Chemico-Biological Interactions 345 (2021) 109524

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

DAEH N-terminal sequence of avian serum albumins as catalytic center of Cu (II)-dependent organophosphorus hydrolyzing A-esterase activity

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ARTICLE INFO

Keywords:
Albumins
Phosphoramidates
Copper
A-esterases
Stereoselectivity
Hydrolysis

ABSTRACT

O-hexyl O-2,5-dichlorophenyl phosphoramidate (HDCP) induces delayed neuropathy. The R (+)-HDCP inhibits and caused the so call "aging reaction" on inhibited-NTE. This enantiomer is not hydrolyzed by Ca(II)-dependent A-esterases in mammal tissues but is hydrolyzed by Cu(II)-dependent chicken serum albumin (CSA). With the aim of identifying HDCP hydrolysis by other vertebrate albumins, we incubated albumin with 400 μM racemic HDCP in the presence of 100 μM copper sulfate. HDCPase activity was assessed by measurement of HDCP with chiral chromatography. Human, sheep, dog, pig, lamprey or cobra serum albumin did not show a significant activity (~10%). Rabbit and bovine albumins hydrolyzed both enantiomers of HDCP (25% and 50% respectively). Turkey serum albumin had more HDCPase activity (~80 μM remaining) than the chicken albumin (~150 μM remaining). No animal albumins other than chicken showed stereoselective hydrolysis. Preincubation of chicken albumin with 1 mM the histidine modifying agents, 100 μM N-bromosuccinimide (NBS) and Zn(II), inhibited its Cu(II)-dependent R (+)-HDCPase activity, where as other mM amino acids modifiers had no inhibitory effects. . These results confirm that the stereoselective hydrolysis of (+)-HDCP is a specific A-esterase catalytic property of chicken albumin. The higher HDCPase activity by turkey albumin suggests the amino-terminal sequence of avian albumins (DAEHK) is the active center of this Cu(II)-dependent A-esterase activity.

1. Introduction

O-hexyl O-2,5-dichlorophenyl phosphoramidate (HDCP) is a racemic compound that induces organophosphate induced delayed polyneuropathy (OPIDP) in hen. This compound has an asymmetric center in the phosphorus atom, therefore it has two enantiomers. The racemic HDCP was designed [1] with the aim to understand the biochemical acute neurotoxicity associated with the inhibition of acetylcholinesterase (AChE) and the mechanisms of OPIDP, which has been associated with inhibition and aging of neuropathy target esterase (NTE). A first ex vivo study showed that the racemic mixture of this phosphoramidate is more potent inhibitor of chicken brain NTE than AChE, but did not age it [1,2]. However, a second study in vivo using hens shown the OPIDP effect of the same racemic mixture of HDCP throught inhibition and aging of the brain NTE. More late, this experimental controversy was clarified by means of an ex vivo study using HDCP enantiomers obtained by a chiral chromatography method [3,4] and identified by optical and polarimetric analyzes. They reported that the R (+)-HDCP is the less potent inhibitor of hen brain NTE but it is able to age it. With this result was established the stereoselective hypothesis: "The R (+)-HDCP is the enantiomer that survive to systemic metabolism to induce the neurotoxic effects". With the purpose of demonstrating this hypothesis, were incubated different avian and mammal tissues, including human serum with racemic HDCP or its enantiomers in the presence of physiological concentration of calcium. All tissues tested shown a significant stereoselective hydrolysis of S (−)-HDCP) [5,6] Monroy_Noyola et al., 1999b) [7], meanly rabbit [8] and human serum [9]. These results reinforces the stereoselective neurotoxic hypothesis of R (+)-HDCP. On the other hand, the racemic HDCP has been used as a tool chemical to looking for other A-esterasa systems non-calcium dependent in animals' tissues. Recently, our research group has reported the Cu(II)-dependent

