

Animal biology and pathology / Biologie et pathologie animales

Midgut protease activities in monophagous larvae of Apollo butterfly, *Parnassius apollo* ssp. *frankenbergeri*

Mirosław Nakonieczny*, Katarzyna Michalczyk, Andrzej Kędziorski

University of Silesia, Department of Animal Physiology and Ecotoxicology, Bankowa 9, 40-007 Katowice, Poland

Received 31 July 2006; accepted after revision 12 December 2006

Available online 16 January 2007

Presented by Pierre Buser

Abstract

We assayed the relative activities of midgut proteolytic enzymes in individuals of the fourth (L₄) and fifth (L₅) instar of Apollo larvae, inhabiting Pieniny Mts (southern Poland). The comparisons between midgut tissue with glicocalyx (MT) and liquid midgut contents with peritrophic membrane (MC) were made. Optimal media pHs of the assayed proteolytic enzymes in *P. apollo* midgut samples were similar to those of other lepidopteran species. Endopeptidases, as well as carboxypeptidases, digested effectively in alkaline environment, while aminopeptidases were active in a broad pH range. Trypsin is probably the main endoprotease (correlation with caseinolytic activity in MC of L₅ larvae: $r = 0.606$; $p = 0.004$); however, its activity was low as compared with that in other leaf-eating Lepidoptera. This suggests a minor role of trypsin and chymotrypsin in protein digestion in Apollo larvae, probably due to limited availability of the leaf proteins. Instead, due to very high carboxypeptidase A activity in midgut tissue, the larvae obtain exogenous amino acids either directly or from oligopeptides and glycoproteins. High and significant positive correlations between the enzyme activity and glucosidase as well as galactosidase activities strongly support this opinion. **To cite this article:** M. Nakonieczny et al., *C. R. Biologies 330* (2007).

© 2006 Académie des sciences. Published by Elsevier Masson SAS. All rights reserved.

Résumé

Les activités des protéases chez les larves du monophage *Parnassius apollo* ssp. *frankenbergeri*. On a analysé les activités relatives des enzymes protéolytiques chez des individus d'Apollon, vivant dans les monts de Pieniny (Sud de la Pologne), aux quatrième (L₄) et cinquième (L₅) stades larvaire. Les tissus contenant le glycocalyx (MT) et le liquide de l'intestin moyen, contenant la pellicule péritrophique (MC), ont été comparés. Les pH optimaux des enzymes protéolytiques dans l'intestin moyen de *P. apollo* sont comparables à ceux des autres Lépidoptères. Les endopeptidases, tout comme les carboxypeptidases, sont plus actives en milieu alcalin ; cependant, les aminopeptidases sont plus actives dans un large domaine du pH. La trypsine constitue probablement l'endoprotéase principale (la corrélation avec l'activité caséinolytique dans la pellicule péritrophique chez les chenilles au stade L₅ est : $r = 0,606$, $p = 0,004$), quoique son activité soit faible en comparaison de celle observée chez les autres Lépidoptères se nourrissant de feuilles. Ceci suggère que la trypsine, comme la chymotrypsine, ne joue pas un rôle significatif dans la digestion des protéines chez les chenilles de *P. apollo*, probablement à cause d'une accessibilité limitée des protéines foliaires. Grâce à la haute activité de la carboxypeptidase, les chenilles reçoivent les aminoacides exogènes, soit directement, soit à partir des oligopeptides et des glycoprotéines. Les corrélations positives et très significatives observées entre l'activité de la carboxypeptidase et celles

* Corresponding author.

E-mail address: nakon@us.edu.pl (M. Nakonieczny).

des glucosidases et des galactosidases rendent cette hypothèse très plausible. **Pour citer cet article :** M. Nakonieczny et al., C. R. Biologies 330 (2007).

© 2006 Académie des sciences. Published by Elsevier Masson SAS. All rights reserved.

