Biochemical Pharmacology, Vol. 46, No. 7, pp. 1223–1227, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

POLYCLONAL ANTIBODY GENERATION IN RABBIT BY ADMINISTRATION OF AN ORGANOPHOSPHORUS ACID ANHYDROLASE (OPAA) FROM SQUID

FRANCIS C. G. HOSKIN,* DIANE M. STEEVES,† JOHN E. WALKER† and CHARLES T. WOODBURY†

Biology Department, Illinois Institute of Technology, Chicago, IL 60616; and †U.S. Army Research, Development and Engineering Center, Natick, MA 01760, U.S.A.

(*Received* 5 *January* 1993; *accepted* 1 *June* 1993)

Abstract—When a nerve gas hydrolyzing enzyme [organophosphorus acid anhydrolase (OPAA), formerly DFPase] purified from squid hepatopancreas was injected into rabbits, the resulting sera (RAS) inhibited OPAA purified from either squid hepatopancreas or squid optic ganglia. The inhibition was non-competitive, with 50% inhibition at a 1:1,000 serum dilution, and with the limit of inhibition (in effect, a "titer") at approximately 1:10,000. This RAS did not inhibit the distinctly different OPAAs from a mammalian and two bacterial sources. The hepatopancreas-generated RAS also reacted positively to the appropriate enzyme-linked immunosorbent assay (ELISA) at a titer of 1:100,000. In marked contrast, when OPAA purified from squid optic ganglion was injected into rabbits, the resulting sera did not inhibit squid OPAA, and did not give a positive ELISA. Control sera taken from the same rabbits prior to any injection (RS) did not inhibit the OPAAs. These results show another major difference between squid type OPAAs and the OPAAs from other sources, sometimes termed "Mazur type" OPAAs.

Since the first report of the enzymatic hydrolysis of diisopropylphosphorofluoridate (DFP‡) [1], it has become apparent that several enzymes with "overlapping specificities" [2] have been included under the term DFPase, now organophosphorus acid anhydrolase, or OPAA§ [3, 4]. From 1966 onward [5], evidence has accumulated that certain cephalopod tissues, notably squid nerve and hepatopancreas, are a rich and probably exclusive source of "squid type" OPAA with sharply defined properties that set it apart from all the other OPAAs, loosely termed "Mazur type" by us [6]. Briefly, the squid enzyme hydrolyzes DFP five times faster than another P-F compound, 1,2,2-trimethylpropyl methylphosphonofluoridate (Soman) (Mazur types, ratio reversed); is not stimulated by Mn^{2+} (Mazur types, many-fold stimulation); is not inhibited by 1,10-phenanthroline or 8-hydroxyquinoline-5-sulfonate (Mazur types inhibited); and has a molecular weight of about 30,000 (Mazur types, 50,000–150,000).

Despite our use of the term "Mazur type," this grouping displays major differences among the several prokaryotic and eukaryotic sources. For example, there is experimental evidence [7–9] and general agreement [3, 4] that the bacterial and mammalian diethyl *p*-nitrophenyl phosphate (Paraoxon)-hydrolyzing enzymes should not be included with the P-F splitting OPAAs. Minor differences even seem to have surfaced in the purification of squid type OPAA from *o*ptic *g*anglion and *h*epato*p*ancreas of the *e*ast *c*oast squid, *Loligo pealei* (hence ECOG and ECHP), and the *w*est *c*oast squid, *Loligo opalescens* (hence WCOG and WCHP).

The narrow distribution of squid type OPAA, particularly in the squid giant axon [10], has sometimes suggested a physiological role [see, for example, Refs 11 and 12] for this seemingly functionless enzyme. A potent and specific inhibitor would provide an obvious approach. There is also the potential for benefits applicable to the disposal of aging munitions stores. This would require a scale of production that could only be achieved by expression of the gene encoding the enzyme. For both of these purposes, antibodies to the enzyme would provide an invaluable tool for the isolation of this gene. Evidence of our success in producing such antibodies is the subject of this report.

