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Substance P Hydrolysis by Human Serum Cholinesterase

Oksana Lockridge

Pharmacology Department, University of Michigan Medical School, Ann Arbor, Michigan, U.S.A.

Abstract: Highly purified human serum cholinesterase (EC 3.1.1.8, also known as pseudocholinesterase and butyrylcholinesterase) had peptidase activity toward substance P. Digestion of substance P was monitored by high performance liquid chromatography, which separated three product peptides. The cleavages occurred sequentially. The first peptide to appear was Arg^1-Pro^2. The K_m for this hydrolysis was 0.3 m*M*; maximum activity was 7.9 nmol min^{-1} mg^{-1} of protein, which corresponded to a turnover number of 0.6 min^{-1}. A second cleavage yielded Lys^3-Pro^4. A third cleavage occurred at the C-terminal, where the amide was removed from Met^{11} to yield a peptide containing residues 5–11. Both the peptidase and esterase activities of the enzyme were completely inhibited by the anticholinesterase agent, diisopropylfluorophosphate. Substance P inhibited the hydrolysis of benzoylcholine (a good ester substrate) with a K_I of 0.17 m*M*, indicating that substance P interacted with cholinesterase rather than with a trace contaminant. Peptidase and amidase activities for serum cholinesterase are novel activities for this enzyme. It was demonstrated previously that the related enzyme acetylcholinesterase (EC 3.1.1.7) catalyzed the hydrolysis of substance P, but at entirely different cleavage sites from those reported in the present work. Since butyrylcholinesterase is present in brain and muscle, as well as in serum, it may be involved in the physiological regulation of substance P. **Key Words:** Substance P—Cholinesterase. **Lockridge O.** Substance P hydrolysis by human serum cholinesterase. *J. Neurochem.* **39,** 106–110 (1982).

The demonstration by Chubb et al. (1980) that acetylcholinesterase (EC 3.1.1.7) catalyzed the hydrolysis of substance P provided the first evidence that this enzyme is capable of hydrolyzing a peptide bond. Until then, acetylcholinesterase was known primarily as an esterase, though it also had amidase activity (Moore and Hess, 1975). Similarly, the cholinesterase isolated from human serum (EC 3.1.1.8–acylcholine acylhydrolase, also known as pseudocholinesterase and butyrylcholinesterase) has until now been known to hydrolyze ester bonds exclusively. We have found that serum cholinesterase has peptidase and amidase activities toward substance P. Acetylcholinesterase and butyrylcholinesterase are both present in brain (Ord and Thompson, 1952), where they may be involved in substance P regulation. The present work shows that the sites cleaved by serum cholinesterase are completely different from those cleaved by acetylcholinesterase (Chubb et al., 1980).

MATERIALS AND METHODS

Serum cholinesterase was purified from outdated human plasma by using diethylaminoethyl cellulose ion-exchange chromatography followed by affinity chromatography on procainamide-Sepharose (Lockridge and La Du, 1978; Lockridge et al., 1979). The cholinesterase used in this study had the "usual" genotype characteristics (Kalow and Genest, 1957). The enzyme preparation was considered to be pure on the basis of the following criteria. (1) It was homogeneous on polyacrylamide gel electrophoresis (Lockridge et al., 1979). (2) Sodium dodecyl sulfate gel electrophoresis revealed dimers and monomers, but no contaminants (Lockridge et al., 1979).

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Address correspondence and reprint requests to: Oksana Lockridge, Pharmacology Department, Medical Science I, Room M7444, University of Michigan Medical School, Ann Arbor, MI 48109, U.S.A.

(3) The reaction of radiolabeled diisopropylfluorophosphate with cholinesterase yielded a binding stoichiometry of 4.0 mol bound per mole of enzyme (Lockridge, unpublished results). This confirmed the stoichiometry found with a fluorescent probe ester (Lockridge and La Du, 1978), which led to the conclusion of one active site per subunit in the tetrameric molecule of molecular weight 340,000. Had the preparation contained significant amounts of noncholinesterase protein, the stoichiometry would have been less than 4.0. (4) A single band was seen on isoelectric focusing gels (Lockridge, unpublished results). (5) The preparation had high specific activities of 198 μmol min^{-1} mg^{-1} of protein when tested with the substrate benzoylcholine (50 μM benzoylcholine in 0.067 M sodium potassium phosphate, pH 7.4, 25°) and 704 μmol min^{-1} mg^{-1} of protein when tested with the substrate butyrylthiocholine (1.0 mM butyrylthiocholine in 0.1 M sodium phosphate, pH 8.0, 25°). These specific activities compared favorably with the highest values reported in the literature, which were 174 μmol benzoylcholine hydrolyzed min^{-1} mg^{-1} (Das and Liddell, 1970) and 600 μmol butyrylcholine hydrolyzed min^{-1} mg^{-1} (Masson et al., 1980).