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https://doi.org/10.1016/j.cbi.2021.109524
Received 24 March 2021; Received in revised form 1 May 2021; Accepted 16 May 2021
Available online 20 May 2021
0009-2797/

Abbreviations

OPs	organophosphorus compounds
PTEs	phosphotriesterases, PTEs
NTE	neuropathy target esterase
HDCP	O-hexyl O-2,5-dichlorophenyl phosphoramidate
HDCPase	O-hexyl O-2,5-dichlorophenyl phosphoramidate hydrolyzing activity
AChE	acetylcholinesterase
OPIDP	organophosphate-induced delayed polyneuropathy
p-NPB	*p*-nitrophenyl butirate
EMM	N-etylmaleimide
DCC	*N*,*N*′-dicyclohexylcarbodiimide
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
DFP	diisopropyl fluorophosphate
paraoxon, PXN	diethyl 4-nitrophenyl phosphate
PA	phenyl acetate
CSA	chicken serum albumin
TSA	turkey serum albumin
HSA	human serum albumin
SSA	sheep serum albumin
DSA	dog serum albumin
PSA	pig serum albumin
RSA	rabbit serum albumin
BSA	bovine serum albumin
CoSA	cobra serum albumin
HPLC	high performance liquid chromatography

hydrolysis of R (+)-HDCP in chicken serum [10], which is about 20-fold greater and opposite to that observed in the presence of Ca(II) in several avian tissues, for this reason it has been called "antagonistic stereoselectivity". Finally, an ex vivo study done by chiral chromatography using different commercial animal cuproproteins and racemic mixture of HDCP revealed that the chicken serum albumin is the protein responsible for stereospecific Cu(II)-dependent R (+)-HDCP hydrolysis in the chicken serum [11]. This study shows the Cu(II)-dependent hydrolysis of racemic HDCP by commercial albumins from avian and mammals serums, mainly.

2. Materials and methods

2.1. Chemicals

HDCP (O-hexyl O-2,5-dichlorophenyl phosphoramidate, purity ca. 95%) was supplied by Dr. Naumman (Bayer Chemical Company, Germany). Chicken serum albumin (CSA) human serum albumin (HSA), sheep serum albumin (SSA), dog serum albumin (DSA), pig serum albumin (PSA), rabbit serum albumin (RSA), bovine serum albumin (BSA), turkey serum albumin (TSA), copper sulfate, zinc sulfate, phenyl acetate (PA), *p*-nitrophenyl butirate (*p*-NPB), N-etylmaleimide (EMM), *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-bromosuccinimide (NBS), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), diisopropyl fluorophosphate (DFP) and diethyl 4-nitrophenyl phosphate (PXN) were purchase Sigma-Aldrich Company. Lamprey serum albumin (LSA) with purity 98% was provided by the Dr. Russell F. Doolittle from Biochemistry Laboratory, University of California, San Diego. Cobra serum albumin (CoSA) with purity 98% was donated by the Dr. Jingyu Shao from Biochemistry and Molecular Laboratory, Zhejiang Chinese Medical University (China). Hexane, 1,2-dichloroethane and ethanol HPLC grades came from Scharlau Chemie S.A.

2.2. HDCP hydrolysis by animal serum albumins

HDCP stereoselective hydrolysis was quantified by the chiral HPLC method previously developed [4]. Briefly, it designated the R (+)-HDCP and S (−)-HDCP enantiomers, which were eluted in first and second places from the chromatographic system, based on a polarized light study with HDCP pure enantiomers (99.5%) isolated by the same chromatographic procedure. According to the Cahn-Ingold-Prelog nomenclature rules, the specific R/S configuration was established by comparing it with the properties of NTE phosphorylated with R (+)-*O*-n-hexyl-S-methylphosphorothioamidate [3]. The quantification of the remaining concentration of both the HDCP enantiomers from this chiral HPLC method was previously used by our research group in enzymatic studies into hydrolysis [5,9–11]. Ten microliters of buffer containing 216 μg of each animal serum albumin were incubated with 400 μM of the HDCP racemic mixture and 100 μM of copper sulfate. The total volume of the reaction was 1 mL which was stopped by adding 40 μL of 0.2 M HCl. The released residual HDCP was removed by liquid-liquid extraction with 2 mL of 1,2-dichloroethane. Afterward this extract was centrifuged at 1000 g for 15 min. The extraction yield was over 98% in all cases. Twenty five μL of the organic solvent extract were finally injected into the HPLC system with an OA-4100 Techocel chiral column (HPLC Technology, Macclesfield, UK).

