Biochemical Pharmacology, Vol. 45, No. 5, pp. 991–998, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00
Pergamon Press Ltd

PURIFICATION AND IMMUNOLOGICAL CHARACTERIZATION OF PIGEON SERUM BUTYRYLCHOLINESTERASE

IMPLICATIONS ON ENVIRONMENTAL MONITORING AND TOXICOLOGICAL TESTING OF BIRDS

AHMED D. KHATTAB,* COLIN H. WALKER, MICHAEL I. MACKNESS and PETER W. SAPHIER

Department of Biochemistry and Physiology, School of Animal and Microbial Sciences, University of Reading, P.O. Box 228, Whiteknights, Reading RG6 2AJ, U.K.

(*Received* 15 *October* 1992; *accepted* 22 *December* 1992)

Abstract—Butyrylcholinesterase (EC 3.1.1.8) (BChE) was purified from pigeon serum to electrophoretic homogeneity by a four-step procedure involving blue sepharose CL-6B chromatography, ion exchange chromatography, procainamide affinity chromatography and gel filtration. An overall 2789-fold purification was achieved, with a final specific activity of 61.35 μmol/min/mg. The purified enzyme separated into two peaks when filtered through a column of Sephacryl S-300, a smaller peak containing the tetrameric form of BChE (C_4) and a larger peak containing the monomeric form of BChE (C_1). Native polyacrylamide gel electrophoresis (PAGE) of both peaks revealed single protein bands which coincided with esterase activity, with approximate M_r values of 84,000 and 340,000, respectively. The C_1 monomer represented 85–90% of the activity found in the pigeon serum. It is not clear whether this polymorphism of BChE in vertebrates contributes to the wider inter-individual variations observed in xenobiotics elimination kinetics and in the response to the pharmacological and toxic effects of pesticides. PAGE of the monomeric form of the enzyme in the presence of sodium dodecyl sulphate showed only one protein band with a M_r of 84,000, while that of the tetrameric form revealed two bands, a major protein band (84,000) and a minor band (170,000), representing the monomer and the dimer of the dissociated tetrameric BChE enzyme under reducing conditions. Highly specific polyclonal antibodies were raised in rabbits against the purified enzyme. These antibodies cross-reacted with other avian BChEs, a criterion which make them useful for the immunopurification of other BChEs from different species as well as for biomonitoring and toxicological studies on the role of esterases as an indicator of avian exposure to organophosphorous pesticides.

Butyrylcholinesterase (BChE†) is described as acylcholine acylhydrolase (EC 3.1.1.8) in the International Union of Biochemistry classification. It is also known as pseudocholinesterase, non-specific cholinesterase or serum cholinesterase, and is a widely distributed enzyme present in cholinergic and non-cholinergic tissues [1] including the pancreas, liver, heart, vascular system and urogenital system, as well as the serum [2]. In spite of the widespread distribution of BChE, its physiological function has not been established and its natural substrates are unknown [3]. However, its pharmacological and toxicological importance is recognised. It participates in the metabolism of drugs which are esters [4] and like acetylcholinesterase (AChE) is irreversibly inhibited by organophosphate compounds [5], although BChE differs from AChE in its substrate specificity and susceptibility to specific inhibitors [6].

Butyrylcholinesterase was first used as a bioindicator in the biological monitoring of pesticide exposure in the 1950s when organophosphorous pesticides were introduced into commercial use and the risk of severe poisoning was recognised. Currently, biological screening for exposure of humans and wild vertebrates to organophosphate and carbamate anticholinesterases is largely restricted to measurements of cholinesterase activity [7]. Determination of BChE activity can be a valuable indicator of exposure if the cholinesterase activity of a particular subject prior to exposure is known. Since normal BChE levels vary widely both between individuals and within an individual with time, it is not possible to determine accurately a general control value with which post-exposure measurements can be compared [8, 9]. The underlying problem is that activities are usually measured on the basis of total turnover of substrate per unit volume of serum. Measured in this way, activities are determined by two factors: (1) the quantity of enzyme present and (2) the specific activity of the enzyme. Organophosphorous compounds can cause changes in both factors [10] and at low levels of exposure

* Corresponding author at present address: Bournemouth University, Institute of Health Services, Ambassador House, Oxford Road, Bournemouth, Dorset BH8 8EY, U.K.

† Abbreviations: BChE, butyrylcholinesterase; AChE, acetylcholinesterase; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; IgG, immunoglobulin; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline–Tween 20.

one effect tends to cancel out the other. An increased activity due to more enzyme being present in the blood is offset by a decrease in specific activity due to inhibition.

