

# ESPRESSIONE DELLE PROTEASI NEGLI STADI LARVALI I-V DI *OSMIA CORNUTA* EVIDENZIATA MEDIANTE ZIMOGRAFIA

## PROTEINASES PATTERN CHANGES IN *OSMIA CORNUTA* I-V INSTAR LARVAE INVESTIGATED BY ZYMOGRAPHY

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### RIASSUNTO

L'allevamento in pieno campo di *Osmia cornuta* è ancora necessario a causa della mancanza di informazioni sui meccanismi molecolari che regolano la nutrizione di tali apoidei. Lo scopo di questo studio è stato quello di dare un contributo nella descrizione della variazione del pattern proteasico così da dirottare, in parte, le strategie di allevamento in pieno campo verso quelle di laboratorio.

Da celle pedotrofiche prelevate da un nido di *Osmia* sono stati campionati gli stadi larvali I, III, V e V maturo di *O. cornuta*. Le proteine delle larve sono state estratte in presenza di un tampone nativo, così da preservare l'eventuale presenza dell'attività enzimatica delle proteasi; l'omogenato così ottenuto è stato centrifugato e il surnatante analizzato mediante elettroforesi per attività. Il gel è stato preparato facendo copolimerizzare l'acrilammide in presenza di gelatina, e dopo la corsa il gel è stato incubato per 1 ora a pH 8 a 37°C. Infine il gel è stato colorato con Blue di Coomassie.

L'analisi degli zimogrammi rileva che il pattern proteasico varia durante l'ontogenesi larvale, in particolare nel I stadio larvale sono risultate ben visibili 3 bande di attività proteasica, nel III stadio tali bande sono diventate 4, nel V stadio larvale sono risultate visibili ancora 4 bande di attività, di cui 3 in comune con il terzo stadio, infine nel V stadio larvale maturo è visibile una sola banda di attività.

Questo approccio molecolare può dare un contributo allo studio dei meccanismi di digestione di questo apoidee solitario nell'ottica della messa a punto di una dieta artificiale.

Parole chiave: *Osmia cornuta*; larve; proteasi; zimografia.

### SUMMARY

Field rearing of *Osmia cornuta* is still necessary due to lack of knowledge in nutrition molecular mechanisms. The aim of this investigation was to give a contribute in describing the changes in the expression of the proteinase pattern in order to direct the osmia bee rearing strategy from nest-trapping (NT) and releasing-and-rearing (RR), towards more con-

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trolled conditions such as the laboratory (captivity), whilst optimising their use for crop pollination in both open field and confined environments.

I, III, V and mature V instar larvae of *Osmia cornuta* were sampled from a pedotrophic nest. Proteins were extracted in a native buffer, in order to preserve the proteolytic activity; homogenate was centrifuged and the supernatant was analysed by zymography. Acrylamide gel has been precasted with gelatine. After electrophoresis, gel was incubated for at 37°C 1 hour in a pH 8 buffer. After incubation gel was stained with Blue Coomassie.

Zymograms show that proteinase pattern change during larval ontogenesis. In particular, in the I instar 3 activity bands are well detectable, in the III and V instar 4 bands are detectable, of these 3 are in common, in the V mature instar only one band is detectable.

This molecular approach could help in investigating the digestion mechanisms of this solitary bee in order to gain more information to project an artificial diet.

Key words: *Osmia cornuta*; larvae; proteinase; zymography.

## INTRODUCTION

Up to now the releasing-and-rearing (RR) strategy has been the most common method to develop an osmia bee population obtained by nest-trapping (Bohart, 1972; Torchio, 1985; Pinzauti, 1991; Bosh, 1994a, b; Krunic' et al., 1995; Krunic' et al., 2005).

The RR strategy is based on the SWOT system (Strength, Weakness, Opportunity and Threat) that focalises on the critical phases of a process (Felicoli et al., 2004b). The Strengths of the RR strategy phases are gregarism, the ease of acceptance of artificial nests and the presence of a diapause period. The Weaknesses are the biotic and abiotic limiting factors on the populations, dispersion after release and the lack of available technology. The Opportunities include the diapause modularity, availability of rearing sites, nest handling and management of food sources. The Threats are represented by pesticides and parasite proliferation (Pinzauti, 2002; Krunic' et al., 2005).

Abiotic and biotic factors still influence field rearing of bees. Field rearing of bees is still necessary due to lack of knowledge in diapause and nutrition molecular mechanisms (Ladurner et al., 1999; Felicoli et al., 2004a).

*Osmia* bees rearing, their management in pollination, their economic values and their diffusion as a commercial available pollinator tool in agriculture could be improved by their year-round rearing that could be obtained with the development of a laboratory rearing conditions. In particular, food availability, luminosity conditions, population density, fat metabolism during diapause, diapause plasticity, sex-ratio and aging are the critical limiting factors when trying to rear osmia bees in laboratory conditions (Ladurner et al., 1999; Felicoli et al., 2004b).

Investigations have been made in setting an artificial diet composed primarily of crude ingredients and by investigating the possible presence of nectar in the pollen provision of *Osmia cornuta* and *Osmia rufa* (Steen van Den, 1997; Ladurner et al., 1999; Maccagnani et al., 2002a,b).

