ACTA CHEMICA SCANDINAVICA 22 (1968) 2183–2190

# Properties of a Cholinesterase from Body Muscles of Plaice (*Pleuronectes platessa*)

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The reactions of a purified cholinesterase from the body muscles of plaice with some substrates and inhibitors were investigated. The enzyme showed some properties typical for an acetylcholinesterase (E.C. 3.1.1.7) and some typical for a cholinesterase (E.C. 3.1.1.8). Thus, it splits acetylcholine faster than propionyl-, butyryl-, and valerylcholine. It does not display a substrate inhibition with acetylcholine or propionylcholine but slight ones with butyryl- and valerylcholine. It is more strongly inhibited by organophosphorus compounds than either acetylcholinesterase or butyrylcholinesterase.

A butyrylcholine splitting cholinesterase * was found in body muscle of plaice (*Pleuronectes platessa*)[2] and a method of purifying this enzyme has been described.[3]

In the present investigation, the reactions of the enzyme with some substrates and inhibitors have been studied in order to compare its properties with those of acetylcholinesterase or butyrylcholinesterase. An acetylcholinesterase splits acetylcholine faster than propionylcholine and butyrylcholine, the latter being split very slowly or not at all. A butyrylcholinesterase splits butyrylcholine faster than propionylcholine and acetylcholine. The two enzymes also differ in other respects. Acetylcholinesterase displays a substrate inhibition with the choline esters split. This is not the case with a butyrylcholinesterase. The one type of cholinesterase is inhibited by certain substances at concentrations, which are lower than those required to inhibit the other type. Such inhibitors are said to be "specific" or selective for the enzyme inhibited at the lowest concentration. These criteria and their historical development are discussed in several textbooks and in recent reviews, *e.g.*, Refs. 4—8.

* In accordance with the recommendations of IUPAC,[1] acetylcholine hydrolase (E.C. 3.1.1.7) is called acetylcholinesterase. Acylcholine acylhydrolase (E.C. 3.1.1.8) is not called cholinesterase, as recommended, but butyrylcholinesterase in order to facilitate the presentation. The cholinesterase investigated in the present work is called plaice cholinesterase.

*Table 1.* Enzyme preparations used. Specific activities for plaice cholinesterase in μmoles $mg^{-1}min^{-1}$ with butyrylcholine iodide, $1.6 \times 10^{-3}$ M, electrometric determination, pH 8.2, 25°C.

| Enzyme preparation | Specific activity | Used for |
|---|---|---|
| I | 10.3 | $K_m$-determinations |
| II | 47 | $K_m$- and $k_2$-determinations and pH-dependency |
| III | 13.0 | $pI_{50}$- and pS-activity curves |

## MATERIALS AND METHODS

*Enzymes.* Pooled cholinesterase fractions prepared as described earlier [3] were used (see Table 1). Small portions hereof (0.5 ml) were kept deep-frozen in 0.1 M ammonium acetate, pH 8.2. It could be thawed and frozen several times without any effects on the activity. Amounts from 10 to 250 μl were used in the experiments.

Erythrocytes and serum from human blood were used as typical sources of acetylcholinesterase and butyrylcholinesterase, respectively. These enzymes were prepared from heparinized blood by centrifugation. The red blood cells were washed twice with saline and diluted to blood volume with distilled water. 0.2 ml of this solution or of plasma was used for each determination.

*Substrates and inhibitors.* Acetylcholine, propionylcholine, butyrylcholine, and valerylcholine, all as iodides, were prepared according to Tammelin,[9] acetyl-β-methylcholine according to Tammelin *et al.*[10] Benzoylcholine chloride was purchased from Fluka (Fluka AG Chem. Fabrik, Switzerland), triacetin from Kebo AB, Sweden. Di-isopropylphosphoryl fluoride (DFP),[11] methyl-isopropoxy-phosphoryl fluoride (Sarin),[12] methyl-isopropoxy-phosphoryl thiocholine (37-$SN^+$)[13] as iodide, and 2.5-bis(*p*-*N*-allyl-*N*-di-methylammonium)phenyl pentan-3-one dibromide (BW 284C51) [14] were synthetized according to the references given. Eserine salicylate was obtained from Sandoz S.A., Switzerland.