These problems can be overcome by developing assay procedures which will determine the level of a particular esterase protein in the blood. This can be achieved by producing antibodies to specific esterases that can then be used to develop immunoassays [e.g. enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay] [11] to establish the amount of protein associated with the activity of the BChE present. The specific activities of particular esterases, which should be fairly constant, can act as controls (Walker CH, personal communication). This should resolve not only the problems referred to above, but also the problem of interindividual variation [12], and variations related to sex, age, race and hormones.

The current study has been concerned with the development of immunoassays for BChE in humans and also birds which are exposed to insecticides on farm land. This paper describes the purification of BChE from pigeon serum and the raising of antibodies to the purified BChE for use in the immunological characterization of pigeon serum BChE, as well as the BChE of other avian species.

MATERIALS AND METHODS

Materials

All chemicals were of analytical grade and purchased from the Sigma Chemical Co. (Poole, U.K.) unless otherwise stated. DE 52 anion exchanger was purchased from Whatman Biosystems Ltd; Coomassie protein assay reagent from Pierce Ltd. Horseradish peroxidase goat anti-rabbit immunoglobulin (IgG) was from Jackson Immunochemicals (West Grove, PA, U.S.A.). Immulon TM2 micro ELISA plates were purchased from Dynatech Laboratories Inc. (Chantilly, Virginia). Nitrocellulose papers 0.45 μm were from Schleicher and Schull GmbH (Dassel, Germany).

Sources of sera

Blood samples (1 mL) were taken from the brachial vein of feral pigeons (*Columba livia*) and red-legged partridges (*Alectoris rufa*) purchased from Lincolnshire pheasantries (U.K.). Samples of serum from starlings (*Sturnus vulgaris*), house sparrows (*Passer domesticus*), magpies (*Pica pica*) and the tawny owl (*Strix aluca*) were all supplied by the Ministry of Agriculture, Fisheries and Food (Worplesdon, Surrey, U.K.).

Blood was cooled (in ice) and serum rapidly separated by centrifugation (12,000 *g* for 30 min at 4°) and assayed immediately for butyrylcholinesterase activity.

Enzyme assays

All assays were done at 37°. BChE activity was assayed by the method of Ellman *et al.* [13], as adapted by Westlake *et al.* [14], in 25 mM Tris–HCl buffer pH 7.6. Butyrylthiocholine iodide was used as the substrate (0.5 mM) and the subsequent detection of released thiocholine by reaction with 5,5-dithio bis (2-nitrobenzoic acid) was monitored over a 2–3 min period with a recording spectrophotometer set at 410 nm.

Purification

All procedures were performed at 4° using Amicon-Wright columns. Serum (8 mL) was dialysed repeatedly against 20 mM potassium phosphate buffer pH 7.0 containing 0.15 M NaCl and pumped onto a Cibacron Blue Sepharose CL-6B column equilibrated with the same dialysing buffer. The BChE enzyme was recovered with the initial wash, while tightly bound albumin was eluted using the same buffer containing 1.5 M NaCl. The enzyme was concentrated and dialysed extensively against 20 mM sodium acetate buffer pH 5.0 containing 1 mM EDTA and then applied to a DE-52 column. Contaminating proteins were eluted with the buffer wash, whilst BChE enzyme was eluted with a linear NaCl gradient from 0 to 0.2 M at 20 mL/hr. The eluted enzyme was concentrated, dialysed overnight against 4 L 20 mM potassium phosphate buffer pH 6.9 containing 1 mM EDTA and chromatographed on a procainamide-Sepharose 4B column prepared as described previously by Lockridge *et al.* [15, 16]. The BChE enzyme was eluted with a linear salt gradient from 0 to 0.8 M NaCl at 20 mL/hr. Only the fractions that contained the highest enzyme activity were used for gel filtration which was performed using Sephacryl S-300 column equilibrated with 50 mM potassium phosphate buffer pH 6.9 containing 0.5 M NaCl at 5 mL/hr. The column was calibrated with dextran blue, ferritin, β-amylase, alcohol dehydrogenase and bovine serum albumin. Those fractions containing BChE were pooled, concentrated and stored at −20°.

Protein determination

Protein concentrations were determined according to the method of Bradford [17] using crystalline bovine serum albumin as a standard. Protein in column fractions was monitored by measuring absorbance at 280 nm.

Electrophoresis

SDS–PAGE was performed according to the method of Laemmli [18] using 7.5–11% SDS gel. Staining of protein bands was done with Coomassie brilliant blue R-250. Relative molecular mass was estimated using a commercially obtained calibration kit (Sigma SDS 7B).

Non-denaturing PAGE was carried out with 6% gel and the BChE activity on the gel was visualized according to the method of Juul [19] using butyrylthiocholine iodide as the substrate.