The above criteria of purity would reveal contaminants present in concentrations greater than 1%. Trace contaminants would not be detected, but could still be present in the "pure" preparation. The possible presence of trace contaminants having protease activity was examined by measuring hydrolysis of casein with the Bio-Rad protease detection kit. The cholinesterase preparation lacked the ability to hydrolyze casein and was therefore considered to be free of plasmin, plasminogen, and other proteases. It was also found to be free of paraoxonase/arylesterase activity (Krisch, 1968).

The stock solution of pure cholinesterase had a concentration of 209 units/ml (1 unit hydrolyzes 1 μmol of benzoylcholine per min, at 25° and pH 7.4), which was 209 times the concentration found in average human sera. The cholinesterase preparation was filtered through a 0.45-μm Millipore filter and stored in 0.02 M potassium phosphate (pH 7.0), containing 1 mM EDTA, at 4°.

Substance P was obtained from Peninsula Laboratories, San Carlos, CA. A 1-mM stock solution was prepared by dissolving the peptide in water.

Separation of peptides by HPLC

The chromatograph was a Varian Instruments model 5060, equipped with a variable wavelength UV-visible detector and a Fluorichrom. The reverse-phase, 30 cm × 4 mm column, MCH-10, which is C_{18} bonded to 10-μm silica, was obtained from Varian Instruments. Double-distilled water was prepared in the laboratory. Sequanal grade trifluoroacetic acid was purchased from Pierce Co., Rockford, IL; HPLC grade acetonitrile from Burdick and Jackson Laboratories Inc., Muskegon, MI.

Amino acid analysis

The isolated peptide peaks were evaporated to dryness in a rotatory evaporator. Constant-boiling 6 M HCl (Pierce Co.) was added, and the sealed, evacuated tubes were heated at 110° for 24 h. Amino acid analysis using the standard ninhydrin method of detection was performed by AAA Laboratory, Mercer Island, WA. Amino acid composition was also determined in our laboratory by reacting amino acids with o-phthaldialdehyde to form fluorescent derivatives, which were separated by HPLC (Lindroth and Mopper, 1979). Results from the two methods agreed.

Kinetic parameters

K_m values for hydrolysis of substance P were determined by incubating variable concentrations of substance P (0.031–0.50 mM) with cholinesterase for 1 h at 37°. The final cholinesterase concentration in these reactions was 105 units/ml, which is 0.5 mg protein/ml. The buffer was 10 mM potassium phosphate plus 0.5 mM EDTA, pH 7.0; the total volume was 80 μl. After 1 h of reaction, the sample was injected into the 50-μl loop of the Valco injector on the chromatograph. Peptides were separated by eluting the reverse-phase column with a linear gradient, as in Fig. 1. Absorbance at 220 nm was recorded. The