*Determination of cholinesterase activity.* Cholinesterase activity was determined at 25°C either electrometrically [15] at pH 8.2, ionic strength 0.125, or in an automatic recording titrator [16,17] in nitrogen atmosphere measuring the amount of NaOH (0.01 or 0.1 M) used per time unit at a pH of 8.0 and an ionic strength of 0.1 by adding potassium chloride. The $pI_{50}$ (— negative logarithm of the inhibitor concentration giving 50 % inhibition) of the various inhibitors was determined electrometrically, generally after 30 min of incubation (2 h with 37-$SN^+$ in order to compare with earlier results in the literature [18]) in the absence of substrate and the concentrations were calculated on the reaction mixtures (6 ml) before adding the substrate (0.6 ml). During the titrimetric experiments to determine the enzymic activities and pH-activity dependence, the volume of the reaction mixture was 20 ml and contained the substrate, enzyme, and inhibitors, these components being added in the order mentioned. The ionic strength was usually 0.1 to allow comparisons between the two methods and with values in the literature (see tables and legends). The inflexion point of the pH-activity curve, here taken to represent the p*K* of an ionizing group of the cholinesterase, the ionization of the substrate being negligible, was determined by graphical derivation of the curve. $K_m$-values were obtained by Lineweaver-Burk plots [19] of the initial activities obtained at the lowest substrate concentrations. The velocity constant, $k_2$ ($l\ mole^{-1}min^{-1}$) for the reaction between enzyme and inhibitors was calculated by plotting the logarithm of the activity against time. For all activities two or four measurements were made and the values given are the means of these results after correcting for the separately determined spontaneous hydrolysis.

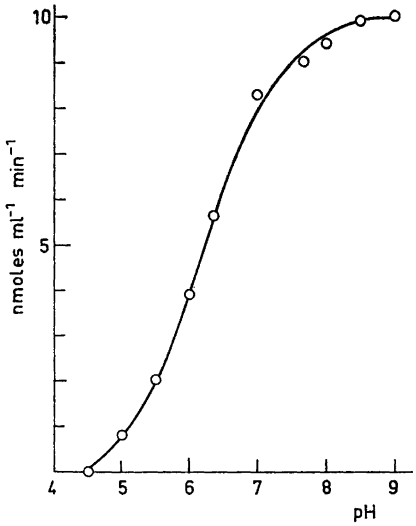


*Fig. 1.* pH-activity relationship of plaice cholinesterase. Titrimetric determination, 0.1 M KCl, acetylcholine $1.5 \times 10^{-2}$ M. Preparation II.

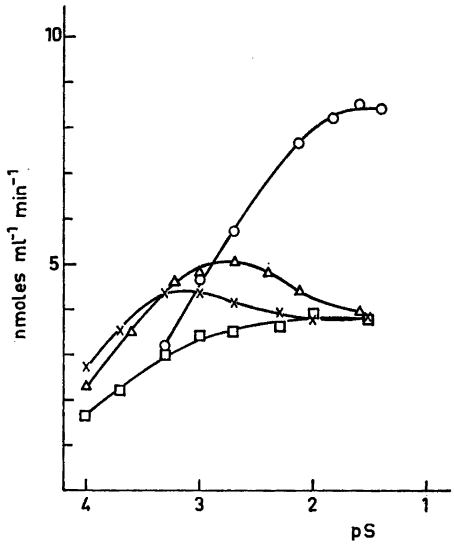


*Fig. 2.* pS-activity relationships of plaice cholinesterase, preparation I. Titrimetric determinations, 0.25 M KCl, pH 8.0. O = acetylcholine, □ = propionylcholine, △ = butyrylcholine, × = valerylcholine.

## RESULTS

*The pH-activity dependence* of the plaice cholinesterase was determined titrimetrically using acetylcholine as a substrate. The result is given in Fig. 1. From this curve a p$K$=6.3 could be calculated (see Methods).

