

TYROSINE STORAGE IN STORED-PRODUCT COLEOPTERA

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ABSTRACT :

In the rice weevil *Sitophilus oryzae*, we identified insoluble proteins with exceptionally high levels in Tyrosine (up to 27 %), which proved to be very different from arylphorins (serine, tyrosine and glycine representing together about 60 % of the total aminoacids). During larval and pupal stages, these polypeptides are stored in dense fat body granules, together with other storage proteins. We proposed to call this new class of Tyrosine-Rich Proteins Tyrostaursins. A survey of other Coleoptera has revealed a contrasted situation. Some species accumulating mainly tyrostaursin-like proteins (*Callosobruchus maculatus*, *Rhizopertha dominica*, *Trogoderma granarium*), while others bear arylphorin as the major form (*Tenebrio molitor*, *Lasioderma serricorne*). *Oryzaephilus surinamensis* and the bark beetle *Ips sexdentatus* have both.

The significance of such an evolutionary choice is unclear, as no evident correlation can be established between storage form and systematic position. Tyrostaursins showed a high polymorphism as reflected by HPLC studies. A survey of possible use as infra-specific marker is underway.

INTRODUCTION :

The aromatic aminoacids are not synthesized by insects (except with the intervention of possible symbionts) and therefore only originate from the food, mainly as tyrosine and phenylalanine. The needs for aromatic ring-containing compounds is great in most insects, mainly because of cuticle sclerotization and melanization through phenolic metabolites.

Insects have established different strategies to store aromatic aminoacids during larval life and to mobilize them during metamorphosis. These strategies may imply low molecular weight compounds (tyrosine, tyrosine-0-phosphate, β alanyl tyrosine, γ -glutamyl-phenylalanine, β -glucosyltyrosine; review by Brunet, 1980) and high molecular weight proteins rich in phenylalanine and tyrosine.

In all the insect orders investigated until now (Diptera, Hymenoptera, Lepidoptera, Dictyoptera), a class of storage proteins called arylphorins has been identified (reviews by Levenbook, 1985; Rahbe et Bonnot, 1986). These highly soluble proteins are hexameric (monomers of 70-80 kDa), with about 10% Tyrosine and 10% Phenylalanine. They are formed in the haemolymph of larva and partly incorporated into the nymphal fat body.

In Coleoptera, no storage form was identified although cuticle is often highly sclerotized and melanized. We found new kinds of storage protein in the Curculionid *Sitophilus oryzae* L. (Rahbe *et al.* 1990), characterized by their insolubility, their lower molecular weight (30-50 kDa), their very high tyrosine level (15-27%) and relatively low phenylalanine level (2-5 %); basic amino acids were low and sulphur aminoacids absent; serine, glycine, glutamine plus glutamic acid represented together more than 40% of the total amino acids. These proteins, named tyrostaourins, are stored in granules of the fat body with associated non-TRP (TRP=tyrosine rich proteins).

The aim of the present work is to investigate the status of tyrosine storage in some other Coleoptera (mostly stored product insects).

MATERIALS AND METHODS

Insects

Pupae of insects were obtained from larvae reared on their natural food : *Tenebrio molitor* L., *Trogoderma granarium* Evert, *Orizaephilus surinamensis* (L.), *Lasioderma serricorne* (F.), *Rhyzopertha dominica* (F.) from P. Pracros -INRA Bordeaux - ; *Ips sexdentatus* (Boerner) on pine from F. Lieutier - INRA Orléans - ; *Metamasius hemipterus* (L.) from P. Zagatti - INRA Brouessy -, *Callosobruchus maculatus* (F.) from A. Delobel - ORSTOM Bondy ; *Rhynchophorus palmarum* (L.) strain, originating from Guadeloupe, were bred in our laboratory on artificial diets. *Sitophilus* species and strains were from our laboratory and reared on untreated "Magali blondo" wheat at $27,5 \pm 0,5$ °C and 70 %RH.

For all species, the newly formed pupae were collected within 2 days after the pupal moult, weighed and stored at -20 °C.

