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Isolation and Partial Characterization of a Peptide from Split Pea (*Pisum sativum*) Toxic for *Sitophilus* Weevils (Coleoptera, Rhynchophoridae)

¹Mbailao Mbaiguinam, ¹Nanadoum Maoura, ¹Mbahidi Milaiti and ²Bernard Delobel

¹Laboratoire de Recherche sur les substances naturels (LRSN) BP 1027 N'Djaména

²Laboratoire de biologie fonctionnelle, Insectes et Interactions BF2I,

UMR INRA/INSAde Lyon, bât Louis Pasteur 20,

Av. A EINSTEIN-69621 Villeurbanne Cedex

Abstract: Everywhere in the world and especially in subsaharian Africa, human food is mainly based upon amylaceous crops, mainly Cereals. Unfortunately, the harvest depends of the rainy season length and stored Cereals are attacked and rapidly destroyed by numerous insects. The weevils of the *Sitophilus* genus are the most dangerous pests of Cereals. Legumes seeds are toxic for these weevils. Traditionally, in Africa and India, mixed legume seeds to cereals permit to prevent them from attacks by *Sitophilus* weevils. Classical extraction and purification techniques allowed establishing that the toxic compound is a small protein of low molecular weight of 5200 Daltons. The characterization of this protein opens the way towards a new method of protection of the stored cereals.

Key words: *Sitophilus*, cereals, pests, legume, purification, protein, toxicity

INTRODUCTION

Cereals are the main food source of Humans. In poor countries, the Cereals are stored on the farm, after harvest, for whole year consumption. Unfortunately, not only the climate and specially the rain are limiting factors to the Cereal production, but also, insects especially in sub-saharian zones, eat or destroy a big part of the grain devoted to human nutrition. The losses are as much important when temperature and humidity are more propitious (Delobel and Tran, 1993).

Among those Insects thriving on Cereals, those belonging to *Sitophilus* genus are the most noxious. *S. oryzae* may be the most dangerous as it may develop on various foods. An experimental study demonstrate that the introduction of 3 couples of *S. oryzae*, in a silo can destroy 5000 kg of wheat in only 28 (Grenier *et al.*, 1986). Legumes seeds are toxic to the 3 most common species: *S. oryzae*, *S. granarius* and *S. zeamais*. Only some strains of *S. oryzae* acquired the ability to develop and reproduce on some Legume seeds. Some scientists, observed the traditional practices of Africa and India consisting in the storage of a mixture of legume and grain in order to protect these. They therefore suggested limiting the post harvest destructions by developing these practices (Rider, 1982; Combs *et al.*, 1977; Thind and Muggleton, 1981; Holloway and Smith, 1985). A

genetic study revealed that the ability to develop on split pea of some rare strains of *S. oryzae* is autosomal and recessive (Grenier *et al.*, 1997). Pretheep-Kumar *et al.* (2004) have admixed milled rice with pea flour extract at 1% concentration and tested it for the toxicity and reproductive effect on *S. oryzae*. They found that mortality of the insects increased and the fecundity suppressed. The compound, responsible of the toxicity in pea is not known.

Legumes, more than Cereals, are rich in secondary compounds such as lectins, enzyme inhibitors, cyanogenic compounds, non proteic amino acids, saponins, alkaloids etc. These substances may have repulsive, antifeedant or toxic properties against herbivores. In this study the toxicity of the split pea towards *Sitophilus* was measured in order to fractionate, purify and identify the responsible molecule.

MATERIALS AND METHODS

First experiments (effects of whole flour of some legume seeds on *Sitophilus* weevils) have been conducted in the Laboratoire de recherche sur les Substances Naturelles (L.R.S.N., Chad) since 1996. Isolation and characterization have been conducted in the Laboratoire de biologie fonctionnelle, Insectes et Interactions INSA de Lyon, France from 2003 to 2005.

