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PARTIAL PURIFICATION AND PROPERTIES OF SHEEP SERUM 'A'-ESTERASES

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Abstract—Using paraoxon and pirimiphos-methyl as substrates, much of the 'A'-esterase activity of sheep serum was found to be in the high-density lipoprotein (HDL) fraction. A method was developed for the partial purification of 'A'-esterases by the preparation of a lipoprotein fraction, followed by preparative polyacrylamide gel electrophoresis. The properties of the partially purified preparations of 'A'-esterase were studied. Although four different preparations all contained a major protein unit, which resembled the core protein of HDL, there was evidence of differences between preparations with regard to substrate specificity, suggesting the existence of multiple enzyme forms. Gel filtration of serum samples indicated that paraoxonase activity is expressed by proteins with mol. wts > 200,000, strongly suggesting that the 'A'-esterase activity of the lipoprotein fraction is present in one or more forms of HDL_2. The dependence of 'A'-esterase activity upon Ca^{2+} and the problem of esterase classification are discussed.

In 1953 Aldridge [1] defined two classes of esterases according to their interaction with organophosphate anticholinesterases. 'A'-Esterases hydrolyse such substances whilst 'B'-esterases are inhibited by them. The active forms of organophosphorus pesticides are inhibitors of 'B'-esterases and are in many cases deactivated by 'A'-esterases [2]. Mammals tend to have high levels of 'A'-esterase activity in the blood and the liver, and this is apparently an important factor in determining their relatively low susceptibility to such organophosphates as diazinon and pirimiphos-methyl. By comparison birds have very low levels of 'A'-esterase activity and are relatively susceptible to these two compounds [3]. In spite of their toxicological importance, 'A'-esterases have not been properly characterised although several attempts have been made to do this [4, 5]; however no preparation of 'A'-esterase has been purified to homogeneity. The aim of the present work was to purify and characterise the 'A'-esterases found in sheep serum [6]. Sheep serum was chosen as the enzyme source on account of its ready availability and high 'A'-esterase activity. Paraoxon (*o,o*-diethyl-*o,p*-nitrophenyl phosphate) and pirimiphos-methyloxon (2-diethylamino-6-methylpyrimidine-4-yl dimethyl phosphate) were used as substrates. In addition a study was undertaken to compare the properties of 'A'-esterases with those of sheep serum 'arylesterase'.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical reagent grade unless otherwise stated. Sucrose, glacial acetic acid, potassium bromide, calcium chloride and methanol were obtained from Fisons. Tris (Sigma 7–9® Trizma biochemical buffer), acrylamide and *N,N'*-methylene-bisacrylamide (both especially pure for electrophoresis), TEMED, ammonium persulphate, β-mercaptoethanol, bromophenol blue, glycine, paraoxon, Triton X-100 and Coomassie brilliant blue R-250 were obtained from Sigma (London) Chemical Co. Pirimiphos-methyloxon was a kind gift from I.C.I. Plant Protection Division.

Sheep serum

Sheep blood was obtained unheparinised from Reading Abattoir and centrifuged at 2100 *g* for 1 hr at 4° to obtain the serum.

Purification of sheep serum 'A'-esterase

All procedures were performed at 4°.

The initial stage of the purification entailed the preparation of a total lipoprotein fraction as described by Mackness and Walker [6]. Potassium bromide (324.6 mg/ml serum) was added to sheep serum to give a sp. g. of 1.21, and the mixture centrifuged at 135,000 *g* for 24 hr at 4°. Lipoproteins (high- plus low-density) separated from the remainder of the serum (termed the soluble fraction) as a pale yellow layer and this layer was removed with a Pasteur pipette. 'A'-Esterase in this lipoprotein fraction was then further purified by preparative polyacrylamide gel electrophoresis (PAGE).