Sample preparation for electrophoresis

Frozen insects were crushed in cold Yeager's buffer, pH 6.9 saturated with phenylthiourea (25 µl buffer /mg f.w.), then centrifuged at 800g for 3 min at 4 °C. The supernatant ("soluble fraction") and the pellet ("insoluble fraction") were collected, lyophilized and stored at -20 °C until electrophoresis.

SDS-PAGE electrophoresis

7.5%-15% acrylamide gradient slab gels were used for both analytical and preparative purposes: 1mm thick, 160 mm wide; 160 mm separating gel/ 30 mm stacking gel. Electrophoreses were run at 10 °C, 110 V constant voltage for 14 h.

For analytical runs, the equivalent of 1 mg of fresh weight were deposited on each lane. Gels were stained by the double-staining tyrosine-specific procedure developed by Rahbe *et al.* (1990).

PVDF electroblotting

For preparative runs, the proteins fractions from approx. 2 mg of insect, equivalent to 150-200 µg of total protein were deposited on the gel. At the end of the run, gels were cut to appropriate size and inserted into a sandwich of Whatman 3MM paper, as described in the BioRad Transblot apparatus protocol. The blot was carried for 8 hours at room temperature, 40V constant voltage in a borate buffer (Boric Acid 50 mM - Tris 50 mM, pH 8.5). The membranes were then stained with Amido-black and the protein spots cut off carefully and stored dry at -20 °C until hydrolysis

Protein hydrolysis and amino-acid analysis

Each protein was treated with two hydrolysis conditions : the whole Immobilon® spot was placed in a glass tube with 1,5 ml either {6N HCl + thioglycolic acid (0,2 %)} or {6N HCl + Phenol (1mg/100 ml)}. The amino acid analyses were performed on a Kontron Liqumat III autoanalyzer with a standard elution program. Complete protocol is described elsewhere (Delobel *et al.*, submitted).

HPLC

Reverse-phase HPLC of insoluble TRP was performed on an Altex gradient device with UV detection (280nm). Proteins were solubilized in Guanidine-HCl 6M, centrifuged and injected (20-100µl; 0.1-5mg). Column was a 300x4.1mm packed with C4 Nucléosil® 300Å 7µ (Interchim, Montluçon FRA). Gradient was from %B 70% to 30% in 40 min. A: H₂O, TFA 0.1% - B: Acetonitrile-H₂O 90-10, TFA 0.1%.

RESULTS

Electrophoretic studies

Table I shows the main characteristics deduced from electrophoresis. The specific staining of Tyrosine Rich Proteins (TRP) allows us to quickly determine their MW, and to test their solubility in Yeager or in 3.5 M Urea.

Table I: Properties of Tyrosine Rich Proteins found in nymphs of 10 Coleoptera, as deduced from SDS-PAGE. *(x)=minor protein **putative types: T=Tyrostaaurin, A=arylphorin.

Species	Solubility* in Yeager	Solubility* in 3.5M Urea	MW kDa	Type**
<i>Rhizopertha dominica</i>	-	-	32	T
<i>Lasioderma serricorne</i>	+	+	72	A
<i>Trogoderma granarium</i>	- (+)	- (+)	10-30 (73)	T (A)
<i>Tenebrio molitor</i>	+ (-)	+/- (-)	71 (45)	A (T)
<i>Oryzaephilus surinamensis</i>	+ -	+ -	70-78 34-36	A T
<i>Callosobruchus maculatus</i>	-	+/-	16-37	T
<i>Metamasius hemipterus</i>	-	-	63	T
<i>Rhynchophorus palmarum</i>	-	-	65	T
<i>Sitophilus oryzae</i>	-	-	31-51	T
<i>Ips sexdentatus</i>	+ -	+ +	76 46	A T

Aminoacid Composition

After transfer, some of the TRP were analyzed for amino acid composition; only characteristic amino acids are presented in table II, expressed in mole per cent.

Table II: Composition, in mol%, of Tyrosine Rich Proteins of 10 Colepteran species
**S.li*=composition of a control arylphorin from the Lepidoptera *Spodoptera litoralis*.