2.3. Effect of amino acid modifier compounds on the hydrolysis of HDCP by CSA and TSA

In order to suggest the amino acids involved in the Cu^{2+}-dependent stereospecific hydrolysis of R (+)-HDCP by the CSA, 216 μg of CSA were preincubated in 1 mL with different concentrations (100 μM or 1 mM) of amino acid modifier compounds as EMM and DTNB (cysteines), DCC (aspartic and glutamic) and NBS (histidine), as well as with other compounds that bind to specific residues in animal serum albumins which included PXN, PA, *p*-NPB, DFP (Tyr 411) and zinc (His 4) for 15 min. Later, they were incubated with an aliquot 400 μM of racemic HDCP in the presence of 100 μM of copper sulfate for 60 min at physiological conditions of pH and temperature. The reaction volume was 1 mL. In the case of TSA, it was only pre-incubated with 100 μM of NBS or zinc sulfate.

2.4. Sequencing of the N-terminal end of the TSA

Since TSA also hydrolyzed HDCP in the presence of copper sulfate, the first 10 amino acids of its N-terminal sequence of this animal albumin were made using the "The Procise" method. It was performed by a contracted external service with *Laboratorio de Química de Proteínas, Consejo Superior de Investigaciones Científicas* (CSIC), Madrid, Spain. This sequence was checked with the N-terminal sequence predicted by computational analysis from a genomic sequence of *Meleagris gallopavo* (turkey) reported in Documentation of NCBI's Annotation Process [12].

2.5. Statistical analysis

The remaining concentrations of HDCP enantiomers of each animal serum albumin in the presence of copper (experimental groups) versus remaining HDCP isomers incubated only with copper (control group) were analyzed by the one-way ANOVA statistical test. While that the comparison between remaining concentrations of HDCP isomers of the same experimental group was carried out through the Student's t-test. $P < 0.05$ was used as a value of statistical significance. These analyzes were carried out with the SPSS v.19 software and the figure plots were designed with the Sigma Plot v.12 software.