Preparative non-denaturing PAGE was performed using the Mark II Shandon–Southern preparative polyacrylamide electrophoresis apparatus. The solutions employed for making gels were as described by Laemmli [7] with the exception that sodium dodecyl sulphate was omitted. The separating gel used was 8 cm of 7.5% (w/v) polyacrylamide gel containing 0.1% (v/v) Triton X-100 overlayed with a 1 cm 3% (w/v) polyacrylamide gel which also con-

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tained 0.1% (v/v) Triton X-100. Five millilitres of sample (4 ml lipoprotein fraction to 1 ml sample buffer) was applied to the column and cold water was circulated through the cooling system and electrode buffer (0.05 M Tris, 0.38 M glycine, pH 8.15) circulated between the upper and lower electrode reservoirs. Electrophoresis was at 30 mA for 1 hr and then at 80 mA for the remainder of the electrophoresis run. A pump transferred ice-cold elution buffer (0.43 M Tris–acetate, pH 8.3) at the rate of 14 ml/hr from a reservoir to a fraction collector which collected fractions at 25-min intervals via the elution plate at the base of the separating gel and a LKB Uvicord II Flow Analyser attached to a recorder.

Samples eluted from the column were immediately assayed for paraoxonase activity and were then stored at −80° until required for further assay.

Fractionation of lipoproteins

In order to determine the distribution of 'A'-esterase in the lipoprotein, high- and low-density lipoprotein fractions were prepared by the following method. Potassium bromide was added to serum (90.7 mg/ml serum) to give a sp. g. of 1.063 and the resultant solution centrifuged at 135,000 *g* for 24 hr at 4° (M.S.E. Superspeed 65 Ultracentrifuge). The upper pale layer of low-density lipoprotein (LDL) was removed using a Pasteur pipette and 234.1 mg/ml potassium bromide was added to the remainder of the serum to increase the sp. g. to 1.21. After centrifugation at 135,000 *g* for 24 hr at 4°, the upper yellow layer of high-density lipoprotein (HDL) was removed. Serum, LDL, HDL and soluble fractions were assayed for A-esterase activity.

Assay of enzyme activity

(i) *Enzyme activity in serum, lipoprotein and soluble fractions.* (a) *'A'-Esterase activity.* 'A'-Esterase activities towards paraoxon (paraoxonase activity) and pirimiphos-methyloxon (pirimiphos-methyloxonase activity) were assayed by the methods described by Brealey *et al.* [3] with the exception that the phosphate buffer used in both assays was changed to 0.02 M Tris–HCl, pH 7.6, buffer.

(b) *Arylesterase activity.* Arylesterase activity towards phenylacetate was assayed using the method of Lorentz *et al.* [8]. The assays were conducted at 37° and 20 μl of undiluted enzyme source was used.

(ii) *Enzyme activity in fractions from preparative PAGE.* (a) *'A'-Esterase activity.* The activity of 'A'-esterase towards paraoxon in fractions from preparative PAGE was measured by mixing 3.9 ml of 0.43 M Tris–acetate buffer, pH 8.3, 0.1 ml of 0.12 M paraoxon in ethanol and 1 ml of the sample. The mixture was incubated at 37° with shaking and the change in absorbance at 400 nm recorded over 20 min, using a Gilford 240 spectrophotometer.

The activity of 'A'-esterase towards pirimiphos-methyloxon was measured by mixing 2.98 ml of 0.43 M Tris–acetate buffer, pH 8.3, containing 10 mM calcium chloride, 0.02 ml of 2 M pirimiphosmethyloxon in ethanol and 0.5 ml of the sample. The mixture was incubated at 37° with shaking. Portions (0.2 ml) of the reaction medium were withdrawn at 5 and 10 min after the substrate had been added and were analysed for hydrolysis products by the method of Brealey and Lawrence [9].

(b) *Arylesterase activity.* Arylesterase activity in fractions from preparative PAGE was measured by the method described earlier (i) except that the sample size used was 250 μl.

(iii) *Activation assays.* The activity of paraoxonase peaks from the preparative PAGE column was measured for activation by Ca^{2+} and the activator solution (all fractions from the preparative column not showing paraoxonase activity combined) by slight modifications of the paraoxonase method described earlier.