Immunological methods

Production of antibodies. Purified BChE enzyme, 100 μg in 1 mL phosphate-buffered saline (PBS), was mixed with 2 mL of Freund's complete adjuvant and 10 μL of 30% Brij.35. The mixture was sonicated and injected subcutaneously at three to four sites on New Zealand white rabbits. The rabbits were bled before immunization. They received booster injections after 4 and 6 weeks (50 μg antigen in 1 mL PBS mixed with 1 mL Freund's incomplete adjuvant).

The rabbits were bled 2 weeks after the final injection. The blood was allowed to clot and antiserum obtained by centrifugation. IgG was isolated from the antiserum using a protein A sepharose CL-4B column.

Determination of antisera specificity and titre. Sera were examined by Ouchterlony double immunodiffusion [20] on 1.5% agarose gel made in PBS containing 0.02% (w/v) sodium azide. The gels were placed in a damp container at room temperature for up to 72 hr. The precipitate lines were visualized either directly or after washing several times with PBS by activity staining according to the method of Martin *et al.* [21] using *N*-methyl indoxyl acetate as a substrate. The same procedure was repeated for cross-reactivity testing, replacing the pigeon serum with serum from the following birds: partridge, magpie, tawny owl, starling and house sparrow.

Immunoprecipitation was performed by mixing equal volumes of either the diluted crude pigeon serum or the purified BChE enzyme with different concentrations (0.025–0.175 mg) of the purified antibody in a total of 0.3 mL 20 mM potassium phosphate buffer pH 7.2. After incubation at 4° for 24 hr, the samples were centrifuged (12,000 *g*, 4°) for 30 min and the supernatant was assayed for the remaining BChE activity.

The control experiment was performed in the same manner using partially purified IgG from the same rabbits before immunization.

Western blotting. Proteins from SDS–polyacrylamide gels were electroblotted onto nitrocellulose filters (0.45 μm pore size) using established methodology [22] with the following minor modifications. The electrode buffer used for the transfer was 25 mM Trizma base, 192 mM glycine, 20% (v/v) methanol and 0.1% (w/v) SDS. The SDS–polyacrylamide gel was pre-equilibrated in this buffer for 30 min prior to transfer, and electroblotting was performed at 0.4 amps for 3 hr using a Trans-Blot cell (Bio-Rad).

After electroblotting, the distribution of BChE was revealed by incubating the nitrocellulose filters with rabbit anti-pigeon BChE antiserum (1/100 dilution) for 2 hr, followed by washing and further incubation with goat anti-rabbit Fc IgG conjugated to horse radish peroxidase (1/1000 dilution) for another 2 hr, and final visualization using a peroxidase detection method.

ELISA. The volume of all the reagents added to the microtitre plate wells was 50 μL and the assays were performed in triplicate. The coating antigen purified BChE enzyme) was diluted in 0.05 M sodium carbonate buffer pH 9.6 to make 10 μg/mL solution and added to each well. The plate was incubated overnight at 4° and then washed three times with PBS containing 0.1% Tween 20 (PBS-T). Subsequent steps were carried out at room temperature; between each step the plate was washed three times with PBS-T.

The remaining sites to which the antigen was not bound were then blocked by addition of PBS containing 5% bovine serum albumin and incubated for 1 hr, followed by washing. Serial dilutions of antiserum were made in PBS-T and added to each well. The plate was incubated for 2 hr, then washed with PBS-T. The goat anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:500 in PBS-T was added to each well and allowed to incubate for 2 hr, followed by washing.

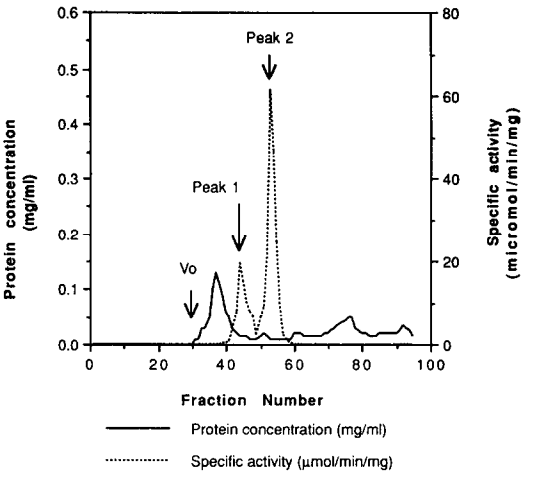

Fig. 1. Gel filtration elution profile of BChE activity and protein content (A_{280}) on Sephacryl S-300 at 4°. Column, 2.2 × 100 cm; eluent, 50 mM potassium phosphate buffer pH 6.9 containing 0.5 M NaCl; flow rate, 5 mL/hr. Fractions (4 mL) were collected and assayed as described. Peak 1 represents the tetrameric form of the BChE enzyme. Peak 2 represents the monomeric form of the BChE enzyme. Vo is the void volume (as determined with dextran blue).