Insects: Strain Sfr of *S. oryzae* has been studied and raised for a long time at Laboratoire de biologie fonctionnelle, Insectes et Interactions (INSA of Lyon, France). This strain is sensitive, like the wild strains, to all the tested legume seeds. Insects are raised in rectangular plastic boxes 8.5 × 5 × 4.5 cm top and bottom are gridded. Boxes are kept in an incubator (temperature 27.5±0.2°C, relative humidity 70%). Insects are grown on wheat, rice, sorghum or maize preliminarily kept at -20°C for 15 days. Trials of extracts, along purification steps, were performed by incorporating extracts to flour pellets (Wicker, 1984).

Plant material: Purification started from split peas cultivated on organic farms. Seeds were grounded and flour was sieved to 200 µm before extraction.

Mortality tests: Thirty non sexed weevils aged 10 to 14 days were kept on 10 g of pellets containing the extracts. Dead Insects were counted every day for 25 days. Mean mortality percentages were generally established on three repeats.

Extraction: Five organic and three aqueous solvents were used in the first experiment. Ten grams of pea flour were mixed with 100 mL of solvent and kept under agitation for 5 h, at 4°C in the case of aqueous solvents and at room temperature in the case of organic solvents. The aqueous extracts were centrifuged for 20 min at 20000 g under refrigeration. The pellet was washed with 3 times 50 mL of the same solvent. Supernatants were pooled and freeze dried. In the case of organic solvents, they were filtered on glass, the residue washed with 50 mL. Solvents were eliminated in a rotative evaporator.

Dialysis: Spectra/Por dialysis membranes with a molecular cut-off of 3500 and 25000 were used. Dialysis was performed against 100 mL of distilled water. Water was changed every 2 h for 6 h and for the last time after a night at 4°C under a low agitation. The pooled waters were evaporated to 100 mL. Retained and not retained fractions were freeze dried and incorporated in pellets to be tested.

Chromatography: Ion exchange chromatography on DEAE-Cellulose used step elution from pH 9.4 to 3.6. Elution was observed at OD 280 nm, 10 mL fractions were collected. Gel filtration with Fractogel TSK HW 50 (F) (molecular cut-off 200 000 Da) was eluted in 0.066 M phosphate buffer pH 7 containing 0.2 M NaCl. Elution was observed at OD 280 nm, 10 mL fractions were collected.

Electrophoresis: Acrylamide gel electrophoresis in presence of SDS as denaturising reagent was performed following the method of Schagger and Jagow (1987) for small peptides. Silver coloration of Blum *et al.* (1987) was used.

Amino acid analyses: Air-dried of purified protein was hydrolysed, under nitrogen, in HCl vapour at 120°C for 24 h using a Pico-Tag work station (Waters). Along with 2-β-mercaptoethanol (4%), to preserve sulphur-containing amino acids, 200 µL of 6 N HCl were placed in the hydrolysis tank. After hydrolysis, 10 nmol of glucosaminic acid per mg of sample were added as an internal standard. The samples were dried under vacuum in a Speedvac apparatus (Savant Instrument Inc., Farmingdale) and taken up with 0.05 M lithium citrate buffer, pH 2.2. The samples were submitted to ion exchange chromatography on an automatic amino acid analyser (Beckman 3600). Amino acids were detected by the ninhydrin reaction, identified by their retention time and wavelength ratio and quantified by their absorption at 570 nm (440 nm for proline).

Statistical analysis: Means were compared using student t-test and the level of significant difference was determined at p<0.05.

RESULTS

Table 1 shows mortality at 7 and 14 days of insects reared on pellets with extracts by the different solvent used. The only significant mortality was obtained with acidified water. Eighty six percent mortality was obtained at 7 days and 100% mortality was reached at 14 days. All the other extracts led to mortality non significant, close to the control.