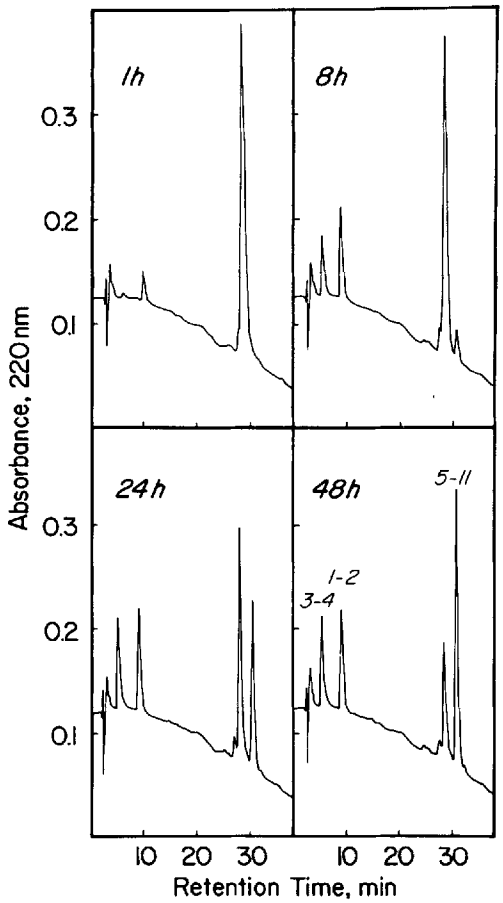


FIG. 1. Digestion of substance P by cholinesterase and separation of peptides by HPLC. Substance P, 0.5 mM, was incubated with 0.5 mg/ml of pure cholinesterase in a total volume of 0.4 ml, at 37°; the buffer was 10 mM potassium phosphate, pH 7.0, containing 0.5 mM EDTA. After 1, 8, 24, and 48 h of incubation, 50-μl aliquots were injected into the chromatograph. Peptides were eluted with a linear gradient between solvent A (0.1% trifluoroacetic acid) and solvent B (acetonitrile). Acetonitrile increased from 0 to 60% during the 40-min gradient; the flow rate was 1.0 ml/min, temperature ambient. Absorbance at 220 nm was recorded. The peaks between 2 and 4 min are the injection peaks; substance P was eluted at 28 min. The peak at 5.5 min contained Lys^3-Pro^4, the peak at 9 min Arg^1-Pro^2, and the peak at 30.5 min residues 5–11. Cholinesterase was not eluted from this column.

amount of each peptide was estimated from peak height. Lineweaver-Burk plots yielded values for K_m and V_{max}. Turnover number, defined as μmol substrate hydrolyzed per min per μmol active site, under conditions in which substrate concentration is saturating, was calculated from V_{max}.

K_I for substance P was measured by its ability to inhibit hydrolysis of benzoylcholine. Six different concentrations of substance P (16.6–166.7 μ*M*) were tested in assays containing 20, 35, and 50 μ*M* benzoylcholine. The cholinesterase concentration was fixed at 0.007 units/ml, which is 0.035 μg protein/ml. The buffer was 50 m*M* sodium potassium phosphate, pH 7.4, at 25°. A Dixon plot yielded a noncompetitive K_I with a value of 0.17 m*M*.

RESULTS

Cholinesterase hydrolysis of substance P

The HPLC traces in Fig. 1 show that incubation of substance P with pure cholinesterase converted the substance P peak, with its retention time of 28 min, into four new peaks eluted at 5.5, 9, 27.5, and 30.5 min. Control incubations of substance P without cholinesterase did not yield product peaks. To assure that microbial contaminants were not contributing to hydrolysis of substance P, the reaction mixtures were kept sterile by filtering through 0.45-μm Millipore membranes in a "bas" (Bioanalytical Systems, West Lafayette, IN) filter apparatus. Further evidence supporting cholinesterase as the catalytic agent included the observations that (1) the rate of hydrolysis depended on the quantity of cholinesterase added, (2) substance P inhibited the reaction of cholinesterase with its ester substrate, benzoylcholine, and (3) the anticholinesterase agent, diisopropylfluorophosphate, completely inhibited substance P hydrolysis. In these experiments, diisopropylfluorophosphate was incubated with cholinesterase for 10–30 min before adding substance P. The concentration of diisopropylfluorophosphate was 100 times the concentration of cholinesterase-active sites. After treatment with diisopropylfluorophosphate, both the esterase and peptidase activities of cholinesterase were zero.

Amino acid analysis

A reaction similar to that in Fig. 1 was allowed to proceed nearly to completion. Three product peaks were collected, dried, and analyzed for amino acid composition. The small shoulder at 27.5 min was not analyzed. The results are shown in Table 1. The peak eluted at 5.5 min contained Lys and Pro, the peak eluted at 9 min contained Arg and Pro, and the peak eluted at 30.5 min contained two Glu, Gly, Leu, Met, and two Phe.

A control reaction of substance P and buffer, without cholinesterase, was incubated and eluted from the HPLC column under the same conditions as the sample. This control was eluted at 28 min. Its amino acid composition was Arg, two Glu, Gly, Leu, Lys, Met, two Phe, and two Pro, which is the composition of authentic substance P.

