INVESTIGATION OF THE POSSIBLE RELATIONSHIP BETWEEN MUTATION AT THREE-DIMENSIONAL STRUCTURAL LEVEL AND RESISTANCE OF *CULEX PIPIENS'S* ESTERASES TO INSECTICIDES

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1. Introduction

Protein modelling was practiced on a specific problem with high health impact: the acquired resistance of mosquitoes to insecticides. It is known that the target of common organophosphate insecticides is esterase. One way resistance is obtained is through esterase overexpression (1). However, point mutations have been noticed in esterase A and B loci of resistant *culex pipiens*, which might play some role in the resistance (2).

2. Materials and methods

The esterases from various insecticide resistant and insecticide sensible culex pipiens populations were studied at the Institut des Sciences de l'Evolution (ISEM), Universite des Sciences et Techniques du Languedoc (France). Overall, 28 proteins have been sequenced for 2 loci A and B as shown below.

A_loci 6 resistant and 8 sensible esterases		B_loci 6 resistant and 8 sensible esterases	
Resistant	**Sensible**	**Resistant**	**Sensible**
SA1prot	Slab1prot	B1	Slab1
SA2prot	Slab2prot	B2	BrugeA
SA4prot	BrugeA	B4	BrugeB
SA5prot	BrugeB	B5	Trans
SA7prot	Transprot	B7	BSA1
SA8prot	Crisse	B8	Bleuet
	Heteren		Heteren
	Bleuet		Slab2

3. Results

3. 1. Alignment

3.1.1. Alignment

The alignment of 28 Esterase was performed using CLUSTALW (Higgins D et al., 1994, Nucleic Acids Res. 22:4673-4680, http://www.ebi.ac.uk/clustalw/) and analyzed using BELVU (http://www.cgr.ki.se/cgr/groups/sonnhammer/Belvu.html) and SEAVIEW (http://pbil.univ-lyon1.fr/software/seaview.html).

Three alignment files in FASTA format were prepared

CoEs.fasta for all Esterase; A_only.fasta for A loci and B_only.fasta for B_loci

3.1. 2. Selection of one reference sequence for 3D modeling

The sequence of SA8prot (resistant) was selected because it is the closest to sensible sequences (only 3 different residues in comparison with sensible Slab1prot)

3.2. Modeling of SA8prot (A_loci)

3. 2.1. Search for a structural template

The sequence SA8prot was submitted to the Internet servers BIOSERV (http://bioserv.cbs.cnrs.fr/) and 3D-PSSM (http://www.sbg.bio.ic.ac.uk/~3dpssm/) to find the best structural templates for modeling.

The following templates were obtained for SA8prot

Top 3D-PSSM	1: 1maa,	2: 1qid,	3: 1eqh,	4: 1dx4,	5: 1k4y
Top PDB-BLAST	1: 1maa,	2: 1mah,	3: 1e2b,	4: 1c2o,	5: 1f8u
Top TITO score	1: 1maa,	2: 1qid,	3: 1eqh,	4: k4y	

3. 2.2. Build initial SA8prot 3D models

Each template was used to built 3 models for SA8prot (Modeller4 through BIOSERV). The scores of these models are as follows:

	PROSA-II			**VERIFY-3D**		
	Model1	Model2	Model3	Model1	Model2	Model3
1eqh	***- 0,503***	- 0,412	- 0,352	**0,350**	0,348	0,340
1maa	- 0,377	- 0,232	***- 0,328***	0,316	0,331	**0,369**
1qid	***- 0,482***	- 0,500	- 0,438	**0,363**	0,331	0,370
1k4y	- 0,420	- 0,300	***- 0,392***	0,263	0,310	**0,310**

From above tables, the best model for 1qeh is Model1
for 1maa is Model3
for 1qid is Model1
for 1k4y is Model3

and among these models, Model 1 from 1qid displays the best scores. Is was named Model1

3.2.3 Cysteines and disulfide bridges

Cysteines can make disulphide bridge with other cysteines and disulfide bridges are present in all selected structural templates. A free cysteine is also observed in the 1qid template. Disulfide bridges and free cysteines observed in the four templates and in the resulting models are summarized below:

1maa template 1maa model	3 bridges (6 CYS) : 69 - 96, 257 - 272, 409 - 529 7 free CYS 61, 296, 306, 328, 362, 436, 508 and 1 bridge 195 - 219
1qid template 1qid model	3 bridges 67 - 94, 254 - 265, 402 - 521 and 1 free CYS 231 7 free CYS 61, 296, 306, 328, 362, 436, 508 and 1 bridge 195 - 219
1k4y template 1k4y model	2 bridges 87 - 116 and 273 – 284 7 free CYS 61, 296, 306, 328, 362, 436, 508 and 1 bridge 195 - 219
1eqh template 1eqh model	4 bridge 36 - 47, 41 - 57, 59 - 69 and 37 – 159 7 free CYS 61, 296, 306, 328, 362, 436, 508 and 1 bridge 195 - 219

It can be observed that (i) disulfide bridges are not fully conserved between templates and, (ii) the number and position of bridges and free cysteines in the models is identical whatever the template used.

The number of free cysteines in the models is large in comparison to the templates. At this time, we have no explanation for this. It may happen that the protein is multimeric and that some disulfide bridges are formed between monomers.

3. 2.4. Analysis and optimization of initial SA8prot Model1

We used InsightII to view and analyze Model1, in comparison with the structural template (PDB ID: 1qid)

We observed an unusual loop conformation for residues 270 to 290 was found loop. The reason for this is apparently due to a too strict alignment. To get better conformations, we relaxed alignment "unaligning" few residues at the C-terminal loop end as indicated below:

Model1 L L N E N E I E N R I L ▶ L L N E N E - - - I E N R I L
1QID V L P - - - F D S I F R V L P - - - F D S - - - I F R

This changes in file model1.ali was save into new file: model2.ali

Files model2.ali and model2.top were used to build Model2 through a standalone version of Modeller4 running on a local SGI workstation.

The .two models were sent to the ***Eval123D server*** (http://bioserv.cbs.cnrs.fr/valid.html). The evaluation scores provide detailed information for each residue and each fragment of the model.

From the Evaluation scores we can see that, generally the structure of model2 is better than model1, except for residues 414 - 434. In this part, evaluation scores of model1 are better.

This part of Model2 was modified according to Model1. The changes in file model2.ali were saved into a new file: model3.ali. File model3.ali and model3.top were used to build Model3.

Model4 was the obtained from Model3 by modifying fragment 62 - 73.

Evaluation scores of 4 models and template 1QID are shown in the table:

	Eval23D	**Verify 3D**	**Prosa II**	**EvTree**	**SFE**
Model 1	**0,052**	0,236	**0,037**	- 0,365	- 540,6
Model 2	0,051	0,219	0,008	- 0,405	- 529,5
Model 3	0,047	0,219	0,021	- 0,385	- 522,2
Model 4	**0,063**	0,210	**0,026**	- 0,403	- 526,0
1QID	0,111	0,321	0,192	0,290	- 569,2

From the above table, we note that Model 4 displays a good Eval23D score, but that Model1 is the better according to all other scores.

We therefore selected Model 1 as our Model and modified it in two regions, where Model4 has better scores (regions 71 - 78 and 85 - 95). The changes in file model1.ali were saved into new file: model5.ali. Files model5.ali; model5.top were used to build Model5

	Eval 23D	**Verify 3D**	**Prosa II**	**EvTree**	**SFE**
Model 1	0,052	0,236	0,037	- 0,365	- 540,6
Model 4	0,063	0,210	0,026	- 0,403	- 526,0
Model 5/1	**0,072**	**0,241**	**0,047**	**- 0,363**	**- 551,3**
Model 5/2	0,066	0,239	0,036	- 0,366	- 542,4
Model 5/3	0,070	0,257	0,045	- 0,369	- 536,1

It can be seen that all of 3 variations of model 5 have better scores than Model 1 and Model 4. The best is Model 5/1. It was named Model 5.

Examination of Model 5 (use Insight II) shows that a very bad loop conformation (knot) is present for fragment 421 - 440. To relax this region, we "unaligned" two residues.

```
Model 5     F S V D S D T Y N H Y R I V F C D
1QID        F N H R A S - - - - - - - N L V W

Model 6     F S V D S D T Y N H Y R I - - V F C D
1QID        F N H R A S - - - - - - - N L - - V W
```

The changes in file model1.ali saved into new file: model6.ali. Files model6.ali; model6.top were used to build Model 6.