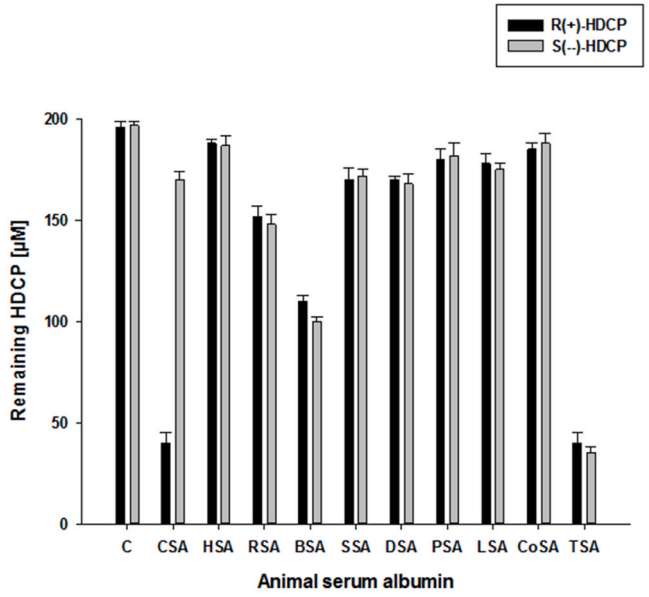

Fig. 1. Copper-dependent hydrolysis of HDCP by animal serum albumins. The values represent the average and SD of three experiments performed with 216 μg of serum albumin from chicken (CSA), human (HAS), rabbit (RSA), bovine (BSA), sheep (SSA), dog (DSA), pig (PSA), lamprey (LSA), cobra (CoSA) and turkey (TSA) in 1 mL were incubated with 400 μM of racemic HDCP and 100 μM of copper sulfate at pH 7.4, 37 °C for 60 min. The 400 μM of racemic HDCP and 100 μM of copper were incubated without albumin as control group (C). *p˂0.05 (ANOVA). **R (+)- HDCP vs S (−)-HDCP, p˂0.05 (t-Student).

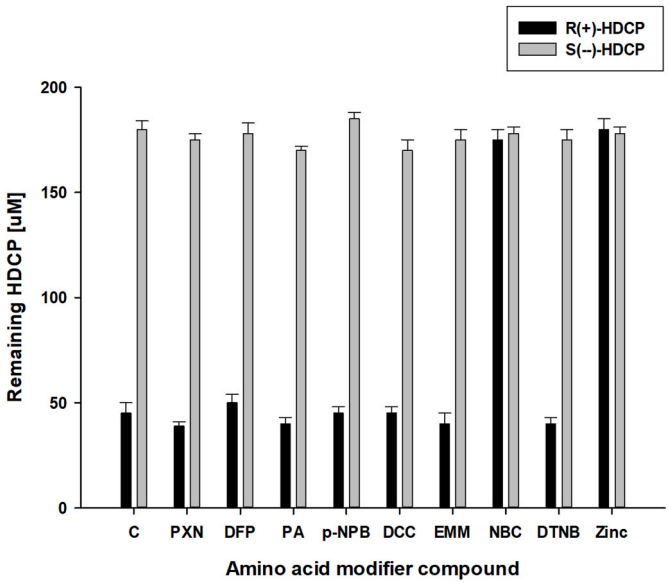

Fig. 2. Effect of amino acid modifier compounds on the "contrary stereospecificity" The bars represent the mean ± SD of three experiments performed with 216 μg of CSA pre-incubated 15 min in 1 mL with 1 mM of PXN, DFP, PA, *p*-NPB, DCC, EMM, DTNB or 100 μM of NBS or Zinc and incubated with 400 μM racemic HDCP and 100 μM copper for 30 min at 37 °C and pH 7.4. Control group (C) correspond to CSA without pre-incubation.

3. Results

3.1. Copper-dependent hydrolysis of racemic HDCP by animal serum albumins

Commercial and available animal serum albumins were incubated with racemic 400 μM HDCP in the presence of 100 μM copper sulfate. As shown in Fig. 1, the CSA was the only tested animal serum albumin that stereoselectivity hydrolyzed R (+)-HDCP ˃ S (−)-HDCP. The R (+)-HDCP was significantly (p˂0.05) more hydrolyzed (80%) (40 μM remaining) than its corresponding form S (−)-HDCP that was hydrolyzed 14% (remaining 172 μM). The total level of HDCP hydrolysis (both enantiomers) by CSA was at 47% (remaining 212 μM). While, RSA and BSA showed 25% and 47% of total hydrolysis, (remaining 300 μM and 210 μM, respectively). These non-stereospecific activities were lower than CSA but statistically significant (p˂0.05) with respect to the control group (C) (racemic HDCP + copper). The HSA, SSA, DSA, PSA, LSA and CoSA serum albumin did not show significant hydrolysis of the racemic mixture of HDCP. Their activity levels were around 10% for each enantiomer (Remaining ~180 μM for each enantiomer). The TSA had the highest total level of copper-dependent hydrolysis of HDCP of all the tested animal serum albumins, showing 80% hydrolysis for each isomer of the racemic mixture (p˂0.05). These residual HDCP enantiomers (remaining 160 μM for each one) were similar to that obtained for the R (+)-HDCP isomer hydrolyzed by CSA in the presence of 100 μM copper sulfate.