The enzyme reactions were started by addition of 50 μL of substrate solution (0.4 mg/mL *O*-phenylenediamine hydrochloride in 25 mM citric acid, 50 mM sodium phosphate pH 5, containing 0.01% (v/v) hydrogen peroxide). The reactions were then stopped after 10 min by the addition of 50 μL 12% sulphuric acid and the absorbance read at 492 nm using a Bio-Rad ELISA plate reader.

RESULTS

Purification

A four-step purification method was used to purify BChE enzyme from pigeon serum. In the first step, selective removal of albumin from pigeon serum prior to processing was achieved by affinity chromatography on immobilized Cibacron Blue F3 G-A. BChE does not interact with the Blue Sepharose CL-6B column and 100% of the activity applied was recovered with the initial wash. In the second step, albumin-depleted serum was chromatographed on DEAE-cellulose at pH 5.0 in the cold as described by Das and Liddell [23], with the modification suggested by Muensch *et al.* [24]. This step typically yielded 40% of the activity applied. The third step was procainamide affinity chromatography at pH 6.9 according to the methods of Lockridge *et al.* [15, 16] except that we operated at 4°. A single peak of BChE activity was eluted and 70% of the activity applied was recovered. In the final step (gel filtration), the BChE activity separated into two peaks when filtered through a column of Sephacryl S-300 (Fig. 1). The smaller peak (peak 1)

Table 1. Purification of pigeon serum BChE

	Specific activity (μmol/min/mg)*	Fold purification†
1. Starting material (pigeon serum)	0.0220	—
2. Blue Sepharose CL-6B chromatography	0.0374	1.7
3. DE 52 pH 5.0	1.5	68
4. Procainamide affinity chromatography	45.4	2063
5. Gel filtration (final product)	61.35	2789

* Value refers to fraction with greatest activity.
† Overall purification, relative to original serum.

representing the high relative molecular mass protein contained the tetrameric form of BChE (C_4), while the larger peak (peak 2) representing the low relative molecular mass protein contained the monomeric form of BChE (C_1) [25]. Table 1 represents a summary of the results of purification. An overall 2789-fold purification was achieved with a final specific activity of 61.35 μmol/min/mg.

Native PAGE of both the monomeric and the tetrameric forms of the purified BChE revealed single protein bands which coincided with esterase activity, with approximate M_r values of 84,000 and 340,000, respectively (Fig. 2).

SDS–PAGE of the monomeric form of the enzyme under reducing conditions showed only one protein band with a M_r of 84,000, while that of the tetrameric form under the same reducing conditions revealed two bands, a major protein band with a M_r of 84,000 and a minor residual slow moving band with a M_r of 170,000. These two bands represent the monomer and dimer of the dissociated BChE enzyme under reducing conditions.

Assessment of antibody specificity and titre

The specificity of the antibodies was tested by a combination of methods including Ouchterlony double immunodiffusion and immunoprecipitation together with western blotting. Ouchterlony double immunodiffusion experiments were done on 1.5% agarose gel, with different dilutions of both antibodies and antigens. Precipitation lines on the gel due to the formation of a BChE–antibody complex were seen, but these lines were not very clear. As the antibodies had little effect on the catalytic function and enzyme activity was fully recovered in the bound phase [26], the BChE–antibody complex was enzymatically active. Accordingly, the precipitates observed with the above immunological method were made more visible and clear using *N*-methyl indoxyl acetate staining (activity staining). No precipitation lines were observed in the control which contained the pre-immune rabbit serum and pigeon serum BChE.

The specificity of antibodies towards pigeon serum BChE was also investigated by precipitation of the BChE activity from both the purified BChE and the