Efforts to direct the osmia bee rearing strategy from nest-trapping (NT) and releasing-and-rearing (RR), towards more controlled conditions such as the laboratory (captivity), whilst optimising their use for crop pollination in both open field and confined environments, can be improved by a molecular approach.

The aim of this investigation was to describe the proteinase expression profile pattern during larval ontogenesis of *Osmia cornuta*.

## MATERIALS AND METHODS

### *Chemicals*

Chemicals and high-purity solvents were obtained from Sigma Chemical (St Louis, MO).

### *Biological samples*

Insect specimens came from a releasing and rearing campaign performed in the coastal area of Tuscany (Italy) during the March-April 2005 in the Laboratory of Apidology of Pisa University. Four female larval stages were identified on the basis of both size and presence of the previous instar exuvia (Rust et al., 1989). Female larvae were separated into the following groups: I instar (sample 1), III instar (sample 2), V instar (sample 3) and mature V instar (sample 4).

### *Crude extracts preparation*

After thawing, insects were homogenised with 50 mM Tris-HCl pH 8.0 (0.1 g of insect/mL of buffer, 0.25 g of pollen/mL of buffer) at room temperature. Sixty specimens of each sample were used for extraction, 3 extractions of 20 specimens each. The homogenates were then centrifuged at 2500 g for 15 min at 4 °C for debris removal and at 30 000 g for 40 min at the same temperature for supernatant clarification. Total protein content was determined by the colorimetric method of Bradford (1976) using ovalbumin as reference protein and equalized in the different samples by dilution with the same buffer. The supernatants were then stored at -20 °C until analysed.

### *Zymography*

Zymography was performed in a Miniprotean II apparatus (Bio-Rad Laboratories, Hercules, CA). Proteins in the clarified extracts from each bee sample underwent Proteolytic activity-staining electrophoresis (Heussen & Dowdle, 1980), using 0.1% gelatine as precast protein substrate in a discontinuous 12% T Laemmli's electrophoresis system under semi-denaturing conditions (before loading, samples were 1:2 diluted with 2% SDS without boiling). No  $\beta$ -mercaptoethanol was added. Ten  $\mu$ L of each clarified extracts (~30  $\mu$ g of proteins) were loaded in each lane. After electrophoresis, gels were shaken gently at room temperature for 30 min in 100 mL 2% Triton X-100 in water to remove SDS and restore full enzyme activity. Gels were then transferred to a bath containing 100 mM Tris-HCl buffer,

pH 8.0 and kept under mild shaking, at 37 °C for 1 hour, then stained with 0.1% Blue R250 Coomassie and de-stained with 40% methanol and 10% acetic acid.

#### *Gel analysis*

Single lanes were densitometrically scanned and data analysed by means of the Quantity One 4.2.3 software (Bio-Rad Laboratories). White bands were identified and circled and the activity was expressed as the sum of the pixel intensities abbreviated as p.i., as already reported in a previous paper (Felicioli et al., 2004a).