Amino acid	S.or	R.pa	M.he	I.se	C.ma	O.su	T.gr	S.or	L.se	T.mo	<i>S.li</i> *	
Serine	17.0	17.6	12.2	6.5	2.8	7.7	5.7	7.5	6.6	5.3	3.8	
Glx	12.7	14.1	11.5	21.6	9.6	11.2	11.6	12.0	17.6	12.2	12.1	
Glycine	14.6	14.5	9.6	10.3	15.5	14.5	10.3	8.1	8.5	5.7	4.5	
Tyrosine	19.1	18.5	18.5	10.2	12.5	24.6	13.8	7.5	11.9	14.0	11.4	
Phenylalanine	5.0	1.8	3.3	8.8	1.5	3.3	5.0	5.9	6.5	7.2	8.4	
Basic am. ac.	2.2	3.0	8.8	4.5	8.4	6.5	9.9	8.4	8.5	13.4	15.8	
	<i>Tyrostaaurins</i>			<i>Tyrostaaurin-like</i>				<i>Arylphorins</i>				

HPLC

HPLC fractionation followed by electrophoresis of the purified fractions allowed us to ascertain (as confirmed by differences in amino acid composition), that at least in *S. oryzae* one

electrophoresis band may be composed of two different TRP (fig. 1). The HPLC analysis of different individuals of the same species shows that a high degree of polymorphism may occur in some TRP (fig. 2).

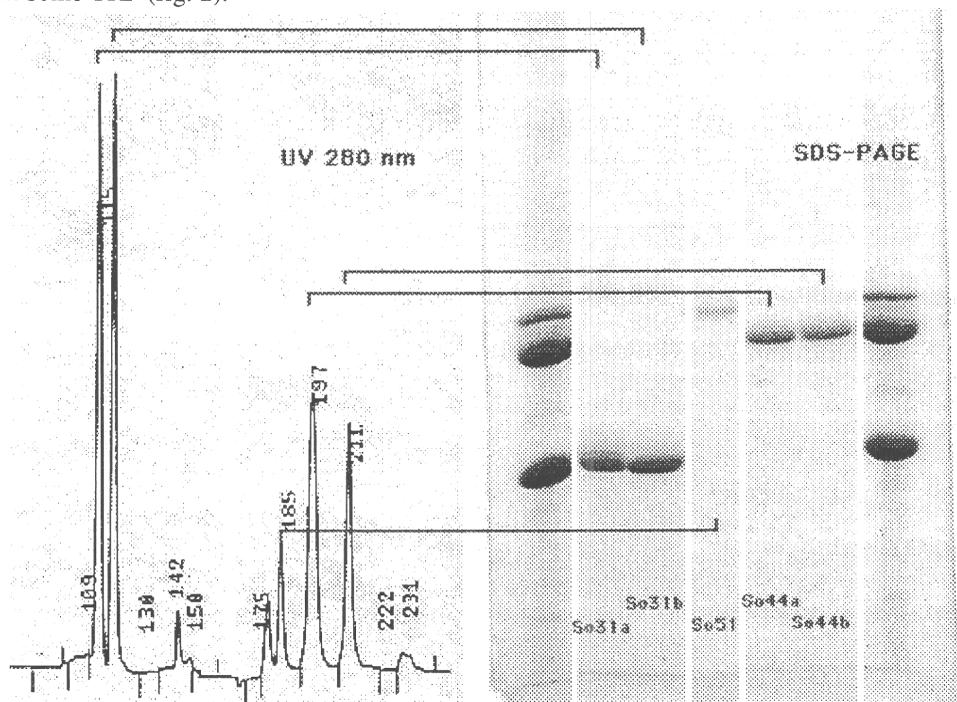


Fig. 1: Electrophoretic analysis of the HPLC purified tyrostaurens of *Sitophilus oryzae*.
* = starting material.

DISCUSSION

Sitophilus oryzae (L.) TRP are present in 5 different forms, all of them insoluble in Yeager and 3.5 M Urea (fig. 1). The amino acid composition is very typical, with Ser, Gly and Tyr near or above 15 % of the molar percentage, and the basic amino acids very low. The sulfur aminoacids are absent. These proteins are stored during larval and nymphal life in dense proteinaceous granules in the fat body (Rahbé *et al.* 1990). They are clearly not of the same family as the arylphorins known in other insects, and we therefore named them tyrostaurens.