Order of cleavage

The peptide containing Arg^1-Pro^2 was cleaved off first (see Scheme 1). This is evident in Fig. 1, where a 1-h incubation yielded a significant amount of Arg^1-Pro^2 (9-min peak), but only a minor amount of Lys^3-Pro^4 (5.5-min peak). The small shoulder at 27.5 min may be peptide 3-11. The peptide containing Lys^3-Pro^4 appeared second. Coincident with the appearance of peptide 3-4 one might have expected peptide 5-11. However, as shown in Fig. 2, this was not the case. Peptide 5-11 appeared later than peptide 3-4, and it continued to increase at a time when the amount of peptide 3-4 had leveled off. This indicated that the peak eluted at 30.5 min might represent a third cleavage, that of amide from the C-terminal. Such a cleavage would yield a peptide with the observed amino acid composition. To confirm this possibility, peptide 5-11 was spotted on a

TABLE 1. *Amino acid analysis of peptides isolated by HPLC*

	μmol detected			
Amino acid	5.5-min peak	9-min peak	30.5-min peak	Control peak
Arginine		0.235		0.235
Glutamic acid			0.432	0.565
Glycine			0.200	0.264
Leucine			0.204	0.273
Lysine	0.198	0.035		0.277
Methionine			0.183	0.266
Phenylalanine			0.456	0.611
Proline	0.214	0.295		0.540

Substance P, 0.48 m*M*, was incubated with 0.05 mg/ml of pure cholinesterase at 37° in 32 m*M* phosphate buffer, pH 7.4, under sterile conditions. After 143 h, when the reaction was nearly complete, the entire 1 ml of incubation mixture was injected into the chromatograph, where peptides were separated as in Fig. 1. Individual peaks were collected and analyzed for amino acid composition. The results above are for the ninhydrin detection method. The control was a separate incubation of substance P with buffer, but no cholinesterase, lasting for 143 h at 37°. The control was eluted as a single peak, at 28 min.

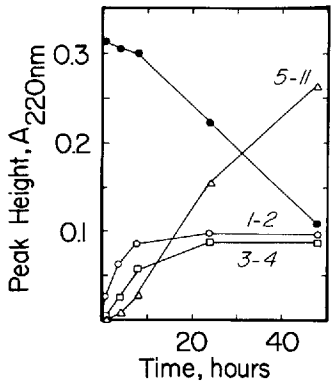


FIG. 2. Time course of hydrolysis of substance P by cholinesterase. Substance P was incubated with cholinesterase as in Fig. 1. Peak heights of the peptides separated by HPLC are plotted as a function of incubation time. ●–●–●, 28-min peak, contains substance P and amidated peptide 5-11; ○–○–○, 9-min peak, contains peptide 1-2; □–□–□, 5.5-min peak, contains peptide 3-4; △–△–△, 30.5-min peak, contains deamidated peptide 5-11.

thin-layer cellulose plate and subjected to electrophoresis in pH 6.5 buffer (pyridine/glacial acetic acid/water, 25:1:225), in which the peptide appeared to have a net neutral charge. A neutral charge is consistent with the explanation that peptide 5-11 had a free carboxyl at the COOH terminal. After electrophoresis, the plate was sprayed with ninhydrin and it was found that peptide 5-11 stained with ninhydrin, indicating that the NH_2-terminal glutamine had not cyclized to pyro-G1N, which could also have resulted in a neutral peptide if the amide were not hydrolyzed. On the basis of the above, it follows that amidated peptide 5-11 was co-eluted with substance P at 28 min (Fig. 1).

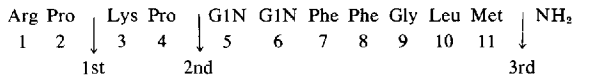
K_m and K_I

The K_m value for the first cleavage of substance P was estimated to be 0.3 m*M*. The maximum rate of hydrolysis, V_{max}, was calculated to be 7.9 nmol min^{-1} mg^{-1} of protein, which corresponded to a turnover number of 0.6 min^{-1} (see Materials and Methods for experimental details).

Because substance P was hydrolyzed at a much slower rate than the ester, benzoylcholine, substance P could be used as an inhibitor. It was found that substance P inhibited the hydrolysis of benzoylcholine by cholinesterase. The noncompetitive K_I value was 0.17 m*M*. The observation of noncompetitive inhibition in place of the expected competitive inhibition can be explained by the large difference in turnover number between substance P (0.6 min^{-1}) and benzoylcholine (15,000 min^{-1}). The slow turnover number for substance P means that the complex between substance P and cholinesterase breaks down very slowly. This reduces the total amount of enzyme available for benzoylcholine binding and makes substance P appear to be an irreversible inhibitor (Segel, 1975).