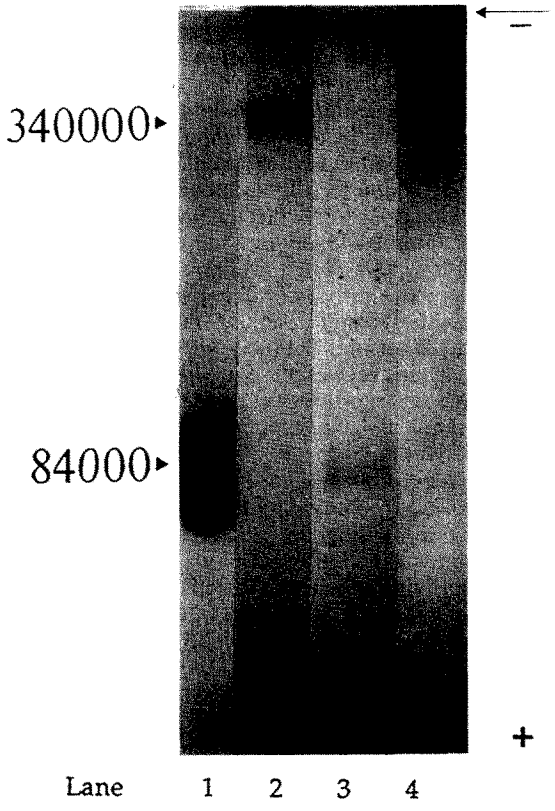

Fig. 2. Non-denaturing gel electrophoresis was performed in 6% polyacrylamide gels in a Tris-glycine, pH 8.8 buffer system (stacking gel was 3% at pH 6.8). Electrophoresis was carried out at 200 mV for 3 hr. Five micrograms of the monomeric form of BChE were applied to lanes 1 and 3; 15 μg of the tetrameric form of BChE were applied to lanes 2 and 4. Lanes 1 and 2 were developed by a BChE specific staining method using butyrylthiocholine iodide as substrate, while lanes 3 and 4 were developed by staining with Coomassie brilliant blue R. The arrow shows the origin of the migration and the orientation of negative (−) and positive (+) electrodes is indicated.

Table 2. Immunoprecipitation of BChE activity

IgG concentration (mg/mL)	% Precipitated activity
0.075	48
0.180	100

* Immunoprecipitation was performed using different concentrations of the antibodies raised against purified pigeon serum BChE. Details of the experiment are given in Materials and Methods. BChE activity in the supernatant was assayed by the Ellman method.

The results are expressed as % of BChE activity precipitated by the antibodies in each tube.

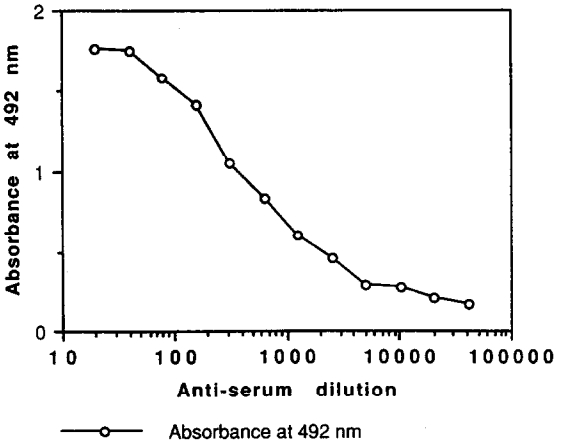

Fig. 3. ELISA for the determination of antiserum dilution using microtitre wells coated with purified pigeon BChE. Serial dilutions of antiserum were made in PBS-T 20. The antigen–antibody complex was then incubated with 1:500 goat anti-rabbit IgG–horseradish peroxidase conjugate. Absorbance was read at 492 nm after incubation with the substrate solution (see text). The results were expressed as the mean of triplicate determinations after subtracting the absorbance values of the blanks.

crude pigeon serum samples, using different concentrations of the purified antibodies. The results indicated precipitation reaction (formation of antigen–antibody precipitate) in the tubes which contained a mixture of rabbit antiserum with either pigeon serum or purified BChE, and there was a 100% reduction in BChE activity from the original sample due to precipitation of antigenic esterase activity by the polyclonal antibody (Table 2). At the same time, there were no precipitation reactions in the tubes which contained a mixture of pre-immune rabbit serum with pigeon serum or purified BChE, as well as no reduction in BChE activity.

Analysis of the BChE enzyme by western blotting indicated the presence of two bands on nitrocellulose paper with M_r values of 84,000 and 170,000, representing the monomer and dimer of the BChE enzyme, respectively. After both the crude pigeon serum and the purified tetrameric form of the enzyme had been transferred to nitrocellulose, the polyclonal antibodies recognized only these two bands, whereas the transfer of the purified monomeric form of the same enzyme resulted in the recognition of only one band with a M_r of 84,000.

Finally, a screening immunoassay method was developed to monitor the titre of specific antibodies in the immunized rabbits. ELISA technique was used for this purpose. Figure 3 shows the antibody titre of the rabbit serum collected on the 56th day after immunization with a total of 200 μg of BChE. The results indicated the presence of antibodies and at 20,000 dilution of the antiserum it was possible to detect antigenic BChE.

Cross-reactivity of antibodies with BChE from different species of birds

The antibodies raised against pigeon serum BChE showed cross-reactivity with BChE from other avian species in the following order: partridge > magpie > tawny owl > starling > house sparrow. Meanwhile, these antibodies did not cross-react with mammalian BChE, namely human, horse and rat. The conditions of these experiments were not standardized for testing the maximum immunoreactivity, so the above order of cross-reactivity is only a rough estimate.

DISCUSSION

Most attempts to purify BChE have focused on the purification of the enzyme from horse and human plasma [27]. The purification of plasma BChE has proven to be difficult [28–30] because of the large size and complex structure of the enzyme, features which it shares with AChE. The fact that plasma BChE is in solution rather than being membrane-bound is a simplifying feature, but its relative scarcity, the complex protein composition of plasma and the sensitivity of plasma BChE to denaturation have made it difficult to obtain adequate amounts of pure enzyme.