Tenebrio molitor TRP is soluble in Yeager and 3.5 M Urea, is not localized in granules, and its composition and molecular weight (71 kDa) show clear similarity with arylphorin.

Ips sexdentatus protein electrophoresis reveals the simultaneous presence of a 76 kDa soluble TRP which is an arylphorin, and a 46 kDa insoluble TRP which is much like tyrostaurens in composition, except for a lower level in Ser and a higher level in Glx.

These three examples show the diversity of situation found in Coleoptera. The comparison with the systematic position (according to Crowson, 1986) reveals that all Curculionidae have true tyrostaurens as main TRP, while *I. sexdentatus*, which also belongs to the Curculionioidea superfamily, has both arylphorin and tyrostaurens-like proteins. In the Bostrychoidea superfamily, *Rhizophorthera* has a tyrostaurens-like TRP, while *Lasioderma* has a typical arylphorin. Figure 4 emphasizes the absence of correlation between TRP status and systematic position. This situation may arise from the presence in all Coleoptera, as in other insect Orders, of the genes encoding for arylphorins, their level of expression being extremely variable. In addition, Coleoptera seem to have developed insoluble modes of storage, through the tyrostaurens or tyrostaurens-like protein families, whose level of expression is also variable.

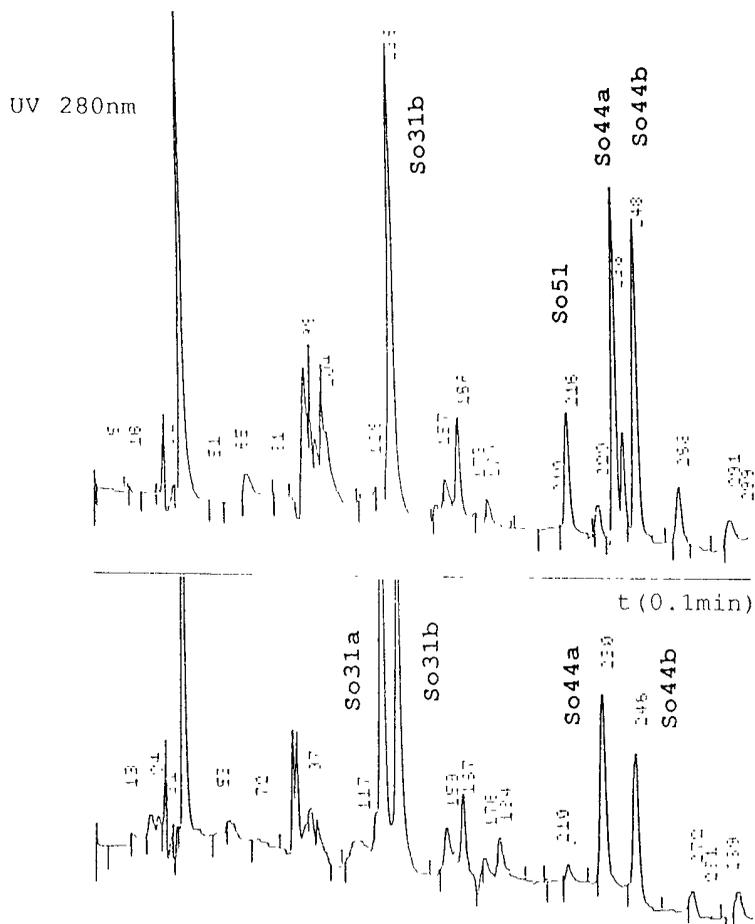


Figure 2: Comparison of HPLC profiles of tyrostaourins from two individual nymphs (A & B) of *S.oryzae*. Labels (Soxx) point to individual tyrostaourin peaks.

Inside the Curculionidae family, where the storage form is typically tyrostaourin, traces of arylphorin were found in the soluble fraction, but its tyrosine content, although high, was lower than in arylphorins from insects using this form as main storage (table II).