DISCUSSION

Pure human serum cholinesterase cleaved substance P at three sites:



The first cleavage was between Pro^2 and Lys^3, followed by cleavage between Pro^4 and $G1N^5$, and then by deamidation at the C-terminal.

In the reaction studied by Chubb et al. (1980), acetylcholinesterase cleaved substance P primarily between Leu^{10} and Met^{11}, and also between Phe^7 and Phe^8, Phe^8 and Gly^9, Arg^1 and Pro^2, and Gly^9 and Leu^{10}. Thus, cholinesterase and acetylcholinesterase have completely different specificities toward substance P.

The fact that serum cholinesterase cleaved peptide bonds adjacent to proline called to mind the post-proline cleaving enzyme isolated from bovine brain (Blumberg et al., 1980) and prolyl endopeptidase isolated from rat brain (Kato et al., 1980); however, both of these enzymes cleaved substance P at only one bond, Pro^4-$G1N^5$. Cholinesterase, post-proline cleaving enzyme, and prolyl endopeptidase are all serine enzymes inhibited by diisopropylfluorophosphate; but their subunit molecular weights on sodium dodecyl sulfate gels are quite different, being 90,000 for human serum cholinesterase (Lockridge et al., 1979) and 57,000 for post-proline cleaving enzyme from lamb kidney (Yoshimoto et al., 1978), while prolyl endopeptidase had a molecular weight of 70,000 on Sephadex G-100 (Kato et al., 1980). This suggests that they are not identical enzymes.

Another enzyme reported to hydrolyze substance P was tonin isolated from rat submaxillary gland (Chrétien et al., 1980). Tonin cleaved the Phe^7-Phe^8 and Phe^8-Gly^9 bonds. The maximum rates of hydrolysis were similar for tonin (450 nmol h^{-1} mg^{-1}), acetylcholinesterase (450 nmol h^{-1} mg^{-1}), and cholinesterase (474 nmol h^{-1} mg^{-1}). Thus, the low rate for cholinesterase is similar to that observed with other enzymes, and suggests that cholinesterase may be involved in the physiological regulation of substance P.

Berger et al. (1979) reported that human plasma contained an enzyme catalyzing the hydrolysis of substance P, with a K_m value of 8 μM. They did not identify their enzyme or suggest that it might be cholinesterase. Their reported K_m is appreciably

lower than the value we found with purified cholinesterase, but the difference might have been influenced by the presence of metal ions or other plasma constituents affecting the enzyme at the much lower concentration of substrate employed by Berger et al.

The concentration of substance P in human plasma is reported to be 180 pmol/L on the average (Nilsson et al., 1975). This extremely low concentration precludes any appreciable binding of substance P by cholinesterase in plasma at this concentration. However, the plasma concentration of substance P may be higher at times; or the concentrations of enzyme and substrate may reach more favorable levels for hydrolysis in localized areas in the blood or tissues.

The possibility that the observed cleavages of substance P were caused by a trace contaminant in the cholinesterase preparation rather than by cholinesterase itself cannot be completely ruled out. Although the cholinesterase was pure by several criteria, contaminants constituting less than 1% of the total protein would have escaped detection. The observation that substance P inhibited the hydrolysis of the cholinesterase substrate, benzoylcholine, strongly supports the argument that cholinesterase catalyzed the hydrolysis of substance P, rather than a trace contaminant.

The hydrolysis of substance P by cholinesterase was very slow in comparison with the rate of hydrolysis of many ester substrates. For example, substance P was hydrolyzed with a turnover number of 0.6 min^{-1}, whereas benzoylcholine was hydrolyzed with a turnover number of 15,000 min^{-1} and heroin with a turnover number of 500 min^{-1} (Lockridge et al., 1980). This was not unexpected as, in general, proteases hydrolyze the peptide bond much more slowly than the ester bond. Our finding that cholinesterase has peptidase and amidase activities toward substance P suggests that there may be other peptide hormones, as well as amide-containing drugs, that can serve as substrates for cholinesterase.

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