*The dependence of the enzymic activity on the substrate concentration* was investigated with four choline esters and is illustrated in Fig. 2. Butyryl- and valerylcholine showed a slight substrate inhibition. The $K_m$-values are given in Table 2. It is observed that $K_m$ decreased with increasing length of the acyl-group.

Any enzymic hydrolysis of acetyl-$\beta$-methylcholine, which is known to be split only by acetylcholinesterase, was not detected at the enzyme concentrations used for the measurements presented in Table 2. However, by increasing the enzyme concentration 5-fold, the hydrolysis could be measured electrometrically. The pS-activity curve is given in Fig. 3 and displays no substrate inhibition. Benzoylcholine is split by butyrylcholinesterase but not by acetylcholinesterase. As is evident from the electrometric determinations with increased concentrations of plaice cholinesterase (Fig. 3), the enzyme splits benzoylcholine slowly displaying a continuous substrate inhibition in the range measured.

Some *inhibitory effects* were studied. p$I_{50}$-values and observed reaction rates ($k_2$-values), the latter in the presence of the substrate acetylcholine (pS=1.8), are given for some well-known cholinesterase inhibitors in Table 3

*Table 2.* Michaelis-Menten constants, $V$-values and optimal substrate concentrations. The former data were obtained from Lineweaver-Burk plots of titrimetric data for some cholinesterase substrates measured at 25°C, pH = 8.0, ionic strength = 0.25, preparation I.

| Substrate | $K_m$ mM | $V$ nmole ml$^{-1}$min$^{-1}$ | Optimal pS |
|---|---|---|---|
| Acetylcholine iodide | 0.92 [a] | 9.2 | 1.5 |
| | 0.87 [ab] | 9.5 | 1.6 |
| Propionylcholine iodide | 0.15 | 3.8 | 2.0 |
| Butyrylcholine iodide | 0.14 | 5.4 | 2.8 |
| Valerylcholine iodide | 0.10 | 3.6 | 3.2 |
| Triacetin | 3.3 | 5.3 | 1.8 |

[a] For two separate preparations, I and II, respectively.
[b] At an ionic strength = 0.1 preparation II resulted in a $K_m = 0.40$ mM, $V = 6.9$ nmole ml$^{-1}$min$^{-1}$, optimal pS = 1.8.

together with some values reported in the literature. It is observed that the plaice cholinesterase is inhibited at lower concentrations and with higher reaction rates by the investigated inhibitors than are the other cholinesterases. Acetylcholinesterase inhibition by BW 284C51 was an exception. The eserine sensitivity of the plaice cholinesterase is evident from the values in Table 3. Complete inhibition was reached at a pI = 6.0.

*Table 3.* $pI_{50}$- and $k_2$-values for some inhibitors of plaice cholinesterase. $k_2$-values were determined in the presence of the substrate, acetylcholine iodide ($1.5 \times 10^{-2}$ M). For comparison corresponding figures are given for some of the substances in relation to acetylcholinesterase and butyrylcholinesterase.

| Inhibitor | Plaice cholinesterase | | Acetylcholinesterase from human erythrocytes | | Butyrylcholinesterase from human plasma | |
|---|---|---|---|---|---|---|
| | $pI_{50}$ [a] | $k_2 \times 10^{-4}$ [b] l mole$^{-1}$min$^{-1}$ | $pI_{50}$ | $k_2 \times 10^{-4}$ l mole$^{-1}$min$^{-1}$ | $pI_{50}$ | $k_2 \times 10^{-4}$ l mole$^{-1}$min$^{-1}$ |
| Eserine[c] | 8.0 | | 7.0[a] | | 6.9[a] | |
| Sarin[c] | 9.5 | 16.8 | 8.8 [18] | 0.6 [18] | 8.4 [18] | 0.7 [18] |
| 37-SN+[d] | 10.4 | 8.0 | 8.4 [13] | | 7.1 [13] | |
| DFP[c] | 9.4 | 7.4 | 6.6[a] | | 8.7[a] | |
| BW 284C51[c] | 6.1 | | 7.3[a] | | 4.2[a] | |

[a] Determined electrometrically, pH = 8.2, ionic strength 0.125, 25°C, preparation III.
[b] Determined titrimetrically, pH = 8.0, ionic strength 0.1, 25°C, preparation I.
[c] Incubation time: 30 min before adding substrate.
[d] Incubation time: 2 h before adding substrate.