Keywords: *Parnassius apollo*; Apollo butterfly; Midgut; Digestive enzymes; Proteases

Mots-clés : *Parnassius apollo* ; Apollon ; Intestin moyen ; Enzymes digestives ; Protéases

1. Introduction

Parnassius apollo L. (Lepidoptera, Papilionidae) is a species of steppe origin, with plenty of subspecies and races. Some of them underwent decline or became extinct during the last several decades [1]. *Parnassius apollo* ssp. *frankenbergeri* SLABÝ inhabiting the Pieniny Mts (southern Poland) has avoided this threat only due to a recovery plan initiated in 1991 (see [1] for detailed references).

Larvae of Apollo butterfly from the Pieniny Mts were considered as oligophagous, since in natural conditions they were observed feeding on *Sedum telephium* ssp. *maximum* and *Sempervivum soboliferum* [2]. Since *S. telephium* is preferred as a food-plant, this subspecies falls into a ‘telephiophagous’ trophic group, together with other Carpathian, as well as Scandinavian and Balkan forms [3]. According to Pekarsky, the ‘albophagous’ group of *P. apollo*, inhabiting the Alps and southwestern Europe, shifted from *S. telephium* to *S. album* because of the disappearance or shortage of the former food-plant in their biotopes as the result of long-term climatic changes. Further observations supported this hypothesis: it has been noticed that ‘telephiophagous’ larvae and imagoes are larger than ‘albophagous’ ones [2]. Moreover, larvae of alpine ‘albophagous’ subspecies that fed on *S. telephium* were larger and their development was faster than on native *S. album* [4]. Recent experiments on *P. apollo* ssp. *frankenbergeri* showed that this subspecies is a monophage only successfully developing on *S. telephium* [1].

Analysis of the chemical contents of *S. telephium* has revealed the presence of organic acids, amino acids and carbohydrates in its aboveground parts [5]. Further phytochemical studies showed low contents of organic substances (app. 8% wet weight of leaves) and the presence of secondary compounds, mainly various phenolic acids (also as glycosides), and small amounts of non-phototoxic coumarins. There were no alkaloids, cyanogenic glycosides or their derivatives [6]. Phenolic acids are known as feeding deterrents, hardly ever as attractants or nutrient source [7]. Phenolic compounds and coumarins seem to constitute the plant defence system, although no clear changes of their concentrations

have been stated so far during the vegetation period or in the response to larval feeding [6]. On the other hand, in natural conditions larvae eat no more than about 30% of the leaves of the single plant, then move to another one [8]. It has also been found that rearing *P. apollo* on artificially cultivated orpine (in changed photoperiod) resulted in digestion disturbances and increased larval mortality [9,10].

A last-instar Apollo larva in hyperphagic phase consumes about 2 g of plant tissues daily, which contain 20–50 µg of phenolic acids [6]. This relatively fast chyme passage through its alimentary tract may be the adaptation to the presence of the secondary compounds in the food plant. Such a response can diminish harmful effects of allochemicals, but simultaneously may decrease nutrients availability, particularly amino acids from the plant proteins.

Adaptation of a herbivore digestive system to a specific food source involves a synthesis of an appropriate set of digestive enzymes [11], regulation of their excretion [12] and maintenance of alkaline condition in the midgut lumen (pH between 9.5–12) [13]. These species-specific changes must ensure obtaining sufficient amounts of necessary nutrients to complete the feeding stage of development [12]. The critical aspect in proteins digestion is the release of exogenous amino acids (e.g., arginine, lysine, and few others) necessary for the synthesis of wide arrays of structural and functional proteins [14]. Broadway has shown that the trypsin:chymotrypsin ratio determines the protein-digestion strategy of an insect. When larvae use only trypsin as endoprotease, their growth is positively correlated with the amount of arginine and lysine in the ingested proteins. A set of endoproteases enables faster and more effective proteins digestion. Hence, larvae of oligophagous *Pieris rapae* (Pieridae), with relatively high protein requirements and limited food sources, use different endoproteases (trypsin:chymotrypsin ratio 1:1), in comparison with polyphagous *Trichoplusia ni* (Noctuidae) larvae, which have a very low chymotrypsin activity (trypsin:chymotrypsin ratio 3:1) [15].