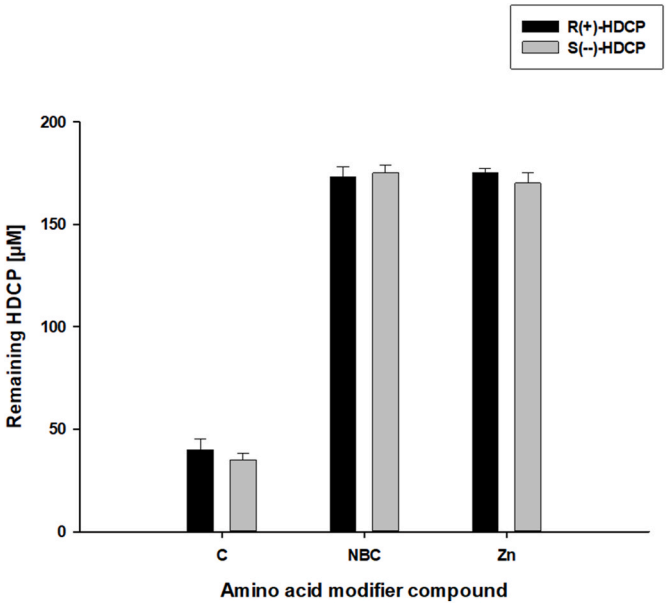

Fig. 3. Effect of *N*-bromosuccinimide (NBSNBC) and zinc sulfate (Zinc) on the Cu^{2+}-dependent hydrolysis of HDCP by TSA. The bars represent the mean ± SD of three experiments performed with 216 μg of TSA pre-incubated 15 min in 1 mL with 100 μM of NBS or Zinc and incubated with 400 μM racemic HDCP and 100 μM copper for 30 min at 37 °C and pH 7.4. Control group (C) correspond to TSA without pre-incubation.

3.2. Effect of amino acid modifier compounds on copper-dependent hydrolysis of racemic HDCP by CSA and TSA

With the purpose to suggest the amino acids involved in the Cu^{2+}-dependent activity, CSA was pre-incubated with different amino acid modifier compounds before to be incubated with racemic HDCP and copper sulfate. Fig. 2 shows that pre-incubation of CSA with 1 mM of paraoxon, DFP, PA, *p*-NPB, EMM, DCC or DTNB did not inhibited the CSA copper-dependent stereoselective hydrolysis. The hydrolysis of R (+)- HDCP was 5 times higher than S (−)-HDCP independently of the amino acid modifier compound pre-incubated. In contrast, 100 μM of NBS or zinc sulfate inhibited 100% the copper-dependent stereoselective hydrolysis of R (+)-HDCP by CSA. This same inhibitor effects of NBS and zinc sulfate was observed on the Cu^{2+}-dependent hydrolysis of both HDCP isomers by TSA in the presence of copper (Fig. 3).

Table 1
N-terminal sequences of serum albumins from several species
The N-terminal chicken-turkey peptide has: DAEHK and follows SE. It can be interpreted either as (a) human, rabbit, bovine, pig, and dog have a deletion of E, or (b) that is inserted E into chicken and turkey that was not in the others. The N-terminal zone from 1 to 31 aa in the preprotein are the same in chicken as in turkey, except that aa 24 is F in chicken and V in turkey, which corresponds to the nucleotide sequence at position 70 is T in chicken and G in turkey. In the peptide of interest the aa sequence is the same in chicken as in turkey (DAEHK at positions 27 to 40) but there is a difference in the nucleotide sequence at position 27 of the aa D that in chicken is GAT-encoded and in turkey by GAC. An appropriate comparison of chicken and turkey albumins with other species, would be as follows (Detail information of some sequences are showed in Supl. Material Document 1 and 2).