(i) For Ca^{2+}—before the assay calcium chloride was added to the assay buffer to give a final Ca^{2+} concn of 10 mM.

(ii) For the activator—prior to assay 1 ml of assay buffer was replaced by 1 ml of activator solution.

(iii) For Ca^{2+} and the activator solution combined—modifications (i) and (ii) were both carried out prior to assay.

Protein determinations

Protein was measured using the Coomassie blue dye binding method as modified by Spector [10].

Analytical denaturing PAGE

Analytical denaturing PAGE was performed by the method of Laemmli [7] adapted for vertical slab gel electrophoresis, in order to determine the purity of the paraoxonase peaks from the preparative PAGE column. The gel used was a 7.5% (w/v) polyacrylamide gel overlayed with a 3% (w/v) stacking gel. Electrophoresis was at 20 mA for 4 hr, after which the gel was stained for protein in 0.1% (w/v) Coomassie brilliant blue R-250 in methanol:glacial acetic acid:water (45:9:46) for 2 hr and destained in methanol:glacial acetic acid:water (5:1:5) to remove background staining.

RESULTS

The distribution of 'A'-esterase and arylesterase activities in the soluble and lipoprotein fractions of sheep serum are shown in Table 1, as is the distribution of 'A'-esterase activity after the fractionation of the total lipoprotein fractions into LDL and HDL fractions. The results show that the majority of paraoxonase activity was in the lipoprotein fraction and pirimiphos-methyloxonase was distributed almost equally between the two fractions, while the majority of arylesterase occurred in the soluble fraction. The total recovery of arylesterase activity from serum in the two fractions at 93.5 ± 6% is larger than the recovery of either of the two 'A'-esterase activities (73 ± 12% for paraoxonase and 51 ± 9% for pirimiphos-methyloxonase), suggesting the existence of different types of esterase with different substrate specificities.

In order to determine the distribution of 'A'-esterase activity in the lipoprotein fraction, HDL and LDL fractions were prepared and assayed for 'A'-esterase activity towards both paraoxon and pirimiphos-methyloxon substrates (Table 1). Nearly all of the paraoxonase activity and all of the

Table 1. Distribution of enzyme activity in soluble, total lipoprotein, high- and low-density lipoprotein fractions of sheep serum

Enzyme	Activity recovery in the soluble fraction as a % of that in sheep serum	Activity recovery in the total lipoprotein fraction as a % of that in sheep serum	Activity recovery in the high-density lipoprotein fraction as a % of that in the total lipoprotein fraction	Activity recovery in the low density lipoprotein fraction as a % of that in the total lipoprotein fraction
Paraoxonase	24.6 ± 7.7	56.2 ± 7.8	98.5 ± 0.1	1.5 ± 0.1
Pirimiphos methyloxonase	31.9 ± 9.4	22.1 ± 6.1	100 ± 0	N.D.
Arylesterase	76.9 ± 5.8	16.5 ± 0.7	—	—

N.D. = not detectable.

pirimiphos-methyloxonase activity is found in the HDL fraction. An essentially similar distribution of serum paraoxonase was found on fractionation of human serum.*

The elution profiles of protein and paraoxonase activity from the preparative PAGE column are shown in Fig. 1. The majority of paraoxonase activity eluted after the main bulk of the protein as four major peaks. Fractions 67–73, 74–107, 108–123 and 124–130 were pooled for further assay of paraoxonase, pirimiphos-methyloxonase and arylesterase activities. Fractions 1–66 and 124–150 were pooled as the recombined inactive fraction or activator solution.