MATERIALS AND METHODS

Enzymes and enzyme measurements. Purification of squid type OPAA [13, 14] from ECOG ECHP, WCOG and WCHP has been improved by the further use of gel filtration resin G-150, anion

* Correspondence should be sent to Dr. Francis Hoskin at his present address: Marine Biological Laboratory, Woods Hole, MA 02543. Tel. (508) 548-3705; FAX (508) 540-6902.

‡ Abbreviations and trivial names: DFP, diisopropylphosphorofluoridate; OPAA, organophosphorus acid anhydrolase (formerly DFPase); Soman, 1,2,2-trimethylpropyl methylphosphonofluoridate; Paraoxon, diethyl *p*-nitrophenyl phosphate; ECOG, ECHP, WCOG, and WCHP, optic ganglia and hepatopancreas from east coast squid, *Loligo pealei*, and west coast squid, *Loligo opalescens*; RS, rabbit serum; RAS, rabbit antibody-containing serum; OT, a specific strain of the obligate thermophilic microorganism, *Bacillus stearothermophilus*; and ELISA, enzyme-linked immunosorbent assay.

§ The recommendation for numbering will probably be for either EC 3.1.8.1 or EC 3.1.8.2. Lack of evidence about a natural substrate continues to be an impediment.

exchange resin A-50 (both Pharmacia), and ultrafiltration membrane PM-10 (Amicon) [15]. Judging by gel electrophoresis, the squid enzymes routinely appear to be about 50% pure. Methods of measurement of OPAA activity by means of the fluoride-sensitive electrode have been published frequently from this laboratory and have been summarized in a recent chapter [6]. When Mn^{2+} was added—particularly in measurements involving the non-squid enzyme sources—buffers, enzymes, sera, and Mn^{2+} were combined by dilution and with maintenance on ice in such a manner that the several components were in contact for about 15–20 min prior to addition of substrate. The final concentration of Mn^{2+} was approximately 10^{-3} M, and the intermediate concentration was not more than twice that.

Sera and antisera. These were produced by Zoin Research, Brighton, MA, typically in the following manner. Control serum was obtained from a male New Zealand white rabbit. The rabbit was then injected with 0.25 mL OPAA from ECHP, 0.35 mg protein and 3.16 U/mL (1 unit = 1 μmol DFP hydrolyzed per min at a [DFP] of 3 mM) in Freund's complete adjuvant, injected again in 3 weeks, and 3 weeks after that with enzyme in incomplete adjuvant. A week later a test bleed was taken, and the rabbit was again injected with enzyme in incomplete adjuvant. A week later a final bleed was taken. Thus, there were, from several rabbits, several control sera (RS), and several potentially antibody-containing sera (ECHP-RAS and ECOG-RAS).

These sera were tested for their effects on the OPAA activity of the two sensitizing enzymes, ECHP and ECOG, and also of the two other squid type OPAAs, namely WCHP and WCOG. They were also tested against "Mazur type" OPAAs purified from hog kidney [16], *Escherichia coli* [17] and the strain of *Bacillus stearothermophilus* termed OT [15, 18]. These enzymes were estimated to be about 25% pure.

Enzyme-linked immunosorbent assay (ELISA). These assays were performed by the indirect method [19], in which antigen (ECHP) is bound to a polystyrene plate (Costar), ECHP-RAS or ECOG-RAS is added next, and goat anti-rabbit IgG labeled with alkaline phosphatase (Sigma) is added, followed by *p*-nitrophenyl phosphate (Sigma). The amounts of these ingredients and the dilutions were varied to obtain a standard curve and finally a titer of the presumed antibody in the rabbit serum.