So far, studies on lepidopteran proteolytic enzymes have focused mainly on their activities in pest species.

The current leading issues are: enzyme inhibition [16–18] with respect to plant protease inhibitors [19,20], determination of the primary protein structure, nucleotide sequence in a corresponding gene [21], and the effects of plant anti-nutritional substances on larval growth of the pests [22].

These investigations, aimed primarily at developing effective biological methods of pests' control, have never been applied to disappearing species like *Parnassius apollo*. The preliminary description of the proteolytic activity in Apollo larvae is the goal of this study. This paper continues our earlier contribution to the digestive strategy in this species [23].

2. Materials and methods

The larvae of *Parnassius apollo* ssp. *frankenbergeri* SLABÝ originated from a semi-natural open-field breeding colony, set up in the Pieniny National Park, for a recovery plan in the Pieniny Mts. Following the permission of the Polish Ministry of Environment, the individuals for enzymatic analyses were obtained from eggs collected in the preceding year. The fourth (L₄) and fifth (L₅) instar larvae were randomly chosen from the stock colony. Additionally L₅ larvae were divided into two subgroups ('young' and 'hyperphagic'), as previously described [23]. For enzymatic assays, two samples from each individual were obtained: midgut tissue with glicocalyx (MT), and liquid gut contents with peritrophic membrane (MC).

The optimal pH for selected proteases (total proteolytic (caseinolytic) activity, trypsin and aminopeptidase) was assessed using 0.1 M phosphate (pH 5.8–7.8), 0.05 M Tris-maleate (pH 5.8–7.8), 0.05 M Tris-HCl (pH 7.4–9.0) and 0.1 M glycine-NaOH (pH 8.6–10.6) buffers. The ionic strength of the buffers was adjusted according to the methods described for other Lepidoptera (see Table 1 for references). The incubation temperature for all the assays was 30 °C. Moreover, the temperature-dependent response for trypsin activity was determined.

The applied colorimetric procedures and used substrates are summarized in Table 1. Protein concentrations were determined according to Bradford [33]. Due to the limited number of individuals for the planned analyses, an enzymatic assay for each sample was duplicated and the obtained mean was taken for further calculations.

Common names of enzymes were simplified according to the last review of Terra and Ferreira [34]. Despite some functional differences between insect and vertebrate endopeptidases, the authors use 'trypsin' and 'chy-

motrypsin' instead of 'trypsin-like' and 'chymotrypsin-like', respectively. In a previous paper, they used both names interchangeably [35].

Statistical analyses were performed with ANOVA/MANOVA, and T Tukey's test for unequal sample size was applied for a *post hoc* analysis. Test *t* for dependent variables was used to estimate the significant differences between the samples containing the midgut tissues (MT) or the liquid gut contents (MC) in the same larval instar ($p < 0.05$). At the same *p* level, Pearson's linear correlations of the coefficients between activities of the assayed enzymes were calculated.

3. Results

The highest total proteolytic (caseinolytic) activity in relation to various media was stated in glycine-NaOH buffer within a pH range of 9.2–9.6. For trypsin, it was noted in the same buffer at pH 10.4. On the contrary, high midgut aminopeptidase activity occurred in a wide pH range: 6.6–9.4 (Fig. 1). According to Houseman and co-workers, Apollo larvae trypsin is of a 'high-alkaline' type (optimal pH > 10.0) [36]. We also noticed that its activity was influenced by buffer compounds: at the same pH (8.5 and 9.0), it was higher in Tris-HCl than in glycine-NaOH buffer (Fig. 1).

Trypsin activity of *P. apollo* larvae increased along with the elevation of incubation temperature (Fig. 2). The dependence was not linear – the highest Q_{10} value (2.28) was stated for the 25–35 °C range.