ESPECIE	SEQUENCE							NCBI ProteinACCESSION NUMBER[a]	REFERENCE (other than NCBI)
Chicken	**D**	**A**	**E**	**H**	**K**	**S**	**E**	NM_205261,2 NP_990592.1	Cassady et al., 1991
Turkey	**D**	**A**	**E**	**H**	**K**	**S**	**E**	XM_003205677.1 XP_010707950.1	Monroy-Noyola et al. (this paper)
Human	**D**	**A**		**H**	**K**	**S**	**E**	AAA98797.1, NP_000468.1, 4LB9_A, 4LB2_B	Minghetti et al., 1986
Rabbit	E	**A**		**H**	**K**	**S**	**E**	3V09_A, NP_001075813.1	Syed et al., 1997
Sheep	**D**	T		**H**	**K**	**S**		NP_001009376.1, 5ORF_A	Brown et al., 1989
Bovine	**D**	T		**H**	**K**	**S**		CAA76847.1, NP_001075972.1	Holowachuk, 1991
Pig	**D**	T		Y	**K**	**S**		AAT98610.1	Weinstock y Baldwin, 1988
Dog	E	**A**		Y	**K**	**S**	**E**	AAB32128.1, CAB64867.1	Holowachuk, 1993
Rat	E	**A**		**H**	**K**	**S**	**E**	AAH85359.1	NCBI
Cobra[b]		T	S	S	T	G		S59517	Havsteen et al., 1994
Lamprey[b]		E	D	E	S	F		AAA49271.1	Gray y Doolittle, 1992

[a] Multiple entries can be found for each protein in NCBI protein data bank. Accession number of some example of sequences which have been checked are shown.

[b] Sequence is so different that it cannot be alienated with the other sequences.

3.3. Sequencing of the amino-terminal sequence of the animal serum albumins

In order to support the copper-dependent A-esterase effect observed meanly in CSA and TSA in this study, a bibliographic review was carried out of the N-terminal amino acid sequences of the tested animal serum albumins associated with Cu^{2+} binding [12]. The TSA albumin N-terminal sequence done in this study was stablished and also checked with the N-terminal sequence predicted from a genomic sequence as indicated in Material and Methods of *Meleagris gallopavo* (turkey) reported in Documentation of NCBI's Annotation Process [13]. Table 1 showed the amino-terminal sequence of ten animal albumins used in this study. In Supplementary Material Document 1 details of the albumin sequences are showed.

It was concluded that the TSA has the same amino-terminal sequence that CSA, including the histidine in four position. The N-terminal chicken-turkey peptide has: DAEHK and follows SE. In the peptide of interest the aa sequence is the same in chicken as in turkey (DAEHK) but there is a difference in the nucleotide sequence at position of the aa D that in chicken is GAT-encoded and in turkey by GAC.