## RESULTS

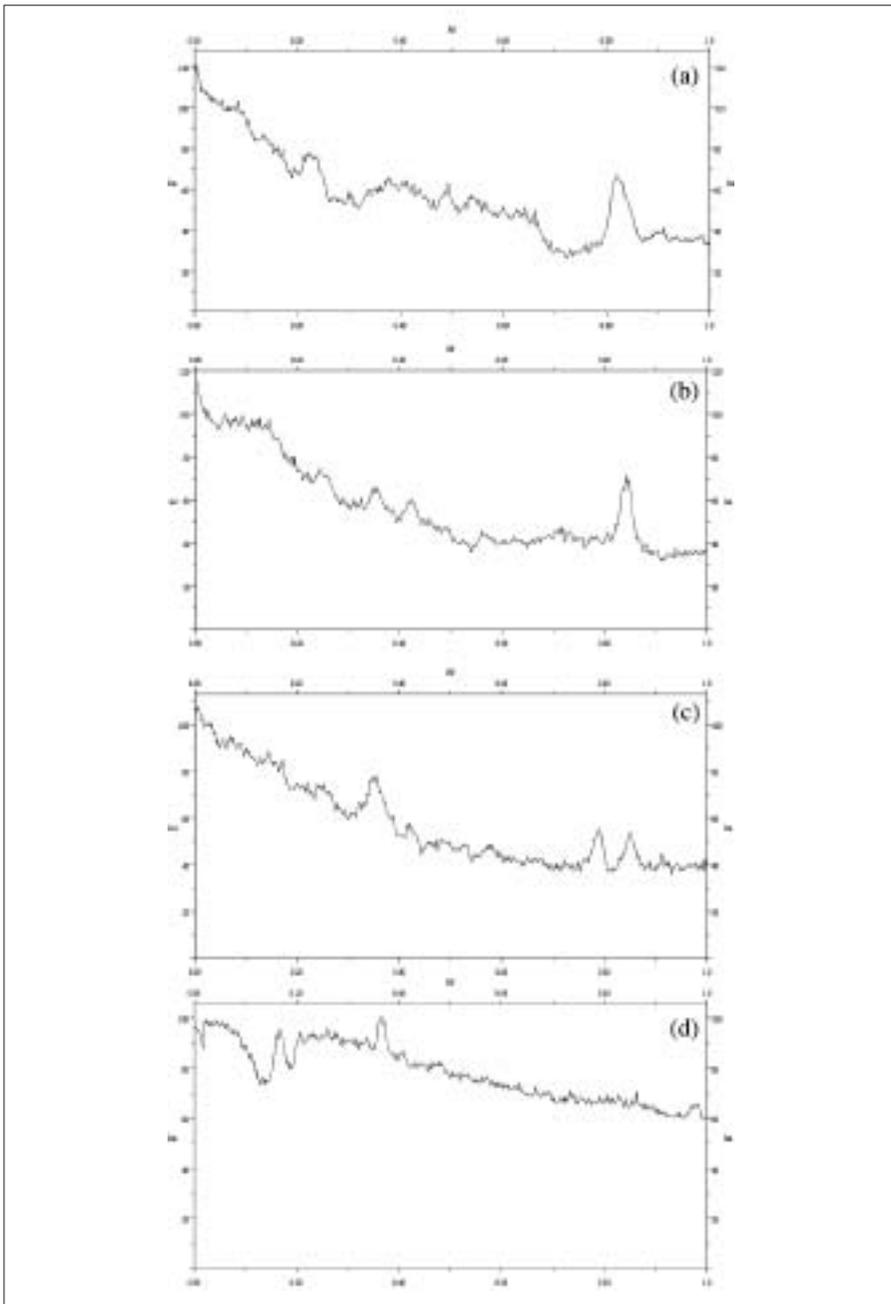
Fig. 1 shows the scanning record (trace) of each lane. In the I instar (sample 1) three activity bands (0.24 Rm; 0.5 Rm; 0.85 Rm) of different intensities were revealed, two diffuse active areas are localised at 0.1 and 0.4 Rm; the major active band is localised at 0.85 Rm (Fig. 1a). In the III instar (sample 2) four activity bands (0.24 Rm; 0.36 Rm; 0.42 Rm; 0.85 Rm) of different intensities were revealed; the major active one is at 0.85 Rm (Fig. 1b). In the V instar (sample 3) four activity bands (0.36 Rm; 0.42 Rm; 0.78 Rm; 0.85 Rm) of different intensities were revealed; the major active band is localised at 0.35 Rm (Fig. 1c). In the V mature instar (sample 4) only one activity band (0.36 Rm) was revealed (Fig. 1d), in this stage, all the other proteinases became undetectable. In Fig. 1 are visible two downward peaks that correspond at two protein bands. The detectability of this two protein bands was possible due to an high expression of these proteins in this larval stage that result in a concentration higher than the gelatine precast in the gel.

## DISCUSSION

In this investigation we compare the variation in the expression profile of several soluble proteinases during ontogeny of the solitary bee *Osmia cornuta* by zymography PAGE. The use of sensitive scanning and quantifiable method renders it a useful tool for a comparative investigation of proteinase patterns in various biological samples (Vaccari et al., 1998; Nanni et al., 2001; Felicioli et al., 2004a). The most active band appeared in the 1-3 samples which are characterised by high growth rate and pollen intake. In the following instar (sample 4) in which no pollen intake occurs only one proteinase activity is still visible

Our results suggest that the 0.24; 0.42; 0.5; 0.78; 0.85 Rm bands are probably related to growth and pollen intake while 0.36 Rm could have another role than the digestive one.

The high number of activities we found in sample 1-3 and their decrease in the sample 4 larval phase strongly suggest that these detectable proteinases may be involved in pollen digestion. This hypothesis is in agreement with data concerning the presence of several proteolytic activities with a digestive role at alkaline pH



**Fig. 1.** Densitometric evaluation of the proteinase activities detected by zymography of I instar (a), III instar (b), V instar (c) and mature V instar (d) crude extract.

detectable in the midgut of several insects (Purcell et al., 1992; Walker et al., 1998; Jimenez & Gilliam, 1989; Felicioli et al., 2004a). The disappearance of some proteolytic activity bands in the sample 4 is in agreement with previous findings in *Apis mellifera*, where two chymotrypsin- and two trypsin-like proteinases with a digestive role have been described in the midgut of larval and adult worker honeybees (Dahlmann et al., 1978) and with the decrease of all the proteolytic activities throughout the investigated instar larvae of *Megachile rotundata* (Felicioli et al., 2004a).

Investigations have been made in setting an artificial diet for *Osmia cornuta* and *Osmia rufa* rearing (Steen van Den, 1997; Ladurner et al., 1999; Maccagnani et al., 2002a,b) and results indicate that something missing in the artificial diet prevented the larvae from completing their development suggesting that *O. cornuta* females produce and add something to the pollen provision, that is called “maternal factor”, essential for the progeny survival. Pollen digestion mechanisms need to be investigated in order to understand if the “maternal factor” is added to pollen provisions.

The characterization by inhibitors or sequencing of the proteinases detected in this investigation could help in setting a suitable artificial diet.

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