Digestion of polypeptides and oligopeptides was stated in the midguts of L₄ and L₅ instars of *P. apollo* larvae (Table 2, Fig. 3). In L₅ larvae total proteolytic activity was lower in the MT than in the MC samples ($p = 0.0002$). Comparison with the MC samples of L₄ larvae revealed a similar level of enzyme activity. Also trypsin and chymotrypsin activities towards B-R-*p*NA (*N*- α -benzoyl-DL-arginine *p*-nitroanilide) and Suc-AAPF-*p*NA (*N*-succinyl-alanine-alanine-proline-phenylalanine *p*-nitroanilide), respectively, were found to be higher in the MC than in the MT ($p = 0.0002$ and $p = 0.0001$, respectively). The activity of chymotrypsin towards G-F-*p*NA (*N*-glutaryl-L-phenylalanine *p*-nitroanilide) was not detected. On the other hand, activities of exoproteases, stated in L₅ larvae, were higher in the MT than in the MC samples ($p = 0.0002$ both for carboxypeptidases and aminopeptidases). Activities of these enzymes in the MC did not differ when compared with those stated in L₄ larvae (Table 2).

In 'young' and 'hyperphagic' larvae, activities of the assayed enzymes were similar, except carboxypep-

Table 1

Assay conditions and methods used in the determination of proteases

Enzyme	Substrate	Buffer for substrate	Homogenate volume [μ l]	Incubation [min]	Termination	Further procedures	Final vol. [ml]	Final product	λ [nm]	Comments	References
Total proteolytic (caseinolytic) activity	50 μ l 1% azocasein	glycine–NaOH: 0.1 M, pH 9.8	50	30 ^a or 60 ^b	0.5 ml 8% TCA	5 min of precipitation of azocasein fragments; centrifugation at 8000 \times g, 10 min	0.6	azopeptides	366	measurement until 10 min	[24,25]
Trypsin	3 ml 1 mM B-R- <i>p</i> NA in 15% DMF	glycine–NaOH: 0.1 M, pH 10.4, 20 mM CaCl ₂	50	30 ^b or 45 ^a	0.6 ml 20% acetic acid	–	3.65	nitroaniline	410	measurement after 30 min; $\epsilon_{410} = 8800$	[26,27] modified after [17,28]
Chymotrypsin	3 ml 1 mM G-F- <i>p</i> NA in 10% DMF	glycine–NaOH: 0.1 M, pH 10.6, 20 mM CaCl ₂	50	60	0.6 ml 20% acetic acid	–	3.65				
	0.6 ml 0.25 mM Suc-AAPF- <i>p</i> NA in 10% DMF			10	60 ^a or 30 ^b	0.12 ml 20% acetic acid	–				
Aminopeptidase	3 ml 1 mM L- <i>p</i> NA in 10% DMF	Tris-HCl: 0.05 M pH 9.0, 5 mM MgCl ₂ \times 6H ₂ O	50	15 ^a (30 ^a) or 60 ^b	0.6 ml 20% acetic acid	–	3.65	nitroaniline	410	measurement after 30 min; $\epsilon_{410} = 8800$	[26,29] modified after [28]
Carboxypeptidase A	0.5 ml 1 mM <i>N</i> -CBZ-V-L	Tris-HCl: 0.05 M pH 9.0, 0.1 M NaCl	50	15 ^a (30 ^a) or 60 ^b	1.5 ml acetate buffer: 0.4 M, pH 5.0	2 ml ninhydrin reagent, 30 min at 100 °C, chilling, 6 ml 50% ethanol	10.05	amino acids	570	measurement after 20–30 min	[30–32] modified after [11]

Abbreviations: B-R-*p*NA (*N*- α -benzoyl-DL-arginine *p*-nitroanilide); DMF (*N,N*-dimethylformamide); G-F-*p*NA (*N*-glutaryl-L-phenylalanine *p*-nitroanilide); L-*p*NA (leucine *p*-nitroanilide); *N*-CBZ-V-L (*N*-carbobenzoxy-L-valine-leucine); Suc-AAPF-*p*NA (*N*-succinyl-alanine-alanine-proline-phenylalanine *p*-nitroanilide); SDS (sodium dodecyl sulphate); TCA (trichloroacetic acid); Tris (Tris-(hydroxy-methyl)-aminomethane).