Mortality tests of retained and non retained fractions after dialysis (Table 2) show that with the 3500 Da MW cut-off membrane, only the retained fraction is toxic, when with the 25000 Da MW cut-off membrane mortality was

Table 1: Total mortality of insects reared on pellets with different extracts

Extraction solvent	Total mortality (%)	
	At 7 days	At 14 days
Control	3.33	6.66
Distilled water	3.33	6.66
Acidified water (pH 2)	86.66	100.00
Diluted ammoniac (pH 10)	3.33	3.33
Ethanol (95%)	6.66	6.66
Diethyl ether	3.33	6.66
Chloroform	3.33	3.33
n-hexane	3.33	6.66
Methanol	0.00	3.33

Each value is the average of three replicated. Levels of significant (Student t-test) p<0.05

observed in the retained and in the non retained fractions. The MW is superior to 3 500 Da and inferior to 25000 Da MW. Mortalities in the retained 25 000 Da fraction could be the consequence of the non exhaustively of the dialysis. The toxic compound is not a small organic compound, which lead the research to protein biochemistry techniques.

Ion exchange chromatography on DEAE-Cellulose led to numerous fraction which were grouped into 6 bigger fractions containing different peaks at 280 nm (Fig. 1). These fractions submitted to tests showed that fractions IV and V (Table 3) were the only ones with significant mortality. The toxic fractions were eluted shortly after the pH 7 buffer application which means a weak retention on the column.

Table 2: Total mortality of insects on fractions after dialysis (3500 and 25000 Cut off)

Fractions	Total mortality (%)	
	At 7 days	At 14 days
Control	0.00	3.33
Non retained (3 500)	3.33	3.33
Retained (3 500)	76.66	100.00
Non retained (25 000)	36.66	76.66
Retained (25 000)	80.00	100.00

Each value is the average of three replicated. Levels of significant (Student t-test) $p < 0.05$

Table 3: Total mortality of insects on pellets with fractions after chromatography on DEAE cellulose

Fractions	Total mortality (%)	
	At 7 days	At 14 days
Control	0.00	3.33
I	0.00	0.00
II	16.66	26.66
III	3.33	16.66
IV	83.33	100.00
V	26.66	76.66
VI	3.33	3.33

Each value is the average of three replicated. Levels of significant (Student t-test) $p < 0.05$

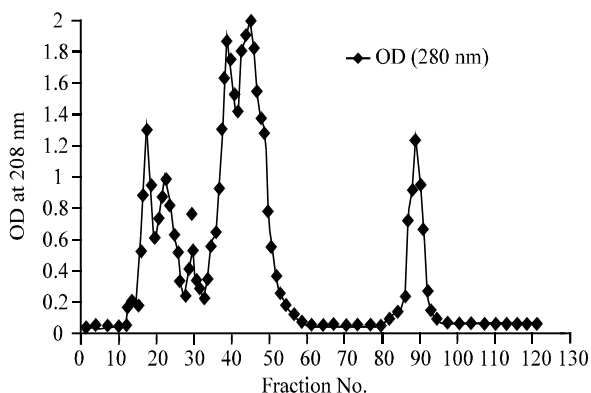


Fig 1: Chromatography on DEAE cellulose

Fractions IV and V from ion exchange were pooled thoroughly dialysed and freeze dried. They were submitted to TSK HW 50 (F) gel filtration to purify in function MW. Five fractions were separated (Fig. 2). Only fraction V showed significant toxicity towards the Insects (Table 4). This fraction is composed of a single symmetric peak at 280 nm and is the last to be eluted, which means the molecular weigh is low and the compound already in good way to complete isolation.

Electrophoresis profile (Fig. 3) shows only one strip in the sample layer. Rf of the molecular weight standards allowed to estimate the log of the MW of the protein at 3.72. Which means a MW of $10^{3.72}$ that is 5248 (Fig. 4). The analysis of its composition of amino acids was calculated according their area (Table 5). This peptide contains from 46 to 48 amino acids

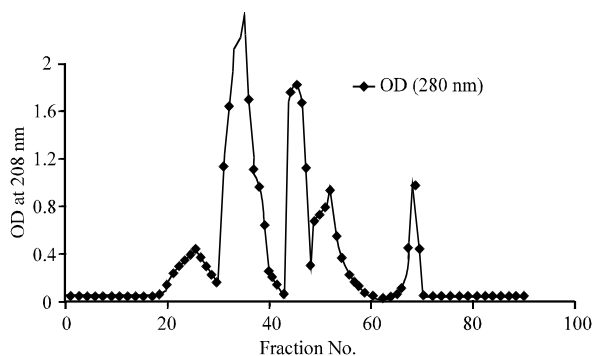


Fig. 2: Chromatography on TSK HW 50 (F)