A study of the tyrostaourin pattern in the *Sitophilus* genera shows close relationships between the three species *oryzae*, *zeamais* and *granarius* (fig. 3). The sibling species *S. oryzae* and *S. zeamais* Mots. are very close but clearly distinguishable: the two main band of *S. oryzae* (31 & 44 kDa) are unchanged in *S. zeamais*., whereas the slowest band of *S. oryzae* (51 kDa) is replaced in *S. zeamais* by a faster band (46 kDa). *S. granarius* (L.) has also 3 groups of bands but their relative importance is different (the faint slowest band of *S. oryzae* and *S. zeamais* becoming very important). Such properties could allow the distinction of possible hybrids (Musgrave and Miller, 1956, Nardon, 1972). Belonging to the same tribe Rhynchophorinae, *Metamasius hemipterus* and *R. palmarum* display a single band in electrophoresis (respectively 63 and 65 kDa), whose solubility and composition is also that of a tyrostaourin. The biochemical evolution having lead from a single protein to many lighter proteins will not be clearly understood until the protein sequences have been determined.

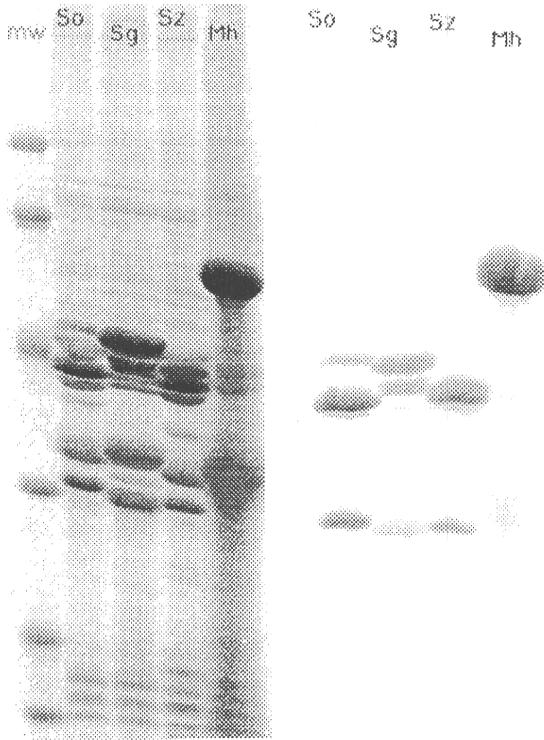


Fig. 3: Electrophoresis of Rhynchophorinae tyrostaurosin (see text for species). Right: tyrosine-specific staining Left: double staining with Coomassie blue. mw=standards.

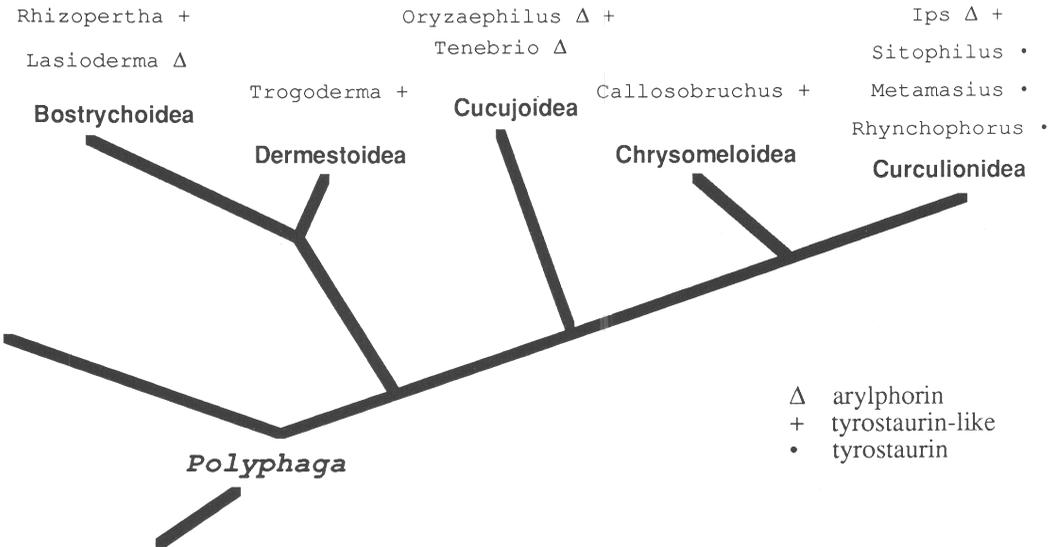


Figure 4: Relations between TRP status of the studied species and the systematic position of their superfamilies (after Crowson, 1986).