RESULTS

Table 1 shows that serum from ECHP-inoculated rabbits (ECHP-RAS) inhibited the enzymatic hydrolysis of DFP or of Soman by any of the four squid type OPAAs (ECHP, ECOG, WCHP, WCOG). The results of nine determinations with DFP have been averaged, and the results of eight determinations with Soman have been averaged because there were no significant differences among the four squid type OPAAs. That is, while the rates of DFP and Soman hydrolysis by squid type OPAA differed (this being a defining property of the squid enzyme), the percentages of inhibition caused by comparable amounts of ECHP-RAS were the same. Rabbit serum prior to inoculation (RS) caused no inhibition. Rather unexpectedly, serum from two ECOG-inoculated rabbits also caused no inhibition of DFP hydrolysis either by the same ECOG enzyme that was used for the rabbit inoculation or by the other squid type OPAAs. The 4% *stimulation* (−4% inhibition in the terminology of Table 1) was insignificant. It may be seen that fewer (or even no) measurements were made with Soman if the point seemed sufficiently demonstrated with DFP. This was due to the much greater hazard involved in using Soman [see, for example, Ref. 6].

Table 1 also shows that two "Mazur type" OPAAs—those from hog kidney and *E. coli*—were not inhibited by ECHP-RAS. While these two sources preferentially hydrolyze Soman, they do hydrolyze DFP at measurable rates, even without added Mn^{2+} [6]. Thus, as shown, they were tested with both substrates, with and without Mn^{2+}. While the hydrolytic rates differed in each combination, the lack of inhibition appeared consistent. If the results of these eight determinations had been averaged, there would have appeared to be an insignificant 5(±10)% *stimulation.*

The response of the OPAA purified from *B. stearothermophilus* (OT) was distinctly different. This is also shown in Table 1. Here, of necessity, the OT-OPAA could only be tested with Soman as substrate, and in the presence of Mn^{2+} [18]. Again, because of the hazard of using Soman, a minimum number of experiments was performed, but the results seem clear. First, the hydrolysis of Soman by this thermophile-derived OPAA was not inhibited by ECHP-RAS. Second, and rather unexpectedly, the OT-OPAA was more than 100% *stimulated* by ECHP-RAS. And third, the same degree of stimulation was seen when control serum (RS) was used.

All of these observations require some additional explanation. It has long been known that mammalian blood hydrolyzes DFP and several other organophosphorus compounds [2]. This is also true of rabbit serum. RAS and RS hydrolyze Soman about five times faster than DFP. By this criterion these rabbit serum OPAAs are "Mazur type" in our terminology. The presence of these additional OPAAs in RAS and RS has complicated the means of obtaining the results presented in Table 1. This is illustrated in the following example. In one experiment, DFP hydrolysis by ECHP was 1.595 ($\Delta[F^-] = 1.595 \times 10^{-5}$ M min^{-1}, linear for 10 min, in a [DFP] of 3 mM and a reaction volume of 5 mL). DFP hydrolysis by RAS was 0.380; by ECHP and RAS mixed together for 10 min before addition of DFP, 0.655. The assumption is that squid type OPAA and the OPAA in RAS (or RS) will not inhibit each other. The question is whether something else in rabbit serum will inhibit squid OPAA. The calculation is:

$$\frac{0.655 - 0.380}{1.595} = 0.172,$$

i.e. 17% activity remaining, or 83% inhibition.

Table 1. Effects of rabbit antiserum (RAS) and control serum (RS) on organophosphorus acid anhydrolases (OPAAs) purified from squid and other sources

Rabbit serum type	Source of OPAA	Substrate for OPAA	Inhibition (%)
ECHP-RAS	Squid*	DFP	77 ± 11 (9)
ECHP-RAS	Squid*	Soman	79 ± 11 (8)
RS	Squid*	DFP	−2 ± 8 (6)
ECOG-RAS†	Squid*	DFP	−4 ± 17 (4)
ECHP-RAS	Hog kidney	DFP; Soman	−8; 0
ECHP-RAS‡	Hog kidney‡	DFP; Soman	0; 2
ECHP-RAS	*E. coli*	DFP; Soman	−8; 9
ECHP-RAS‡	*E. coli*‡	DFP; Soman	−19; −17
ECHP-RAS‡	*B. stearo. (OT)*§	Soman§	−143; −112
RS‡	*B. stearo. (OT)*§	Soman§	−106

* Results with two rabbits and four squid OPAA sources (ECHP, ECOG, WCHP, WCOG) have been averaged since no significant or consistent trends were evident. Values are means ± SD, with the number of determinations given in parentheses.