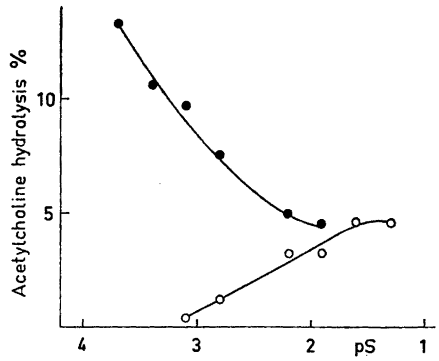


*Fig. 3.* pS-activity relationships of plaice cholinesterase with acetyl-β-methylcholine (○) and benzoylcholine (●), preparation III. Activity is expressed in per cent of the acetylcholine hydrolysis at the optimal acetylcholine concentration $1.5 \times 10^{-2}$ M.

pI-activity curves are given in Fig. 4 for the action of the specific inhibitor of butyrylcholinesterase, DFP, and in Fig. 5 for the specific inhibitor of acetylcholinesterase, BW 284C51, on plaice cholinesterase, acetylcholinesterase, and butyrylcholinesterase from human blood. The curves demonstrate in greater detail the higher sensitivity of the plaice cholinesterase to DFP than that of both acetylcholinesterase and butyrylcholinesterase, but show that this sensitivity is less than that of acetylcholinesterase to BW 284C51.

## DISCUSSION

*pH-activity dependence.* The pH-activity dependence with acetylcholine showed the plaice cholinesterase to be less sensitive to lower pH's than butyrylcholinesterase. From the pH-activity curve (Fig. 1) a p$K$=6.3 for an ionizing group necessary for the enzymic activity [7] was obtained. The corresponding value was 6.9 for purified butyrylcholinesterase from human serum as calculated from the curve published by Heilbronn.[20] Bergmann *et al.*[21] have reported the pH-activity dependence below pH=8 for acetylcholinesterase from the electric organ of electric eel and from their data a p$K$ of 6.2 can be calculated. Thus the pH-activity dependence of the plaice cholinesterase seems to be more similar to that of acetylcholinesterase than to that of butyrylcholinesterase.

*Substrate relationships.* The fact that acetylcholine was split faster by the plaice cholinesterase than butyrylcholine, would by definition imply that this enzyme is an acetylcholinesterase.[6] However, acetylcholinesterase from the sources previously studied should also display a substrate inhibition with acetylcholine.[7] This was not found with the plaice cholinesterase although it was shown that butyrylcholine and valerylcholine displayed a certain substrate inhibition (Fig. 2). Acetyl-β-methylcholine should be split by an acetylcholinesterase and benzoylcholine by a butyrylcholinesterase.[6] The plaice cholinesterase thus behaved more like a butyrylcholinesterase in this respect, since benzoylcholine was split, but acetyl-β-methylcholine being split only very slowly (Fig. 3). Triacetin was split as it is by an acetylcholinesterase.

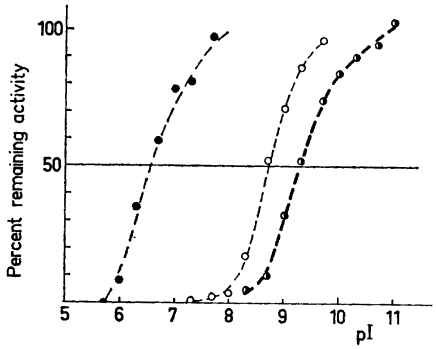


*Fig. 4.* Inhibition-curves for DFP, the specific inhibitor of butyrylcholinesterase with this enzyme and acetylcholinesterase from human blood and with plaice cholinesterase. Electrometric determinations, 0.1 M KCl, pH 8.2, 25°C, incubation time 30 min, acetylcholine $1.5 \times 10^{-2}$ M. ○ = butyrylcholinesterase, ◑ = plaice cholinesterase (heavy line), preparation III, ● = acetylcholinesterase.