Table 4: Total mortality of insects on pellets with fractions after chromatography from TSK HW 50 (F)

Fractions	Total mortality (%)	
	At 7 days	At 14 days
Control	3.33	3.33
I	3.33	6.66
II	0.00	3.33
III	0.00	0.00
IV	3.33	3.33
V	76.66	100.00

Each value is the average of three replicated. Levels of significant (Student t-test) $p < 0.05$

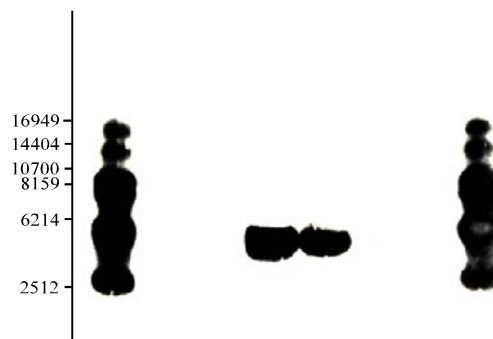


Fig. 3: Electrophoresis profile (left and right = standard of MW; Middle = purified protein)

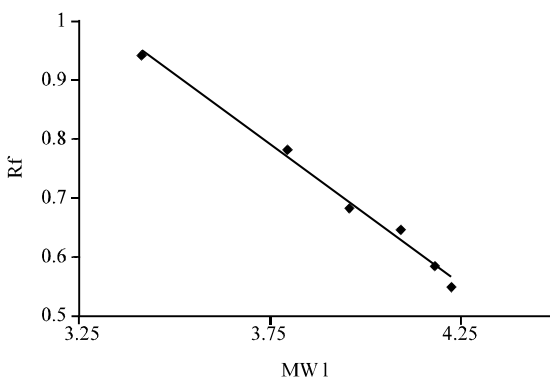


Fig. 4: Estimation of the MW of the purified protein

Table 5: Amino acids composition of the protein based on their area profile

Amino acid	Number
Asx	3
Thr	1 or 2
Ser	5
Glx	5
Pro	1
Gly	17
Ala	5
Val	1 or 2
Cys	-
Met	0
Ile 1	
Leu	2
Tyr	1
Phe	1
Trp	-
Lys	1
His	1
Arg	1
Total number	46 to 48

DISCUSSION

The study shows that the toxicity of the pea for *Sitophilus* weevils is therefore the result of the activity of a small protein. The use of different solvents showed that the toxic compound is only extractible in acidic water and that it is not extracted or destroyed in organic solvents. The electrophoresis profile shows that these results are in good agreement with the hypothesis of a protein base of the toxicity.

This work confirms those done by former researchers. Earlier studies showed that some proteins from legumes are toxic to insects. Lectins, glycosylated proteins: (*P. vulgaris*, *P. coccineus* and *P. acutifolius*) are toxic to bruchid beetles (Gatehouse *et al.*, 1984; Murdock *et al.*, 1990). Another protein named arcelin, a lectin like from *P. acutifolius* is toxic to *Acanthoscelides obtectus*, a seed feeding bruchid commonly known as bean weevil (Shade *et al.*, 1986; Pratt *et al.*, 1990). Enzyme inhibitors like protease inhibitors or amylase inhibitors are also toxic to insects (Gatehouse and Boulter, 1983; Broadway *et al.*,

1986; Laroque and Houseman, 1990; Chrispeels and Raihkel, 1991; Ishimoto and Chrispeels, 1996; Ussuf *et al.*, 2001).