† In view of the results, this should be regarded as *tentative* RAS.

‡ Mn^{2+} added.

§ Does not hydrolyze DFP; Mn^{2+} requiring [15, 18].

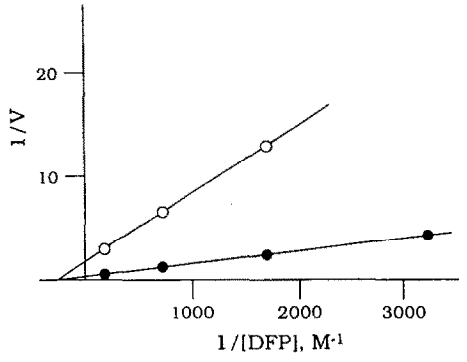

Fig. 1. Reciprocal plot of activity of organophosphorus acid anhydrolase (OPAA) purified from hepatopancreas of east coast squid, *L. pealei* (ECHP), as a function of substrate (DFP) concentration. Key: (●) OPAA alone; and (○) OPAA in the presence of ECHP-generated rabbit antibody-containing serum (RAS). The former (●) was a direct observation; the latter (○) was derived according to the example given in the text. Ordinate scale: 10 = increase in $[F^-]$ of 0.1×10^{-5} M per min in a 5-mL reaction volume.

Because the response of the OT OPAA was rather extreme, an example of that calculation is also given:

$$\frac{1.74 - 0.28}{0.71} = 2.06,$$

i.e. 206% of OT alone, or 106% *stimulation*.

These three determinations were made for each experiment. By dilutions of the various OPAA and rabbit serum sources, with allowance made for the inherently different rates of hydrolysis of the two substrates, with and without Mn^{2+}, values were obtained that stayed reasonably well within the limits shown in the two examples, but for any one set—the first line of Table 1, for example—dilutions, volumes, etc., were kept constant.

Figure 1 shows that the inhibition of ECHP OPAA by ECHP-RAS with DFP as the substrate was probably non-competitive. Only three points are shown for the upper line (OPAA + RAS) because, although the determination at a substrate concentration of 3×10^{-4} M was attempted, the activity was too small to be reliable. The K_m for the uninhibited enzyme was well within the range found repeatedly in this laboratory. While a K_i cannot be given for the ECHP-RAS, Table 2 shows that 50% inhibition was attained at approximately a 1:1,000 dilution of the serum; it may be speculated from the data in Table 2 that the limit of inhibition of the ECHP by the ECHP-RAS would provide a "titer" of 1:10,000.

Table 2. Inhibition of ECHP OPAA as a function of dilution of ECHP-RAS*

Dilution factor	Inhibition (%)
1:200	83
1:2,000	30
1:20,000	3

* See Table 1 for abbreviations.

The term titer is more properly applied to the degree of dilution that will still permit the detection of antibody. By means of ELISA this was at an ECHP-RAS dilution of 1:100,000, as shown in Fig. 2. When the same determinations were attempted with sera or the IgG fractions from two ECOG-inoculated rabbits, it was not possible to obtain a standard curve (ECOG on plate, variable ECOG and constant presumed ECOG-RAS or IgG fraction mixed separately and then applied to plate) and thus, in parallel with the lack of inhibition of squid OPAA by sera from ECOG-inoculated rabbits (see Table 1), no titer comparable to that shown in Fig. 2 was determinable for these sera.