	Eval23D	**Verify 3D**	**Prosa II**	**EvTree**	**SFE**
Model 5	0,072	0,241	0,047	- 0,363	- 551,3
Model 6/1	0,046	0,239	0,014	- 0,381	- 548,8
Model 6/2	**0,056**	**0,235**	**0,045**	**- 0,389**	**- 541,1**
Model 6/3	0,055	0,232	0,032	- 0,376	- 535,7

Using Insight II we can see that in Model 6 the strange loop conformation has disappeared. This means that our loop relaxation was well done. However, results in table show that all variations of Model 6 are worse than Model 5. Only the best of them, Model 6/2, is nearly close to Model 5 (especially by the most important score, Prosa II). This indicates that we could try to improve again Model 6/2. However, due to time limitation, the modeling of SA8prot was stopped.

From all of this, we chose Model6/2 as our Model 6 for SA8prot (resistant in A_loci). This model has correct backbone conformation and relatively good scores (Pic.1).

3. 3. Modelling of a Protein from B_loci:

A similar strategy was used to build a reference 3D model for B_loci esterases.

Using belvu and seaview we chose from B_loci B8 (resistant) for modeling (It has highest identity score to SA8prot).

The B8 sequence was sent to BIOSERV and 3D-PSSM and the 1QID structural template was selected as previously done for A_loci.

B8 were built with either one of 2 ways:

Standalone Modeller4: Output Model M1/1, M1/2, M1/3

BIOSERV Modeller4: Output Model M2/1, M2/2, M2/3, M2/4

All models were sent to the Eval123D server

	Eval 23D	Verify 3D	Prosa II	EvTree	SFE
Model 1/1	**0,085**	**0,228**	**0,037**	**- 0,366**	**- 553,2**
Model 1/2	0,070	0,207	0,025	- 0,394	- 546,6
Model 1/3	0,068	0,219	0,029	- 0,405	- 523,4
Model 2/1	0,066	0,207	0,021	- 0,378	- 540,7
Model 2/2	0,051	0,221	0,019	- 0,377	- 545,7
Model 2/3	0,079	0,225	0,017	- 0,373	- 545,5
Model 2/4	0,082	0,208	0,036	- 0,399	- 543,0

From results in table, the best model is Model 1/1. Using Insight II we can see that the model has no apparent error. From all of this, we chose Model 1/1 as our Model 1 for B8 (resistant in B_loci). This model has a correct backbone conformation and relatively good scores.

3.4. Modelling and analysis of all Proteins in A_loci

Using seaview we checked all differences between SA8prot and each Protein in A_loci. Then, using SPDBViewer, we mutated the 3D model of SA8prot to obtain models of all other A_loci proteins.

	Protein	*Mutations*
Resi	**SA8prot**	
	SA1prot	**43**, **76**, 95, 175, **303**, 364, 504, **505**, 528
	SA2prot	**81**, **295**, 493, 504
	SA4prot	21, **182**, 364, 492, 504, 528, 540
	SA5prot	**39**, 95, 175, **227**, 250, 269, 292, 364, 500, 504, 528, 540
	SA7prot	**9**, **44**, 262, **356**, **503**, 504
Sensible	**BrugeA**	98, 364, 504, 528
	BrugeB	98, 269, 292, 364, 504, 528
	Slab1	177, 504, 540
	Slab2	21, 98, 269, 361, 364, 439, 492, 504, 528, 540
	Crisse	95, 152, 175, 250, 292, 337, 364, 492, 493, 504, 528
	Heteren	21, 98, 269, 361, 364, 492, 504, 528
	Transprot	56, 261, 500, 512
	Bleuet	21, 98, 492, 504

Since the aim of work is *"To investigate the possible relation between structural mutation and resistance of Esterase to insecticides"*, so our interest is focusing in mutations, which are observed only in resistant proteins. For A_loci these mutations are residues 9, 39, 43, 44, 76, 81, 182, 227, 295, 303, 356, 503 and 505.

All mutations were carefully analyzed using Insight II. All mutations are located on the surface of the protein, and reasonably far from the active site. None of the mutation appears to have a significant impact on structure and stability of the protein. It is likely that the mutations observed in the sequences of the A_loci esterases of resistant *culex pipiens* are not responsible for the resistance.