In this study a four-step purification method was used to purify BChE from pigeon serum to apparent homogeneity. Albumin represents more than 50% of the total protein in plasma and it is often the major contaminant in preparations of other plasma proteins [31]. Therefore, Blue Sepharose CL-6B chromatography was included as a first step in this purification procedure. This was followed by ion exchange chromatography, procainamide affinity chromatography and finally gel filtration. A 2789-fold purification was achieved. Purification factors between 3000 and 14,000 have been reported by different workers for both human and horse plasma BChE [32]; however, the purification factor reported here is not directly comparable to those reported by other workers because of different means of determining the protein content of the products at various stages of purification and the difficulty of drying specimens to constant weight after lyophilization, apart from the conventional colourimetric methods of assay.

The purity and the relative molecular mass of our BChE preparation were tested by PAGE under both reducing and non-reducing conditions. Electrophoresis of BChE from different species under

non-denaturing conditions reveals large differences in mobility. These probably reflect differences in the charges of the polypeptides themselves or in their glycosylation [33]. Little is known about possible heterogeneity in the glycosylation of BChE [34]. Nevertheless, the possibility of differences in the charges of the polypeptides could be ruled out by the observation that this tetramer eluted before the monomer on gel filtration, where separation was based on size alone and did not depend on charge. The findings that the approximate M_r (by gel filtration) of the tetrameric form of the pigeon serum BChE enzyme was 340,000 and that of the monomeric form of the same enzyme was 84,000 represented an additional support for our conclusion that the slow moving band in the non-SDS gels represents a tetramer and does not reflect differences in the charges.

In human plasma, 90% of the enzyme activity is present in the so-called C_4 band (tetrameric form) [34], while this study reveals that 85–90% of BChE activity in samples obtained from either fresh pigeon serum or purified BChE is present in the C_1 band (monomeric form).

These results raise the question of the significance of the heterogeneity of BChE which is of particular interest because not only are multiple isoenzymic and polymorphic forms frequently observed [35–39], but the substrate specificity of BChE and the quantity differ greatly from species to species [40]. Massoulie [41] believes that the complex polymorphism of the cholinesterases, in general, is a common feature of these enzymes, and their existence provides a means of adapting the cholinesterases to all kinds of possible subcellular and extracellular localizations according to the physiological demands of each system or species, while others suggest that species differences in the type and quantity of plasma enzymes could be expected to modify toxicity [42]. Since there is no conclusive evidence for a definite physiological function of BChE, the significance of the molecular polymorphism of this enzyme is obscure.

On SDS–PAGE electrophoresis, pigeon serum BChE dissociated into two bands with apparent M_r values of 84,000 and 170,000. Since these values are also consistent with the relative molecular masses of the monomer and dimer determined by other workers for other vertebrates [16, 24, 43], we concluded that the two bands were the monomer and dimer of pigeon BChE. Although such a residual dimer band was also reported for human and horse serum BChE [16, 44, 45], no satisfactory explanation for this phenomenon is available [16].

Among the more interesting aspects of the cholinesterases are their regulation, distribution and fate. These dynamic properties are currently less well understood than the catalytic function and structure in both man and animals [46]. The use of an immunological approach has provided new insights concerning the analysis of structural and functional significance of cholinesterase polymorphism [47], as well as the measurement of tissue levels of BChE enzyme protein [48].

The purification of BChE from pigeon serum enabled us to prepare highly specific polyclonal antibodies to this enzyme. These antibodies do cross-react with other avian BChE such as partridge, magpie, tawny owl, starling and house sparrow, but they do not cross-react with mammalian BChE, namely human, rat and horse. Immunological cross-reactivity is commonly applied as a sensitive criterion for structural homology. This study clearly demonstrates that pigeon BChE has antigenic determinants in common with other avian BChE. Such cross-reactivity is very important in field research, because these antibodies can be used in immunoassays to study the impact of environmental pollution by organophosphorous pesticides on a range of bird species. Moreover, they can be used in immunoaffinity purification of other avian BChE using a very small amount of serum instead of going through the purification procedures as we have, which requires much time and effort, as well as more serum, to obtain very small amounts of purified enzyme.

Western blotting was used to establish whether these antibodies raised in the rabbit will recognize the BChE enzyme alone or whether they will identify other proteins too. Two bands were recognized by the polyclonal antibodies in the run of both the purified tetrameric form of BChE and the crude pigeon serum samples, indicating that they are the monomer and the dimer of the same enzyme. These results support the concept that antibodies prepared against BChE have a high specificity. Consequently, these antibodies could be used to develop an ELISA technique by which the amount of antigenic esterase protein present in a serum sample could be measured. Ultimately, this would enable us to measure the specific activity of the enzyme. Therefore, BChE activity may be replaced by a strategy based on the measurement of BChE specific activity that within healthy individuals or animals exhibits insignificant biological intra-individual variations.

