further inhibited by neostigmine and physostigmine, which showed half-maximal inhibitions of 5 and 100 µM (Fig. 7).

DISCUSSION

Dwarf bean seedlings which were grown in red light were used and enzyme preparations were made from excised hypocotyl
hooks. The hypocotyl hook is a very sensitive plant tissue which shows light-induced action potentials (15, 16) and bending growth reactions (22). This region contains the highest concentration of phytochrome (13) and acetylcholine (Hartmann, unpublished) of the whole seedling. Phytochrome is one of the most important plant pigments inducing photomorphogenic reactions (3, 5). The acetylcholine content of different plant tissues (14, 19) is regulated via phytochrome. Kasturi (20) further reported that the cholinesterase synthesis of pea seedlings is regulated by phytochrome. Unfortunately, we have no information at present about the significance of the interactions between phytochrome and acetylcholine. In animals cholinesterase-hydrolyzing enzymes can be divided into two classes: cholinesterases (EC 3.1.1.8) and acetylcholinesterases (EC 3.1.1.7). In plants, the question arises of whether this is a convenient method of classification; there is the problem of differentiating between the two types of enzymes. Fluck and Jaffe (12) characterized acetylcholine-hydrolyzing enzymes from mung beans (P. aureus Rox.) eggplant (Solanum melongena L.), and corn (Zea mays L.) with significant differences for each enzyme from the three plant species. They suggest that the enzyme prepared from corn seedlings is not a cholinesterase. Kasturi and Vasantharajan (21) prepared an enzyme from peas which was characterized as a cholinesterase. The main criterion used to classify the acetylcholine-hydrolyzing enzymes is the relative velocity of hydrolysis of different choline esters. Cholinesterases (EC 3.1.1.8) have the highest hydrolysis rate for butyrylcholine and much lower rates for acetylcholine or other choline esters. The enzyme which was prepared in this study hydrolyzed butrylthiocholine and propionylthiocholine with 12 and 80%, respectively, of the velocity determined for acetylthiocholine. Therefore we must conclude that it is better to classify the described enzyme as an acetylcholinesterase (EC 3.1.1.7) rather than a cholinesterase (EC 3.1.1.8).

There is still the question if this plant enzyme is really specific for cholinesters. We have tested various substrates commonly used for plant hydrolyases and in no cases could hydrolyzing activity be found. The data of Fluck and Jaffe (10) further showed that this enzyme could not be a pectinase.

Although plant and animal cholinesterases have some important characteristics in common, there are a number of contradictory results which show that they differ in some respects. Hollunger and Niklasson (18) reported a mol wt of 80,000 daltons for an
acetylcholinesterase from mammalian brain. They confirmed that an aggregation of these enzyme molecules took place during storage to produce units with a mol wt of 1 million daltons. We observed similar effects, but the aggregation could be overcome by using glycerol as a preservative. However, we always used newly prepared enzyme for mol wt determinations, which showed a mol wt of 65,000 daltons. Riow and Jaffe (27) reported a mol wt of 80,000 daltons as a “smaller form”, and 160,000 daltons and greater than 200,000 daltons as “bigger forms” of the enzyme prepared from mung beans. The enzyme from peas also had a mol wt higher than 200,000 daltons; separation of this enzyme with SDS gels showed two bands. Thus, cholinesterases from plant and from animal tissues show the same tendency to aggregate.

The temperature optimum with a plateau between 30 and 36 C and the pH optimum of about pH 8.0 coincide with values reported for acetylcholinesterases prepared from animal tissues (1, 6, 18). The Km found (460 μM) is comparable.

An important difference between plant and animal cholinesterases was found regarding substrate inhibition. Our enzyme showed no substrate inhibition at high concentrations of substrate, which is typical of acetylcholinesterases but not of cholinesterases (2). This also conflicts with data from other plant enzyme preparations (27). High concentrations of choline stimulated the enzyme activity. A similar effect was reported for mung beans, but using lower concentrations of choline (27). In our preparations the hydrolyzing activity was inhibited by low choline concentrations, and at 20 μM choline maximum inhibition was found. As found with animal enzymes (8, 23) physostigmine and neostigmine were inhibitory, but in contrast to results obtained in animals neostigmine was more potent than physostigmine.

One of the main differences between plant and animal cholinesterases could be demonstrated for bivalent cations. Many authors (24, 28) reported a strong activation of enzymes prepared from different tissues by Ca, Mg, and Mn ions, the strength of activation was Ca2+ > Mg2+ > Mn2+. Our enzyme preparation showed no changes in activity after treatment with Mg2+, Mn2+, and Ca2+ strongly inhibited the enzyme activity. We need comparable data for other plant acetylcholinesterases to see if this is true in general. Our next step must be to make a more detailed study of the chemical organization of the enzyme to elucidate the differences between plant and animal enzymes.

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LITERATURE CITED

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