^a MT (midgut tissue); ^b MC (liquid midgut content).

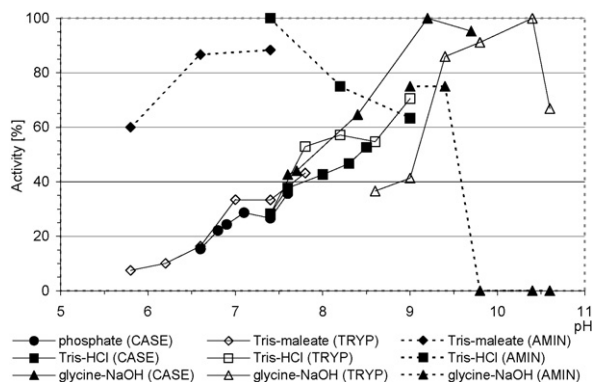


Fig. 1. Total proteolytic (caseinolytic), trypsin, and aminopeptidase activities in midgut of *Parnassius apollo* larvae in relation to buffers pH. Abbreviations: AMIN, aminopeptidase activity; CASE, caseinolytic activity; TRYP, trypsin activity.

tidase, which was higher in the MT samples obtained from 'hyperphagic' individuals (Table 2, Fig. 4). However, there was a significant negative correlation between body mass and caseinolytic activity both in the MT and the MC samples (Table 2). Total protein contents in these samples correlated positively, but insignificantly, with body mass in both age groups (data not shown). This indicates then that the relative importance of protein digestion was decreasing as the larvae were growing up. On the other hand, in the same individ-

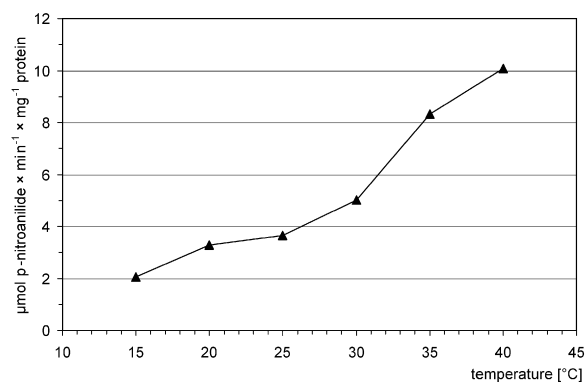


Fig. 2. Trypsin activity of *P. apollo* larvae in relation to incubation temperature.

uals, positive correlations were found between body mass and chymotrypsin activity (towards Suc-AAPF-pNA): significant in the MT and insignificant in the MC. A significant positive correlation with body mass was also found for carboxypeptidase activities in the MT (Table 3). To elucidate whether amino acids could be obtained by Apollo larvae from non-protein, low-molecular-weight compounds, comparisons were made between activities of exopeptidases and those of specific glycosidases. Strong positive correlations in midgut tissue of L₅ larvae were found only for carboxypeptidases (Table 3).

Table 2

Protease activities in the midgut of *Parnassius apollo* larvae (mean ± SD)

Enzyme [μmol of product × min ⁻¹ × mg ⁻¹ protein]*	L ₄ **		L ₅	
	MC (N = 8)		MC (N = 21)	MT (N = 14)
Total proteolytic (caseinolytic) activity***	1551.162 ± 839.893		1284.886 ^a ± 479.031	220.811 ^a ± 121.090
Trypsin	1.089 ± 0.838		1.167 ^a ± 0.493	0.049 ^a ± 0.025
Chymotrypsin ¹	0.037 ± 0.054		0.009 ± 0.008	0.007 ± 0.001
Chymotrypsin ²	0.420 ± 0.234		0.862 ^a ± 0.725	0.046 ^a ± 0.034
Aminopeptidase	0.159 ± 0.099		0.091 ^a ± 0.042	0.676 ^a ± 0.132
Carboxypeptidase ³	64.159 ± 32.221		21.738 ^a ± 18.247	110.530 ^a ± 53.879
Carboxypeptidase (in 'young' subgroups: N = 12 and 6)			22.427 ± 22.305	54.182 ^b ± 6.103
Carboxypeptidase (in 'hyperphagic' subgroups: N = 9 and 8)			20.821 ^a ± 12.110	152.791 ^{ab} ± 24.535