4. Discussion

The results of the present study show that the vertebrate serum albumins show A-esterase activity in the presence of Cu(II). The birds serum albumins (CSA and TSA) were the highest hydrolyzing activation (HDCPase) compared to the other vertebrates serum albumins. This copper activating effect (100 μM) on the hydrolysis of oxo and thio form chiral OPs has been previously observed in both avian serum cuproproteins ([11,14]. Particularly, the N-terminal sequence (N-Asp-Ala--Glu-His-Lys (DAEHK)) was reported to be responsible for the non-specific binding of Cu(II) and Zn(II) in the CSA [15]. However have been demonstrated [16]that the first Cu(II) ion occupies a specific site in the amino-terminal sequence of animal serum albumin trough the α-NH2 nitrogen, the nitrogen atoms of the first two peptide bonds and the 3-nitrogen of the histidine imidazole ring when this amino acid is in the third position, as in the case of BSA and HSA. The Cu(II)-dependent HDCPase activity observed significantly in the birds serum albumins with respect to the other vertebrates serum albumins assayed suggests as a possible metal-dependent esterase catalytic center in the N-terminal sequence DAEHK from CSA and TSA [15]. Because both sequences have conserved the glutamic amino acid in position three and histidine in position 4 in both proteins as report in this study. While, mammals' albumins (HSA, RSA, SSA, and BSA) that showed low Cu (II)-dependent HDCPase do not contain glutamic acid in their N-amino terminal sequence. Therefore, the amino acid histidine is in the third position. The others mammals' albumins (PSA and DSA), fish albumin (CoSA) and reptile albumin (CoSA) assayed that showed no-activity do not contain either of these two amino acids in the N-sequence terminal in to the first five amino acids of their albumins [16]. For this reason, we suggest that the N-terminal sequence of vertebrate albumin is part of this catalytic center of this Cu(II)-dependent A-esterase activity.

On the other hand, our results of CSA pre-incubation assays with amino acid modifier compounds of albumin as paraoxon, DFP [17], PA, *p*-NPB (tyrosine amino acid) [18,19], EMM [20], DTNB (cysteine amino acid) [21]DCC (aspartic and glutamic amino acid) [22], NBS [23] did not show an inhibitory effect on Cu(II)-dependent hydrolysis of R (+)-HDCP by CSA, except the histidine modifier compounds (NBS and Zn(II)) [24,25]. These results suggest the participation of histidine in position four as the N-terminal of CSA as part of the catalytic center of this A-esterase activity. Peters (1996) reported that Zn(II) ion is transported in the first site of Cu(II) transport in vertebrate serum albumins.

The hydrolysis of HDCP by TSA reinforces the participation of the amino-terminal sequence of CSA (DAEHK) in the active center of this copper-dependent A-esterase activity because the TSA has the same N-terminal sequence and it was the other protein with high HDCP hydrolysis but non-stereoselective. This catalytic difference between these two avian albumins may be due to the specific binding of endogenous molecules, such as fatty acids. Which are also transported by albumin in the serum of vertebrates [16] Possibly, the fatty acids-TSA binding could induce an optimum conformational change of the structure protein that leads to its catalytic activity. On the contrary, CSA is unable to bound the same fatty acids at the catalytic center for the S-(−)-HDCP enantiomer hydrolysis. This increased hydrolyzing capacity of TSA has also been observed with the racemic insecticide trichloronate [14].

5. Conclusion

In conclusion, this study reports for the first time the A-esterase activity of TSA on this analogous compound of insecticide methamidophos, known as HDCP and reinforces its stereospecificity ((+)-HDCP › (−)-HDCP) observed in CSA [11]. While mammal's serum albumin has not significant HDCPase activity. The specific site of Cu(II) transport in the N-terminal sequence (DAEHK) of avian albumins [16]

suggests that these amino acids sequence as the possibly catalytic center of metal-dependent A-esterase activity. Finally, both birds' serum albumins could be useful as an experimental model for the degradation of OPs compounds, as well as for other biotechnological applications in clinical and environmental toxicology.

Author contribution

Miguel Angel Sogorb: designed objectives and experimental approaches. Eugenio Vilanova: designed objectives and experimental approaches, Writing – original draft. Antonio Monroy: designed objectives and experimental approaches, performed the experimental assays, Writing – original draft. Damianys Almenares-Lopez: made the statistical analysis. All authors read and approved the final version of the manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interestsThe scientific information shown in this study has been included in The Spanish Office of the Patents and Brand; ES 265192 B2.

Acknowledgements

This project has been supported by CONACYT grant FORDECYT-PRONACES 840801. Thanks to Dr. Russell F. Doolittle for giving us lamprey serum albumin and Dr. Jingyu Shao for the donation of the cobra serum albumin.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2021.109524.

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