Number	9	39	43	44	76	81	182	227	295	303	356	503	505
Original aa	P	P	E	L	N	S	P	V	N	V	V	T	P
Mutated aa	R	S	K	W	S	R	S	I	K	L	I	A	L
aa in template	N	P	N	M	N	S	P	V	T	E	A	E	K

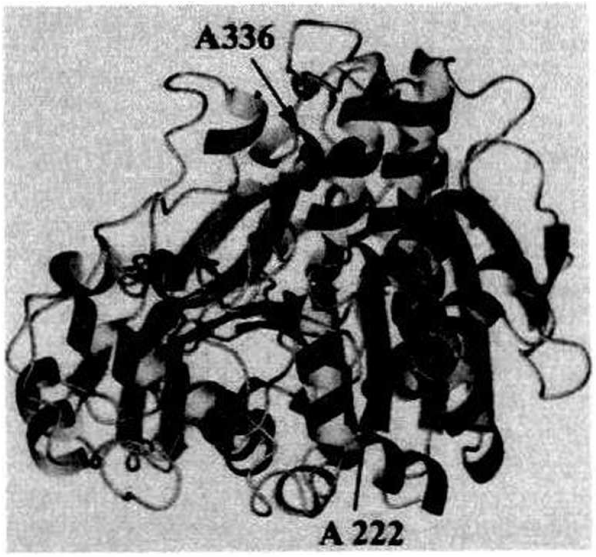
3.5. Modelling and analysis of all Proteins in B_loci

Using seaview we check all differences between B8 and each of Protein in B_loci. Then, using SPDBViewer, we mutated the 3D model of B8 to obtain models of all other B_loci proteins.

	Protein	*Mutations*
Resistant	**B8**	
	B1	20, 38, 98, 188, **213**, 222, **257**, **301**, **335**, 376, 461, 476
	B2	38, **62**, 256, 266, 461
	B4	20, 38, 54, 98, **100**, **127**, 188, 222, 297, 376, 461, 476
	B5	20, 38, 54, 98, **127**, 176, 188, 222, 376, 461, 476, **511**,
	B7	25, 38, 98, **151**, 176, **202**, **246**, 461
Sensible	**BrugeA**	20, 26, 38, 93, 98, 176, 188, 266, 297, 303, 407, 461, 476, 517
	BrugeB	20, 38, 98, 176, 222, 266, 288, 297, 303, 376, 461, 476, 481, 517
	Slab1	25, 38, 169, 176, 262, 459, 461, 500
	Slab2	20, 38, 98, 176, 222, 266, 297, 303, 376, 460, 461, 476
	BSA1	20, 38, 54, 93, 98, 188, 239, 266, 297, 376, 461, 476, 492, 497
	Heteren	3, 20, 38, 54, 95, 98, 176, 222, 266, 297, 303, 376, 460, 461, 476, 517
	Trans	38, 256, 461
	Bleuet	25, 38, 169, 176, 297, 320, 375, 461, 476, 517

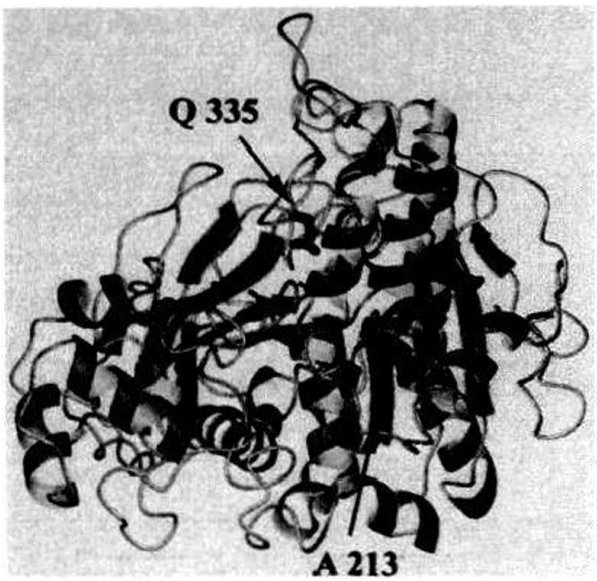
With the same aim of work as in A_loci, our interest is focusing in mutations, which are happening only in resistant proteins. For B_loci these mutations are residues 62, 100, 127, 151, 202, 213, 246, 257, 301, 335 and 511.

All mutations were carefully analyzed using Insight II.