The results of the assays of the activity peaks for paraoxonase, pirimiphos-methyloxonase and arylesterase activities are presented in Table 2. The results presented for paraoxonase are those where activation took place (see later). Pirimiphos-methyloxonase activity was found only in peaks 67–74 and 124–130, i.e. the first and last paraoxonase peaks eluted from the preparative column. This differential distribution of paraoxonase and pirimiphos-methyloxonase suggests that there are multiple forms of 'A'-esterase which hydrolyse the two substrates. The ratios of the activities for the two substrates are different from the ratio found in the lipoprotein fraction. This indicates that the fractions may contain two or more forms of 'A'-esterase.

All four paraoxonase activity peaks are also active towards phenylacetate and therefore show arylesterase activity but the ratio between activities changes markedly from peak to peak. A major difference between 'A'-esterase and arylesterase is evident from Table 2, namely that arylesterase activity is found in the activator solution where no 'A'-esterase activity occurs.

The pooled peaks of paraoxonase activity were also assayed for activation by Ca^{2+}, the activator solution and these two in combination; these results are presented in Table 3. The fractions representing peak 74–107 were activated by calcium ($P < 0.01$).

* S. Woodhouse, unpublished data.

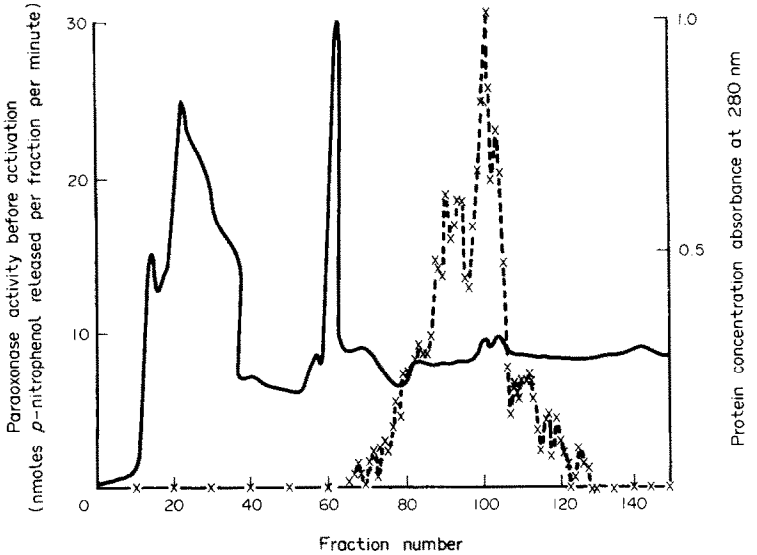

Fig. 1. Profile of paraoxonase activity eluted by preparative polyacrylamide gel electrophoresis after electrophoresis of sheep serum lipoprotein fraction. PAGE is described in Materials and Methods. (——) Protein; (----) paraoxonase activity.

Table 2. Paraoxonase, pirimiphos-methyloxonase and arylesterase activities eluted from the preparative PAGE column

Paraoxonase activity peak	Pirimiphos-methyloxonase			Paraoxonase			Arylesterase			Ratio of activities		
	Sp. act.*	Recovery of activity (%)	Purification factor	Sp. act.*	Recovery of activity (%)	Purification factor	Sp. act.†	Recovery of activity (%)	Purification factor	Paraoxonase to pirimiphos-methyloxonase	Paraoxonase to arylesterase	Pirimiphos-methyloxonase to arylesterase
Lipoprotein fraction	67.8	100	1	1.96	100	1	1635	100	1	1:35	1:83	1:24
67–73	244 ± 36	16.1	3.6	2.25 ± 0.41	0.61	1.2	478 ± 25	1.3	—	1:108	1:39	1:20
74–107	N.D.	—	—	12.3 ± 0.4	22.51	6.3	1980 ± 7	36.1	1.21	—	1:20	—
108–123	N.D.	—	—	3.99 ± 0.67	3.39	2.1	1770 ± 22	15.1	1.08	—	1:45	—
124–130	386 ± 68.6	22.7	5.7	3.18 ± 0.29	0.78	1.6	1660 ± 26	2.8	1.02	1:122	1:68	1:30
Activator solution	N.D.	—	—	N.D.	—	—	410 ± 10	17.7	—	—	—	—
Total activity recovery (%)	—	38.8	—	—	27.29	—	—	73.07	—	—	—	—

N.D. = not detectable.
* Sp. act. = nmoles product released · minute^{-1} · mg protein^{-1}.
† Sp. act. = μmoles product released · minute^{-1} · mg protein^{-1}.