Abbreviations: A.U. (activity unit – 0.001 increase of absorbance in relation to blank), MC (liquid midgut contents), MT (midgut tissue).

* For product description see Table 1.

** Due to shortage of L₄ specimens, there is lack of data for the enzyme activities in the MT samples.

*** Total proteolytic activity is expressed in A.U. × min⁻¹ × mg⁻¹ protein × ml⁻¹.

¹ Assayed with G-F-pNA (*N*-glutaryl-L-phenylalanine *p*-nitroanilide).

² Assayed with Suc-AAPF-pNA (*N*-succinyl-alanine-alanine-proline-phenylalanine *p*-nitroanilide).

³ Mean ± SD for 'young' and 'hyperphagic' subgroups together.

^a Significant differences ($p < 0.05$, T Tukey's test for unequal sample size) between enzyme activity in the MT and the MC samples of L₅ stage.

^b Significant differences ($p < 0.05$, T Tukey's test for unequal sample size) between enzyme activity in the MT samples of 'young' and 'hyperphagic' L₅ larvae.

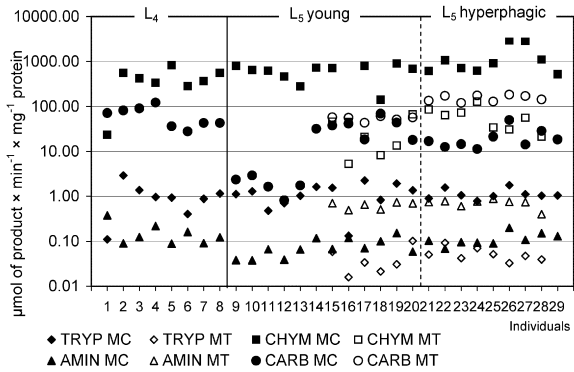


Fig. 3. The endo- and exoproteolytic activity in midgut tissues (MT) and liquid midgut contents (MC) in individuals of *Parnassius apollo*. Abbreviations: AMIN, aminopeptidase activity; CARB, carboxypeptidase activity; CHYM, chymotrypsin activity towards Suc-AAPF-pNA; TRYP, trypsin activity.

4. Discussion

Azocasein and casein are widely used as a measure of total proteolytic digestive capacity, hence allowing comparison among different insects. Christeller and co-workers pointed out that casein, as a milk protein, may not be a suitable measure for herbivores but, even in leaf-eating species, it was digested more effectively than rubisco – a major soluble leaf protein. However, they found that caseinolytic activity was relatively low in leaf-eaters, and in oligophagous species it was nearly two times lower than in polyphagous ones [15,19]. Using the same assay procedure, we have stated that caseinolytic activity of Apollo larvae was

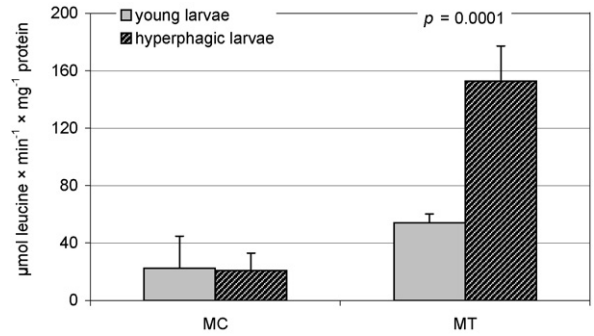


Fig. 4. Carboxypeptidase activity in midgut tissue (MT) and liquid midgut contents (MC) in ‘young’ and ‘hyperphagic’ *L5 Parnassius apollo* larvae (‘subgroups’). The value of *p* (T Tukey’s test for unequal sample size) shows a difference in the enzyme activity between the ‘subgroups’.