A rapid survey of polymorphism within the species *S. oryzae* was also undertaken. HPLC is best adapted for such comparison: the two fastest bands in electrophoresis are resolved into two proteins peaks each, of slightly different composition, although belonging to the same family (fig 1). The slowest band (So 51) may be completely absent in some individuals, as is one of the two components of the fastest band (So 31a). With So44a and b, the situation is much more complex: each of the two proteins may be present under at least two isoforms. This polymorphism depends on the origin of the strain and might be used as a tool for population genetics.

CONCLUSION

Tyrosine Rich Proteins are mainly involved in storage, thus allowing the conservation of many mutations that do not affect this storage function. Such mutations would probably be eliminated in functional proteins, which cannot support these changes without alteration of their properties.

In Coleoptera, the long existing arylphorins are still present at different levels of expression. These polypeptides, derived from hemocyanins (Willott *et al.*, 1989), coexist with new TRP families: tyrostaurens or tyrostauren-like proteins. The survey of Coleoptera superfamilies has to be completed to clarify correlations with systematic position. The polymorphism observed in the same species and the variations observed in the same family could be a powerful tool for the study of population genetics.

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LE STOCKAGE DE LA TYROSINE CHEZ LES COLEOPTERES DES DENREES STOCKEES

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RESUME

Les insectes ont besoin d'un taux élevé de tyrosine pour la sclérotisation et la mélanisation de leur cuticule. Seules d'éventuelles associations symbiotiques et l'alimentation peuvent fournir le groupe des acides aminés aromatiques essentiels. Les insectes holométaboles ont élaboré de nombreuses stratégies pour emmagasiner et mobiliser ces acides aminés pendant la métamorphose. Dans tous les ordres d'insectes déjà examinés, nous avons identifié une classe particulière de protéines de stockage appelée Arylphorine. Ces polypeptides hexamériques très solubles contiennent 10 % Tyr et 10 % Phe. Ils sont transportés dans l'hémolymphe larvaire et incorporés dans les corps gras à la mue de la nymphe. En dépit de leur cuticule adulte foncée et dure, aucune donnée sur le stockage aromatique n'a pu être obtenue chez les coléoptères. Chez le charançon du riz *Sitophilus oryzae*, nous avons identifié trois protéines solubles principales avec des taux exceptionnellement élevés de tyrosine (jusqu'à 27 %). Après purification et analyse, elles se sont avérées très différentes des arylphorines (taux élevés en Sérine, Tyrosine et Glycine représentant à peu près 60 % du total des acides aminés). Pendant les étapes larvaire et nymphale, ces polypeptides sont stockés dans des granules denses du corps gras en même temps que d'autres protéines. Nous avons proposé de nommer cette classe nouvelle de protéines riches en Tyrosine les Tyrostaourines. L'Arylphorine se trouve également présente dans l'hémolymphe chez les Curculionides mais à des taux nettement inférieurs à ceux des diptères ou des lépidoptères. Des études par HPLC ont montré que les tyrostaourines présentent un degré élevé de polymorphisme. Une étude entreprise chez un autre coléoptère des stocks a révélé le contraste de la situation. Certaines espèces du type Curculionide accumulent surtout les Tyrostaourines (*Callosobruchus maculatus*, *Rhyzoperta dominica*, *Trogoderma granarium*) pendant que d'autres contiennent surtout la forme arylphorine (*Tenebrio molitor*, *Lasioderma serricornis*). On ne comprend pas bien la signification d'un tel choix d'évolution, étant donné qu'on ne peut établir un rapport entre la forme de stockage et la position systématique. Une étude est en cours sur la possibilité de l'utiliser comme marqueur de sous-espèces.