Table 3. Activity of fractions from preparative PAGE towards paraoxonase in the presence of various effector solutions

Paraoxonase activity peak	% increase in paraoxonase with 10 mM Ca^{2+}	% increase in paraoxonase with activator solution	% increase in paraoxonase with 10 mM Ca^{2+} and activator solution in combination
63–73	+84.4*	N.S.	N.S.
74–107	+23.5†	N.S.	N.S.
108–123	N.S.	N.S.	N.S.
124–130	N.S.	N.S.	+86.0‡

N.S. = no significant change in paraoxonase activity compared to control.
* Significantly different from control at the $P < 0.01$ level.
† Significantly different from control at the $P < 0.02$ level.
‡ Significantly different from control at the $P < 1$ level.

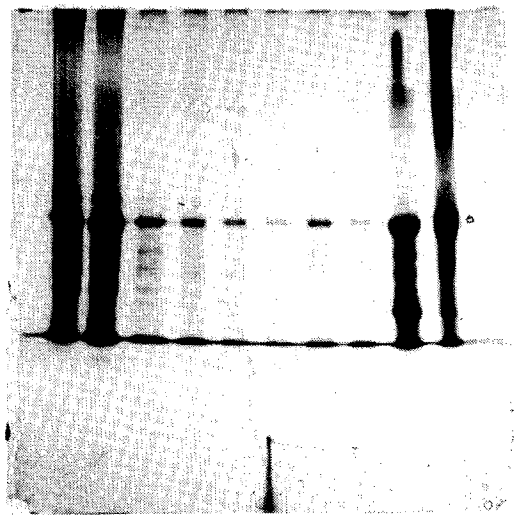

Fig. 2. Analysis of proteins in paraoxonase peaks from preparative electrophoresis. PAGE and protein staining are described in Materials and Methods. Lanes (left to right): (1) sheep serum; (2) lipoprotein fraction; (3) first preparative PAGE, first paraoxonase peak; (4) first preparative PAGE, second paraoxonase peak; (5) first preparative PAGE, third paraoxonase peak; (6) first preparative PAGE, fourth paraoxonase peak; (7) second preparative PAGE, second paraoxonase peak; (8) second preparative PAGE, third paraoxonase peak; (9) standard protein mixture (trypsinogen, bovine serum albumin and ovalbumin); (10) standard protein mixture (cross-linked bovine serum albumin with mol. wts 66,000, 132,000, 198,000 and 264,000).

Increases in activity were also observed after Ca^{2+} treatment of peak 67–73, and Ca^{2+}/activator treatment of peak 124–130, but these were not statistically significant. In a second column run which also gave four peaks of paraoxonase activity, three of the four peaks were activated by Ca^{2+} and the activator solution in combination. This suggests that the enzyme was deactivated by the column separation to a greater degree in the second run and strongly indicates the requirement for another activator apart from Ca^{2+}.

Analytical denaturing PAGE (Fig. 2) indicated that all four paraoxonase peaks had very similar composition. The predominant band in all the peaks was one running in the position expected of a protein with a mol. wt of 71,000. The peaks also contained several other bands of lower mol. wt ranging down to 18,000. The existence of several different protein units could merely be an indication of the impurity of these preparations. On the other hand, in view of the consistency of the picture in different fractions, it might indicate a subunit structure of a number of related proteins with 'A'-esterase activity.