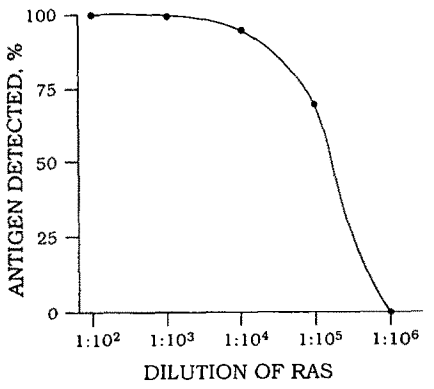

Fig. 2. Indirect enzyme-linked immunosorbent assay (ELISA) of antibody in rabbit serum (RAS) after injection of organophosphorus acid anhydrolase (OPAA) purified from hepatopancreas of east coast squid, *L. pealei* (ECHP). In the indirect method [19], a constant amount of antigen (ECHP OPAA) is bound to the assay plate, and the greatest dilution of RAS that can still detect antigen, in this case 1:100,000, is a measure of the concentration of antibody.

DISCUSSION

Antibodies to squid type OPAA have been produced by the injection of this enzyme purified from hepatopancreas of *L. pealei* (ECHP) into rabbit. The antibody-containing serum, ECHP-RAS, inhibited the enzymatic activity of the OPAA antigen, ECHP, at RAS dilutions in the 10^3- to 10^4-fold range. The ECHP-RAS also inhibited OPAA from optic ganglion (ECOG) and from these two organs in another species, *L. opalescens* (WCHP, WCOG). ECHP-RAS did not inhibit the OPAAs of hog kidney or *E. coli*, or of *B. stearothermophilus (OT)*. Rather unexpectedly, this OT enzyme was stimulated markedly (100%+) by both ECHP-RAS and by RS alone, suggesting that the stimulation was caused by a component of rabbit serum other than the ECHP antibodies. In view of some distinctive properties of the OT enzyme [18], this stimulation warrants further investigation.

Antibody production by ECHP was also confirmed by the positive ELISA reaction at ECHP-RAS dilutions in the 10^4- to 10^5-fold range.

In two attempts, ECOG did not elicit antibodies in rabbit although the antigen, ECHP, and the potential antigen, ECOG, had been purified to similar specific activities. Indeed, this level of purification is attained more easily with ECOG because hepatopancreas starts with a much higher content of other proteins and pigmented materials. On the other hand, it is generally known that nerve tissue is rich in phospholipids, some of which have carbohydrate moieties that may interfere with antibody generation.

The results presented here further confirm the marked difference between the P-F splitting enzyme termed squid type OPAA [6] and a broad selection of other OPAAs. The discovery of a specific inhibitor for the squid enzyme may have a bearing on the question of its function in such a highly specialized tissue as the squid giant axon [5, 10].

Acknowledgements—We are indebted to Dr. K. S. Rajan and the Illinois Institute of Technology Research Institute for encouragement and financial support. We also thank the Academy of Applied Science for providing support, through their Research and Engineering Apprenticeship Program (REAP), for Carol L. Chappell, who was involved in the collection of squid organs from which enzymes were purified.