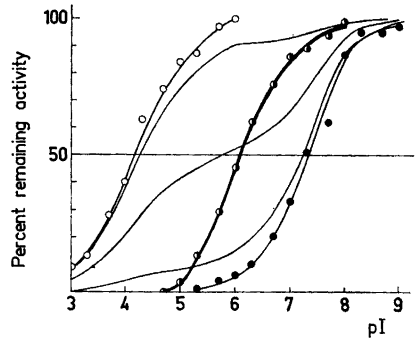


*Fig. 5.* Inhibition-curves for BW 284C51, the specific inhibitor of acetylcholinesterase, with this enzyme and butyrylcholinesterase from human blood and with plaice cholinesterase. Conditions and signs as given in the legend to Fig. 4.

The $K_m$-values decreased with the length of the acyl-group (Table 2). This fact indicates an increased affinity for the enzyme with the length of the acyl-group.

*Inhibitor effects.* There may be several explanations for the relatively large inhibitory effect of eserine as compared with its effect on other cholinesterases, $pI_{50}$ being about 7 for both acetylcholinesterase and butyrylcholinesterase from different sources (see also Long [22]). Possibly the plaice cholinesterase may be more rapidly carbamylated [23] as it is more rapidly phosphorylated (see below) than other types of cholinesterase. The high eserine sensitivity (as well as the cholinester splitting ability) characterizes the enzyme investigated as a cholinesterase.[6]

The rate of phosphorylation ($k_2$) of the plaice cholinesterase by Sarin in the presence of substrate is higher than that of both acetylcholinesterase [18] and butyrylcholinesterase [18] by this inhibitor. The same is valid for 37-$SN^+$ and DFP, Table 3. The phosphorylation rate of 37-$SN^+$ with the plaice cholinesterase is, however, only about half as great as that of Sarin although the phosphorylated enzyme should be the same with the two substances. The difference might be attributed to the more rapid hydrolysis of Sarin.[24] It would seem that the increased sensitivity of the plaice cholinesterase for organophosphorus compounds is a feature that distinguishes the enzyme from both acetylcholinesterase and butyrylcholinesterase. Thus, the fact that the enzyme is still more sensitive to DFP than butyrylcholinesterase, for which DFP should be a selective inhibitor [6] (Fig. 4), may not constitute a reason to consider the plaice cholinesterase as a butyrylcholinesterase.

BW 284C51 contains two quaternary nitrogens and is a reversible, selective inhibitor of acetylcholinesterase.[25] It was not quite as effective with the plaice

cholinesterase as with acetylcholinesterase from red blood corpuscles, the $pI_{50}$ being 6.1 *versus* 7.3 (Fig. 5). However, $pI_{50}$ with BW 284C51 was only 4.2 for butyrylcholinesterase from serum.

The $pI_{50}$-curve for the plaice cholinesterase shown in Fig. 4 cannot be explained as the result of the plaice cholinesterase being a mixture of acetylcholinesterase and butyrylcholinesterase. This is evident from the curves calculated for different mixtures of these latter enzymes, and which are also given in Fig. 5. It is obvious that even if a corresponding $pI_{50}$-value could have been obtained from a mixture, the form of the $pI_{50}$-curve for the plaice cholinesterase excluded this possibility.

From the results with BW 284C51 showing that the plaice cholinesterase was inhibited by lower concentrations of BW 284C51 than butyrylcholinesterase, it appears that the plaice cholinesterase behaves more like an acetylcholinesterase in this respect.

The plaice cholinesterase thus exhibits some features showing similarity to an *acetylcholinesterase*, such as the pH-activity dependence, the fact that acetylcholine is split faster than any of the cholinesters, and the sensitivity to inhibition by BW 284C51.

*Nontypical* for an acetylcholinesterase is that propionyl-, butyryl-, and valerylcholine all are split comparatively quickly, that the maximum velocities obtained at saturating substrate concentrations were about equal; that benzoylcholine is split faster than acetyl-$\beta$-methylcholine which is also split but only at a very low rate; that DFP inhibits the plaice cholinesterase at concentrations still lower than those necessary even for butyrylcholinesterase; that inhibition requires higher concentrations of BW 284C51 than are needed to inhibit acetylcholinesterase.