Pea flour, introduced into pellets caused significant mortality of *Sitophilus* weevils and reduced progeny (Combs *et al.*, 1977; Wicker, 1984; Holloway and Smith, 1985; Grenier *et al.*, 1997). Mohan and Fields (2002) have demonstrated that the movement of *S. oryzae* increased when the adults of this beetle are exposed on pea protein treated wheat for a period of 24 h. Hou and Fields (2003) found that barley, treated with pea flour extract at 0.1% reduced the number of *S. oryzae* by 93%. Hou and Fields (2004) have conducted tests with combination of wheat treated with protein-rich pea flour and a parasitoid of *S. oryzae* (*Cryptolestes ferrugineus*). The study found that the protein-rich flour was toxic and repellent to *S. oryzae* but not toxic to the parasitoid. Jouvansal *et al.* (2003) have purified and determine the 3-D structure of PA1b (a pea albumin 1, subunit b) which contains 37 amino acids and induce a short term mortality of several insects among which the cereals weevils. PA1b contains 37 amino acids and is cysteine-rich. This one contains 46 to 48 amino acids and is glycine-rich. Is it the same protein or an isoform of the PA1b?. Further studies of this purified protein are necessary to answer to that question.

Preservation of food has always been Man worry. Many methods of preservation of cereals have been or are used, some are expensive and some have important drawbacks. Physical methods (cooling of silos, ionisation, gas using) need expensive equipments and energy sources which very difficult to make use of in poor African countries. Use of chemical pesticides is very useful but remains expensive and needs good practices to be safely used. Moreover, it may create long term effects on environment and on man health. At last, after all the hopes of years 1950 to 1970, enthusiasm has decreased with the increasing appearance of resistant insect strains (Heather, 1986; Basode and Bhatia, 1990).

Besides biological control, researches develop on transgenic plants with resistance to insects. It may bring a valuable plant protection if it is used with the required cautions. This small molecular weight protein from Legumes, already eaten by man, could be a good candidate to these new techniques.