Among the environmental stresses to which birds and other wildlife are exposed are those caused by pesticides. Unfortunately, it is particularly difficult to assess either the exposure to, or the effects of, pesticides in the field. Therefore, reliable and cost-effective techniques for evaluating pesticide impact are in high demand, and immunoassays are rapidly replacing many other methods used to detect and quantify substances with important biological or pharmacological properties.

REFERENCES

1. Masson P and Laurentie M, Stability of butyrylcholinesterase: thermal inactivation in water and deuterium oxide. *Biochim Biophys Acta* **957**: 111–121, 1988.
2. Trundle D and Marcial G, Detection of cholinesterase inhibition: the significance of cholinesterase measurements. *Ann Clin Lab Sci* **18**: 345–358, 1988.
3. Kutty KM, Review: biological function of cholinesterase. *Clin Biochem* **13**: 239–243, 1980.
4. Whittaker M, *Cholinesterase*. Karger, Basel, 1986.
5. Karczmar AG, Usdin E and Wills JH, *Anticholinesterase Agents*. Pergamon Press, Oxford, 1970.
6. Layer PG, Alber R and Sporns O, Quantitative development and molecular forms of Acetyl and Butyryl cholinesterase during morphogenesis and synaptogenesis of chick brain and retina. *J Neurochem* **49**: 175–181, 1987.