REFERENCES

1. Mazur A, An enzyme in animal tissue capable of hydrolyzing the phosphorus-fluorine bond of alkyl fluorophosphates. *J Biol Chem* **164**: 271–289, 1946.
2. Mounter LA, Metabolism of organophosphorus anticholinesterase agents. In: *Handbuch der experimentallen Pharmakologie: Cholinesterases and Anticholinesterase Agents* (Ed. Koelle GB), pp. 486–504. Springer, Berlin, 1963.
3. Aldridge WN, Hoskin FCG, Reiner E and Walker CH, Suggestions for a nomenclature and classification of enzymes hydrolyzing organophosphorus compounds. In: *Enzymes Hydrolyzing Organophosphorus Compounds* (Eds. Reiner E, Hoskin FCG and Aldridge NW), pp. 246–253. Ellis Horwood, Chichester, England, 1989.
4. Walker CH, The classification of esterases which hydrolyze organophosphates: Recent developments. *Chem Biol Interact*, in press.
5. Hoskin FCG, Rosenberg P and Brzin M, Re-examination of the effect of DFP on electrical and cholinesterase activity of squid giant axon. *Proc Natl Acad Sci USA* **55**: 1231–1234, 1966.
6. Hoskin FCG, An organophosphorus detoxifying enzyme unique to squid. In: *Squid as Experimental Animals* (Eds. Gilbert DJ, Adelman WJ Jr and Arnold JM), pp. 469–480. Plenum Press, New York, 1990.
7. Hoskin FCG, Possible significance of "DFP-ase" in squid nerve. *Biol Bull* **137**: 389–390, 1969.
8. Pogell BM, Rowland SS, Steinmann KE, Speedie MK and Hoskin FCG, Genetic and biochemical evidence for the lack of significant hydrolysis of soman by a *Flavobacterium* parathion hydrolase. *Appl Environ Microbiol* **57**: 610–611, 1991.
9. Adkins S, Gan KN, Mody M and LaDu BN, Molecular basis for the polymorphic forms of human serum paraoxonase/arylesterase: Glutamine or arginine at position 191, for the respective A or B allozymes. *Am J Hum Genet* **52**: 598–608, 1993.
10. Hoskin FCG, Distribution of diisopropylphosphorofluoridate hydrolyzing enzyme between sheath and axoplasm of squid giant axon. *J Neurochem* **26**: 1043–1045, 1976.
11. Hoskin FCG and Kordik ER, Hydrogen sulfide as a precursor for the synthesis of isethionate in the squid giant axon. *Arch Biochem Biophys* **180**: 583–586, 1977.
12. Hoskin FCG and Noonan PK, Taurine and isethionate in squid nerve. In: *Natural Sulfur Compounds* (Eds. Cavallini D, Gaull GE and Zappia V), pp. 253–263. Plenum Press, New York, 1980.
13. Hoskin FCG and Long RJ, Purification of a DFP-hydrolyzing enzyme from squid head ganglion. *Arch Biochem Biophys* **150**: 548–555, 1972.
14. Garden JM, Hause SK, Hoskin FCG and Roush AH, Comparison of DFP-hydrolyzing enzyme purified from head ganglion and hepatopancreas of squid (*Loligo pealei*) by means of isoelectric focusing. *Comp Biochem Physiol* **52C**: 95–98, 1975.

15. Hoskin FCG, Chettur G, Mainer S, Steinmann KE, DeFrank JJ, Gallo BJ, Robbins FM and Walker JE, Soman hydrolysis and detoxication by a thermophilic bacterial enzyme. In: *Enzymes Hydrolyzing Organophosphorus Compounds* (Eds. Reiner E, Hoskin FCG and Aldridge NW), pp. 53–64. Ellis Horwood, Chichester, England, 1989.
16. Storkebaum W and Witzel H, Study on the enzyme catalyzed splitting of triphosphates. *Forschungsber Landes Nordrhein-Westfalen* No. 2523: 1–22, 1975.
17. Hoskin FCG, Gallo BJ, Steeves DM and Walker JE, Stereoselectivity of Soman detoxication by organophosphorus acid anhydrases from *Escherichia coli*. *Chem Biol Interact*, in press.
18. Chettur G, DeFrank JJ, Gallo BJ, Hoskin FCG, Mainer S, Robbins FM, Steinmann KE and Walker JE, Soman-hydrolyzing and -detoxifying properties of an enzyme from a thermophilic bacterium. *Fundam Appl Toxicol* **11**: 373–380, 1988.
19. Monroe D, Enzyme immunoassay. *Anal Chem* **56**: 920A–931A, 1984.