much higher than in a leaf-eating chrysomelid beetle (*Chrysolina* sp.: Coleoptera), leaf-mining lepidopteran larvae (*Cameraria ohridella*: Lepidoptera), grasshopper larvae and adults (*Stenocepa* sp.: Orthoptera), and omnivorous house cricket adults (*Acheta domesticus*: Orthoptera) (unpublished data).

The optimal pH conditions for assayed proteolytic enzymes in *P. apollo* midgut samples were similar to those found in other lepidopteran species [15]. Endopeptidases hydrolyzed effectively in alkaline environment, while aminopeptidases operated in a broad pH range. The pH profile of caseinolytic activity resembled that of trypsin. Similar results, obtained for *Ostrinia nubilalis* (Pyralidae) larvae, led Houseman and co-workers to the suggestion that trypsin is the major protease

Table 3

Significant correlations between assayed protease activities, and between their activities and body mass (bm) or gluco- and galactosidases, in midgut tissues (MT) and liquid midgut contents (MC) of *Parnassius apollo* L₅ larvae

Midgut compartment	N	Regression equation	r	p	t
MT	14	$Y_{CHYM} = 0.906X_{TRYP} + 0.002$	0.654	0.01	3.00
MC	21	$Y_{CARB} = 283.25X_{AMIN} - 4.810$	0.645	0.002	3.68
MC	21	$Y_{TRYP} = 0.001X_{CASE} + 0.365$	0.606	0.004	3.32
MC	21	$Y_{CHYM} = 8.259X_{AMIN} + 0.088$	0.473	0.030	2.34
MT	13	$Y_{CARB} = 126.215X_{bm} - 31.612$	0.727	0.005	3.51
MT	13	$Y_{CHYM} = 0.079X_{bm} - 0.041$	0.717	0.006	3.41
MT	13	$Y_{CASE} = -264.696X_{bm} + 521.614$	-0.686	0.01	-3.12
MC	20	$Y_{CASE} = -686.908X_{bm} + 2048.906$	-0.548	0.01	-2.78
MT	14	$Y_{CARB} = 8.08X_{\alpha-GLU} - 24.076^*$	0.956	7.6×10^{-8}	11.53
MT	14	$Y_{CARB} = 35.803X_{\beta-GLU} + 17.174^*$	0.815	4.0×10^{-4}	4.88
MT	14	$Y_{CARB} = 123.916X_{\beta-GAL} + 7.694^*$	0.718	0.004	3.57
MT	14	$Y_{CARB} = 283.44X_{\alpha-GAL} + 15.761^*$	0.702	0.005	3.42

Abbreviations: α -GAL (α -galactosidase); α -GLU (α -glucosidase); β -GAL (β -galactosidase); β -GLU (β -glucosidase); CARB (carboxypeptidase); CASE (caseinolytic activity); CHYM (chymotrypsin assayed with Suc-AAPF-pNA (*N*-succinyl-alanine-alanine-proline-phenylalanine *p*-nitroanilide)); TRYP (trypsin).

* Data for gluco- and galactosidases taken from [23].

in that species [36]. We have stated a strong positive correlation between trypsin and caseinolytic activity in midgut contents ($r = 0.606$; $p = 0.004$) (Table 3). Contribution of chymotrypsin was a minor one ($r = 0.378$; $p = 0.09$). Multiple regression analysis revealed that both enzymes only accounted for about 40% of the total proteolytic activity in this compartment ($p < 0.01$). No such correlations were stated in the midgut tissue (MT) samples.