DISCUSSION

The use of the lipoprotein fraction from sheep serum as the starting material for purification of 'A'-esterase followed by preparative electrophoresis provides a relatively simple method of purification which results in a recovery of 15% of the starting activity of paraoxonase and a 53-fold maximum purification (8.4-fold in the lipoprotein fraction with respect to serum and a further 6.3-fold in pooled peak 74–107). In the case of pirimiphos-methyloxonase, the recovery of activity (10%) and the purification factor (26-fold) with respect to the serum are lower than the corresponding values for paraoxonase. An interesting feature of this method of purification is that it appears to achieve some separation of multiple forms. Further work is needed to establish the purity of the paraoxonase peaks eluted by preparative PAGE and further purification may be necessary, e.g. by ion exchange chromatography. It should be noted that 'A'-esterase activity also occurs in the soluble fraction of sheep serum and this form of the enzyme needs to be further purified and compared with that in the lipoprotein fraction.

The results indicate that paraoxonase may require a cofactor other than Ca^{2+} and suggest that an enzyme–Ca^{2+}–activator complex may be required for optimum activity. Mounter and coworkers [4, 11] have also postulated that another 'A'-esterase, hog kidney DFP'ase,* required an enzyme–metal ion–cofactor complex for optimal activity. The predominant distribution of 'A'-esterase activity in the HDL fraction of sheep serum would suggest that 'A'-esterase is an HDL or HDL-associated enzyme (see later).

The differential distribution of paraoxonase, pirimiphosmethyloxonase and arylesterase activities in the soluble and total lipoprotein fractions of sheep serum, and the differential distributions of these activities in preparative PAGE suggests two things. Firstly, they indicate the presence of multiple forms of 'A'-esterase in sheep serum as has been previously suggested [12]. Secondly, it is clear from these results that 'A'-esterase activity should not be confused with arylesterase activity. The latter is measured by a non-specific assay which, in spite of the implication of its name, is not restricted to esterases which hydrolyse aryl esters.

Analysis of paraoxonase peaks from preparative PAGE by denaturing gel electrophoresis showed that the main protein band in all cases ran in the position expected for a protein of mol. wt of about 71,000. There were also minor components of apparently lower mol. wt in all cases, going down to about 18,000. The estimated mol. wt of the main component is very close to that of the core protein of HDL, which is estimated to be 74,000 [13]. Thus, on the basis of the restriction of activity to the HDL fraction, and the mol. wt of the main protein unit isolated by denaturing gel electrophoresis, it seems clear that the 'A'-esterase activity of serum lipoprotein is mainly associated with one or more species of HDL. The minor components could be either subunits produced by denaturation of the core protein, other proteins, or a combination of the two.

When sheep serum samples were run on a Sephadex G-200 column the mol. wt of the separated protein which showed paraoxonase activity was estimated to be $< 200,000$ [14]. Other workers report that A-type esterases from a variety of mammalian sera have mol. wts of the order of 350,000 [15].

* DFP'ase = diisopropyl fluorophosphatase (DFP = diisopropylfluorophosphate).

HDL_2 particles have mol. wts of approximately 360,000, compared with 150,000–180,000 for HDL_3 [13]. It therefore seems likely that 'A'-esterase activity is being expressed by one or more species of HDL_2 particle. The dependence upon Ca^{2+} for activation of 'A'-esterase in the present study could be explained on the basis of the structure of the HDL core protein. The core protein unit is believed to contain two A-I polypeptides and two A-II polypeptides, the integration of the whole unit depending upon the presence of diacyl phospholipids [13]. Ca^{2+} may be involved in the formation of bonds between phospholipid and protein structure within the core protein and the expression of 'A'-esterase activity may depend upon the integrity of the core protein. The action of the 'activator' solution on certain fractions remains purely speculative. It might be due to the presence of either a phospholipid or a peptide which is involved in the formation of the core protein.

Although much evidence suggests the existence of different species of lipoprotein, all expressing 'A'-esterase activity, but with different substrate specificities [14], some doubts must remain. It is still not clear whether the differences found between fractions after electrophoresis are genuine, or whether there are artefacts due to the partial modification of a single species of lipoprotein particle during separation. Further work is required to elucidate this problem.

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