The high sensitivity of plaice cholinesterase to organophosphorus compounds distinguishes the enzyme from both acetylcholinesterase and butyrylcholinesterase.

As the plaice cholinesterase splits acetylcholine faster than other choline esters it may be said to be an acetylcholinesterase. The presence of acetylcholinesterase has also been demonstrated in the body muscles of several fish species.[2,26] However, the plaice cholinesterase has some properties in common also with a butyrylcholinesterase, as shown in the present investigation. It may thus be misleading to assign the plaice cholinesterase to one of the two types and it is better to name it simply by its source as is strongly recommended in the literature [6] in corresponding cases.

*Acknowledgements.* Substrates and inhibitors were synthetized by Dr. Inger Enander, Mr. Lars Fagerlind and Dr. L-E. Tammelin. Their valuable help is gratefully acknowledged as is also the skilful technical assistance of Mrs. Kerstin Hördell and Miss Ingela Jansson.

## REFERENCES


1. *Enzyme Nomenclature*, Recommendations 1964 of the International Union of Biochemistry, Elsevier, Amsterdam 1965.
2. Lundin, S. J. *J. Cellular Comp. Physiol.* **59** (1962) 93.
3. Lundin, S. J. *Acta Chem. Scand.* **21** (1967) 2663.
4. Wilson, I. B. In Boyer, P. D., Lardy, H. and Myrbäck, K., (Eds.), *The Enzymes*, Academic, New York 1960, Vol. 4, Part A, p. 501.
5. Augustinsson, K.-B. *Ibid.* p. 521.
6. Augustinsson, K-B. In Koelle, G. B., (Ed.), *Handbuch der experimentellen Pharmakologie*, Springer, Berlin 1963, Ergänzungswerk XV, Chapt. 4, p. 89.
7. Krupka, R. M. *Can. J. Biochem.* **42** (1964) 677.
8. Engelhardt, N., Prchal, K. and Nenner, M. *Angew. Chem.* **79** (1967) 604.
9. Tammelin, L.-E. *Acta Chem. Scand.* **10** (1956) 145.
10. Tammelin, L.-E. and Enander, I. *Biochem. Prep.* **11** (1966) 4.
11. Saunders, B. C. and Stacey, G. J. *J. Chem. Soc.* **1948** 699.
12. Bryani, P. J. R., Ford-Moore, A. H., Perry, B. J., Wardrop, A. W. H. and Watkins, T. F. *J. Chem. Soc.* **1960** 1553.
13. Tammelin, L.-E. *Acta Chem. Scand.* **11** (1957) 1340.
14. Austin, L. and Berry, W. K. *Biochem. J.* **54** (1953) 695.
15. Tammelin, L.-E. *Scand. J. Clin. Lab. Invest.* **5** (1953) 267.
16. Larsson, L. and Hansen, B. *Svensk Kem. Tidskr.* **68** (1956) 521.
17. Heilbronn, E. *Acta Chem. Scand.* **13** (1959) 1547.
18. Tammelin, L.-E. *Arkiv Kemi* **12** (1958) 287.
19. Lineweaver, H. and Burk, D. *J. Am. Chem. Soc.* **56** (1934) 658.
20. Heilbronn, E. *Acta Chem. Scand.* **13** (1959) 1255.
21. Bergmann, F., Rimon, S. and Segal, R. *Biochem. J.* **68** (1958) 493.
22. Long, J. P. In Koelle, G. B., (Ed.), *Handbuch der experimentellen Pharmakologie*, Springer, Berlin 1963, Ergänzungswerk XV, Chapt. 8, p. 374.
23. Main, A. R. and Hastings, F. L. *Science* **154** (1966) 400.
24. Heilbronn, E. *Svensk Kem. Tidskr.* **11** (1965) 3.
25. Nachmansohn, D. and Wilson, I. B. *Advan. Enzymol.* **12** (1951) 259.
26. Lundin, S. J. *Biochem. J.* **72** (1959) 210.


Received February 10, 1968.