7. Coye MJ, Lowe JA and Maddy KT, Biological monitoring of agricultural workers exposed to pesticides: 1. Cholinesterase activity determination. *J Occup Med* **28**: 619–627, 1986.
8. Moseman RF and Oswald EO, Development of analytical methodology for assessment of human exposure to pesticides. In: *Pesticide Analytical Methodology*, Chap. 13, pp. 251–257. American Chemical Society, Washington, DC, 1980.
9. Coye MJ, Barnett PG, Midtling JE, Velasco AR, Romero P, Clements CL, Malley MAC, Tobin MW and Lowry L, Clinical confirmation of organophosphate poisoning of agricultural workers. *Am J Ind Med* **10**: 399–409, 1986.
10. Walker CH, Some biochemical responses of vertebrates to sublethal doses of pesticides. *Environ Aspects Appl Biol* **17**: 257–263, 1988.
11. Thompson HM, Walker CH and Hardy AR, Esterases as indicators of avian exposure to insecticides. In: *BCPC Monograph No. 40. Field Methods for the study of Environmental Effects of Pesticides* (Eds. Greaves MP, Smith BD and Greig-Smith PW), pp. 39–45. British Crop Protection Council, Croydon, 1988.
12. NIOSH, *Hazard Evaluation*. National Institute for Occupational Safety and Health, No. 82-248-1472: 1–11, 1984.
13. Ellman GL, Courtney KD, Andreas V Jr and Featherstone RM, A new and rapid colorimetric determination of acetyl cholinesterase activity. *Biochem Pharmacol* **7**: 88–95, 1961.
14. Westlake GE, Blunden CA, Brown PM, Bunyan PJ, Martin AD, Sayers PE, Stanley PI and Tarrant KA, Residues and enzyme changes in wood mice from the use of chlorfenvinphos and an organomercurial fungicide on winter wheat seed. *Ecotoxicol Environ Safety* **4**: 1–16, 1980.
15. Lockridge O and La Du BN, Comparison of atypical and usual human serum cholinesterase. *J Biol Chem* **253**: 361–366, 1978.
16. Lockridge O, Eckerson HW and La Du BN, Interchain disulfide bonds and subunit organisation in human serum cholinesterase. *J Biol Chem* **254**: 8324–8330, 1979.
17. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
18. Laemmli UK, Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**: 680–685, 1970.
19. Juul P, Human plasma cholinesterase isoenzymes. *Clin Chim Acta* **19**: 205–213, 1968.
20. Ouchterlony O, Diffusion-in-gel methods for immunological analysis. *Prog Allergy* **4**: 1–78, 1958.
21. Martin AD, Blunder CA, Fletcher MR, Fletcher WJ, Stanley PI and Westlake GE, Electrophoretic profiles of esterases in starling plasma; an apparent simple genetic variant. *Bull Environ Contam Toxicol* **30**: 373–377, 1983.
22. Towbin H, Staehelin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354, 1979.
23. Das PK and Liddell J, Purification and properties of human serum cholinesterase. *Biochem J* **116**: 875–881, 1970.
24. Muensch H, Goedde HW and Youshida A, Human serum cholinesterase subunits and number of active sites of the major component. *Eur J Biochem* **70**: 217–223, 1976.
25. Biagioni S, Scarsella G and Toschi G, Studies on human cholinesterases in relation to genetics, pharmacology and neuromuscular pathology. In: *Cholinesterases, Fundamental and Applied Aspects* (Eds. Brzin M, Barnanrd EA and Sket D), pp. 331–343. Walter de Gruyter, Berlin, 1984.
26. Brimijoin S, Mintz KP and Alley M, Production and characterization of separate monoclonal antibodies to human acetylcholinesterase and butyrylcholinesterase. *Mol Pharmacol* **24**: 513–520, 1983.
27. Main AR, Purification and properties of plasma cholinesterases. *Rev Biochem Toxicol* **6**: 117–154, 1984.
28. Silvers A, *The Biology of Cholinesterases*. North Holland Publishing Co, Amsterdam, 1974.
29. Svensmark O, Molecular properties of cholinesterases. *Acta Physiol Scand [Suppl]* **245**: 10, 1965.
30. Augustinsson KB, Classification and comparative enzymology of the cholinesterases and methods for their determination. *Exp Pharmacol* **15**: 89, 1963.
31. Subramanian S, Dye-ligand affinity chromatography: the interaction of cibacron blue F3GA with proteins and enzymes. *CRC Crit Rev Biochem* **16**: 169–205, 1984.
32. Brown SS, Kalow W, Pilz W, Whittaker M and Woronick CL, The plasma cholinesterases. A new perspective. *Adv Clin Chem* **22**: 1–121, 1981.
33. Massoulie J, Bon S, Lazar M, Grassi J, Marsh D, Meflah K, Toutant JP, Vallette F and Vigny M, The polymorphism of cholinesterases; classification of molecular forms; interactions and solubilization characteristics; metabolic relationships and regulations. In: *Cholinesterases, Fundamental and Applied Aspects* (Eds. Brzin M, Barnanrd EA and Sket D), pp. 73–97. Walter de Gruyter, Berlin, 1984.
34. Chatonnet A and Lockridge O, Comparison of butyrylcholinesterase and acetylcholinesterase (Review). *Biochem J* **260**: 625–634, 1989.
35. Bernsohn J, Barron KD and Hess A, Cholinesterases in serum demonstrated by starch gel electrophoresis. *Proc Soc Exp Biol Med* **108**: 71–73, 1961.
36. Main AR, Kinetic evidence of multiple reversible cholinesterases based on inhibition by organophosphates. *J Biol Chem* **244**: 829–840, 1969.
37. Ecobichon DJ, Characterization of the esterases of canine serum. *Can J Biochem* **48**: 1359–1367, 1970.
38. La Motta RV and Wronick CL, Molecular heterogeneity of human serum cholinesterases. *Clin Chem* **17**: 135–144, 1971.
39. Chow AYK and Ecobichon DJ, The esterases of cavian plasma. *Rev Can Biol* **31**: 21–29, 1972.
40. Augustinsson KB, Electrophoresis studies on blood plasma esterase. 1. Mammalian plasma. *Acta Chem Scand* **13**: 571–592, 1959.
41. Massoulie J, The polymorphism of cholinesterases and its physiological significance. *TIBS* **5**: 160–164, 1980.
42. Ecobichon DJ and Comeau AM, Pseudocholinesterases of mammalian plasma: physicochemical properties and organophosphate inhibition in eleven species. *Toxicol Appl Pharmacol* **24**: 92–100, 1973.
43. La Motta RV, Wronick CL and Reinfrank RF, Multiple forms of serum cholinesterase: molecular weights of the isoenzymes. *Arch Biochem Biophys* **136**: 448–451, 1970.
44. Teng TL, Harpst JA, Lee JC, Zinn A and Carlson DM, Composition and molecular weights of butyrylcholinesterase from horse serum. *Arch Biochem Biophys* **176**: 71–81, 1976.
45. Main AR, Tarkan E, Aull JL and Soucie WG, Purification of horse serum cholinesterase by preparative polyacrylamide gel electrophoresis. *J Biol Chem* **247**: 566–571, 1972.
46. Brimijoin S and Mintz KP, Immunochemical approaches to the study of mammalian cholinesterases. In *Cholinesterases, Fundamental and Applied Aspects*

(Eds. Brzin M, Barnanrd EA and Sket D), pp. 247–258. Walter de Gruyter, Berlin, 1984.
47. Brimijoin S, Rakonczay Z and Mintz KP, Immunochemistry of mammalian cholinesterases. *Fed Proc* **45**: 2960–2964, 1986.
48. Brock A, Mortensen V, Loft AGR and Norgaard-Pedersen B, Enzyme immunoassay of human cholinesterase (EC 3.1.1.8): comparison of immunoreactive substance concentration with catalytic activity concentration in randomly selected serum samples from healthy individuals. *J Clin Chem Clin Biochem* **28**: 221–224, 1990.