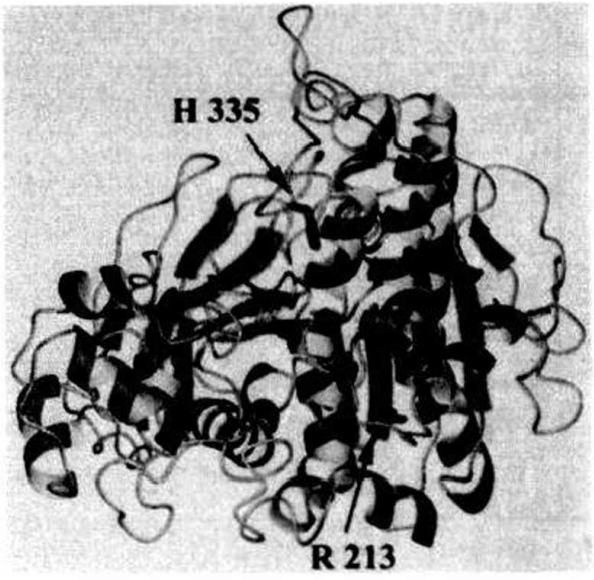


Pic. 1. 3D Model of template

As shown below, several mutations are observed for buried residues. One of them, position 213 is proximal to active site. Most mutations are conservative. However, in position 151, an exposed polar residue is mutated to a valine. In position 213, a small buried alanine is mutated to arginine. The corresponding residue in template is an alanine and significant steric hindrance appears in the arginine mutant. This point should deserve further studies (Pic. 2). Similarly, the mutation to histidine at buried position 335 deserves further studies regarding charge complementarities. This position is moreover in close proximity to a loop region in which alignment between the esterase sequence and the template is questionable (Pic. 3).



Pic. 2. 3D Model of B8



Pic. 3. 3D Model of B1 with mutations

Therefore, although most mutations observed in the sequences of the B_loci esterases of resistant culex pipiens appear unrelated to the resistance phenomenon, three mutations at position 151, 213 and 335 may have significant structural impact. Further investigation will be necessary to more carefully analyze the possible impact of these three mutations.

Number	Original aa	Mutated aa	aa in template	Comment
62	T	S	S	exposed
100	L	F	T	exposed
127	V	I	A	buried
151	E	V	G	exposed, hydrophobic
202	I	V	L	buried
213	A	R	A	**buried, steric hindrance**
246	G	D	N	exposed
257	A	R	E	exposed
301	E	A	S	exposed
335	Q	H	A	**buried, alignment**
511	I	V	I	buried

4. Conclusion

1. 28 three-dimensional models of insecticide sensible and insecticide resistant culex pipiens esterases were built (14 for A_loci and 14 for B_loci).
2. All of mutations in A_loci, which are specific to resistant populations appeared insignificant and probably do not play any role in resistance of Culex pipiens to insecticides.
3. Most mutations in B_loci also appeared unrelated to insecticide resistance, except mutations in positions 151, 213 and 335 (B1). These mutations might have significant impact on structure or stability of the esterase in the resistant population. More detailed discussion of the possible role of these mutations in resistance of *Culex pipiens* to insecticides should await further investigations.

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References

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NGHIÊN CỨU SƠ BỘ KHẢ NĂNG LIÊN QUAN GIỮA ĐỘT BIẾN CẤU TRÚC KHÔNG GIAN CỦA PHÂN TỬ *ESTERASES* VÀ TÍNH KHÁNG THUỐC TRỪ CÔN TRÙNG Ở LOÀI *CULEX PIPIÉN*

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Loài muỗi *Culex pipiens* có khả năng kháng thuốc trừ côn trùng nhờ vào sự thay đổi tính chất của protein Esterase. Việc nghiên cứu khả năng liên quan giữa các đột biến cấu trúc khôn gian của phân tử này và tính kháng thuốc đã được tiến hành tại Trung tâm cấu trúc sinh hóa Montpellier (Pháp).

Mô thình phân tử 3D của 28 protein thuộc nhóm Esterase đã được xây dựng. Qua phân tích các đột biến cấu trúc, có thể kết luận rằng mọi đột biến tại nhóm A_loci đều không gây ảnh hưởng đến tính chất của protein. Điều này có nghĩa là những đột biến này không liên quan đến tính kháng thuốc của *Culex pipiens*.

Đối với nhóm B_loci, có 3 đột biến cấu trúc tại các điểm 151, 213 và 335 có thể gây ra sự thay đổi tính chất của protein. Tuy vậy, việc tìm ra câu trả lời cuối cùng về sự liên quan giữa những đột biến cấu trúc này với tính khangs thuốc trừ côn trùng đòi hỏi phải tiến hành thêm các nghiên cứu khác nữa.