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REFERENCES

- Basode, P.C. and S.K. Bhatia, 1990. Nature of malathion resistance in a laboratory selected strain of *Sitophilus oryzae* L. J. Entomol. Res., 14 : 189-191.
- Blum, H., H. Beier and J.H. Gross, 1987. Improve silver staining of plant proteins, RNA and DNA in acrylamide gels. Electrophoresis, 8: 93-99.
- Broadway, R.M., S.S. Duffey, G. Pearce and C.A. Ryan, 1986. Plant proteinase inhibitors: A defense against herbivorous insects? Entomologia Experimentalis Applicata, 41: 33-38.
- Combs, C.W., C.J. Bilings and J.E. Porter, 1977. The effect of yellow split peas (*Pisum sativum* L.) and other pulses on the productivity of certain strains of *Sitophilus oryzae* L. (Coleoptera: Curculionidae) and the ability of other strains to breed thereon. J. Stored Prod. Res., 13: 53-58.
- Chrispeels, M.J. and N.V. Raikhel, 1991. Lectins, lectin genes and their role in plant defense. The Plant Cell, 3: 1-19.
- Delobel, A. and M. Tran, 1993. Coleopterous pests of food staff and other agriculture products in hot regions. Paris, ORSTOM, CTA, faune tropicale, pp: 411.
- Gatehouse, A.M.R. and D. Boulter, 1983. Assessment of the antimetabolic effects of trypsin inhibitors from cowpea (*Vigna unguiculata*) and other legumes on development of the bruchid beetle *callosobruchus maculatus*. J. Sci. Food Agric., 34: 345-350.
- Gatehouse, A.M.R., F.M. Dewey, J. Dove, K.A. Fenton and A. Putzai, 1984. Effect of seed lectins from *Phaseolus vulgaris* on the development of larvae of *Callosobruchus maculatus*; mechanism of toxicity. J. Sci. Food Agric., 35: 373-380.
- Grenier, A.M., P. Nardon and C. Wicker, 1986. Effect of food change on the development, N-acetylglucosaminidase activity and symbionts of *Sitophilus oryzae* L. (Col. Curculionidae). Bulletin de la Société Entomologique de France, 88: 324-332.
- Grenier, A.M., M. Mbaiguinam and B. Delobel, 1997. Genetical analysis of the ability of the rice weevil *Sitophilus oryzae* L. (Coleoptera: Curculionidae) to breed on split peas. Heredity, 79: 15-23.
- Heather, N.W., 1986. Sex-linked resistance to pyrethroids in *Sitophilus oryzae* L. (Coleoptera : Curculionidae). J. Stored Prod. Res., 22: 15-20.
- Holloway, G.J. and R.H. Smith 1985. Inheritance of the ability of *S. oryzae* L. (Coleoptera: Curculionidae) to feed and breed on yellow split pea (*Pisum sativum*). Bull. Entomol. Res., 75: 367-375.
- Hou, X. and P. Fields, 2003. Granary trial of protein-enriched pea flour for the control of three stored-product insects in barley. J. Econ. Entomol., 96: 1005-1015.
- Hou, X. and P. Fields, 2004. Control of stored product beetles with combinations of protein-rich pea flour and parasitoids. Environ. Entomol., 33: 671-680.
- Ishimoto, M. and M.J. Chrispeels, 1996. Protective mechanism of the Mexican bean weevil against high levels of α -amylase inhibitor in the common bean. Plant Physiol., 111: 393-401.
- Jouvensal, L., L. Quillien, E. Ferrasson, Y. Rhabe, J. Gueguen and F. Vovelle, 2003. PA1b, an insecticidal protein extracted from pea seeds (*Pisum sativum*): 1H-2-DNMR study and molecular modeling. Biochemistry, 42: 11915-11923.
- Laroque, A.M. and J.G. Houseman, 1990. Effect of ingest soybean, ovomucoid and corn protease inhibitors on digestive processes of the European corn borer, *Ostrinia nubilalis* (Lepidoptera:Pyralidae). J.Insect Physiol., 36: 691-697.
- Mohan, S. and P. Fields, 2002. A simple technique to assess compounds that are repellent or attractive to stored product insects. J. Stored Products Res., 38: 23-31.
- Murdock, L.M., J.E. Huessing, S.S. Nielszen, R.C. Pratt and R.E. Shade, 1990. Biological effects of plant lectins on the cowpea weevil. Phytochemistry, 29: 85-89.
- Pratt, R.C., N.K. Singh, R.E. Shade, L.L. Murdock and R.A. Bressan, 1990. Isolation and partial characterization of a seed lectin from Tepary Bean that delays bruchid beetle development. Plant Physiol., 93: 1453-1459.
- Pretheep-Kumar, P., S. Mohan and K. Ramaraju, 2004. Protein-enriched pea flour extract protects stored milled rice against the rice weevil, *Sitophilus oryzae*. J. Insect Sci., 4: 26-29.
- Rider, W., 1982. Agriculture: The roots of deterioration. New Scientist, 54: 567-568.
- Putzai, A., R.R.D. Croy, G. Grant and J.C. Stewart, 1983. Seed lectins: Distribution, Location and Biological Role. In Daussant, J.J. Mosse J. Vaughn Eds., Academic Press, New York, 53-82.
- Schagger, H. and G.V. Jagow, 1987. Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis for separations of proteins in the range from 1 to 10 kDa. Anal. Biochem., 166: 368-379.
- Shade, R.E., L.L. Murdock, D.E. Foard and M.A. Pomeroy, 1986. Artificial seed system for bioassay of cowpea weevil (Coleoptera:Bruchidae) growth and development. Environ. Entomol., 15: 1286-1291.

- Thind, B.B. and J. Muggleton, 1981. Inheritance of the ability of strains of *Sitophilus oryzae* L. (Coleoptera: Curculionidae) to breed on split pea (*Pisum sativum*). Bull. Entomol. Res., 71: 419-424.
- Ussuf, K.K., N.H. Laxmi and R. Mitra, 2001. Proteinase inhibitors: Plant-derived genes of insecticidal protein for developing insect-resistant transgenic plants. Current Sci., 80: 8847-853.
- Wicker, C., 1984. Nutritional and Enzymatic interactions between *Sitophilus oryzae* (Coleoptera: Curculionidae) and his symbiotic bacteria. INSA de Lyon, Ph. D Thesis.