Previous studies on insects' endoproteases assumed their similarities to mammalian enzymes, and numerous data on protein digestion in insects was gathered by methods applied for studies on mammals. Optimal pH allowed a classification of trypsin into high (> 10.0) and low (~ 9.0) alkaline [36]. The highest activity of Apollo larvae trypsin stated in glycine–NaOH buffer (pH 10.4) indicates that this endoprotease belongs to a high-alkaline type.

Lee and Anstee [17] noticed that concentrations of DMF (*N,N*-dimethylformamide) in the assay mixture higher than 7.7% v/v decreased trypsin activity. We used the substrate from the same supplier (SIGMA) but, to obtain the same final concentration of B-R-*p*NA, we had to use 15% v/v DMF (see also Baker [28]). We cannot estimate whether any inhibition of trypsin activity resulted from the applied concentration of DMF. Nevertheless, it is clear from the available data that at least trypsin amidase activity in *P. apollo* larvae falls into the lower range of the activities stated for other leaf-eating Lepidoptera [15,18,19]. This data strengthens our suggestion that trypsin plays a minor role in protein digestion in this species.

An increase in endoprotease activity, in midgut of lepidopteran larvae, along with the increase of incubation temperature, with a maximum at around 50 °C, has been documented in various species (e.g., [17,20,37]). A discontinuity ('break point') at approximately 30 °C in the Arrhenius plots of endoprotease activities was found [17]. Our data did not enable us to make such a plot for comparison; however, Q_{10} values for trypsin in Apollo larvae were the highest in the 25–35 °C temperature range. Similar properties were found for α -glucosidase ($Q_{10} = 3.1$) [23].

The use of G-F-*p*NA (*N*-glutaryl-L-phenylalanine *p*-nitroanilide) – substrate for vertebrate chymotrypsin, once commonly used for detection of the enzyme also in arthropods, revealed negligibly low activity both in the MT and the MC of the Apollo midgut. Tsai and co-workers [38] have shown that chymotrypsin in some arthropods has an extended binding site that requires a longer peptide substrate (up to five amino acid residues instead of two). Lee and Anstee confirmed this obser-

vation in *Spodoptera littoralis* (Noctuidae) larvae [17]. Recently, this finding was generalized for insects [34]. Higher chymotrypsin activity towards Suc-AAPF-*p*NA (than towards G-F-*p*NA) suggests that *P. apollo* also has this type of enzyme. The ratio of trypsin to chymotrypsin activity (towards B-R-*p*NA and Suc-AAPF-*p*NA, respectively), as shown by a regression analysis (Table 3), resembled that stated in other oligophagous species [15].

Relatively low activities of endoproteases in midgut of *P. apollo* larvae suggest that plant proteins are poorly utilized. Mandibles of the last-instar larvae cut relatively large bites of the leaves. Negligible activity of cellulase [23] enables access of digestive enzymes only to the content of mechanically destroyed cells and to the nutrients leaking through pores of the cell wall (symplast transport) [39]. Virtually all the chloroplast proteins (including rubisco) of the intact cells are unavailable for the larvae. Hence, fast passage of the chyme through the gut of Apollo larvae, enabling increased consumption, may be a compensatory mechanism, which allows obtaining sufficient amounts of nutrients and energy from available sources. In consequence, in leaf-eating insects with grazing mouth-parts, a decrease in mechanical digestion efficiency in subsequent larval instars may be expected. If this is not compensated by enhanced enzymatic digestion, decreased assimilation efficiency (assimilation to consumption ratio) should be observed. In fact, this was documented for numerous insect species, belonging to various taxa [40]. We have made similar observation in the last-instar *P. apollo* larvae when compared to the previous one (L₄) [41]. On the other hand, late-instar larvae of sap-feeders (Hemiptera) have increased assimilation efficiency in comparison with the young ones [40].

Limited availability of the leaf proteins points out that the feeding larva must obtain exogenous amino acids from protoplast either directly or from oligopeptides. These molecules easily pass through the peritrophic membrane into the exoperitrophic space; hence their digestion occurs mainly in the midgut wall (MT samples). Some molecules of aminopeptidases and carboxypeptidases are released into midgut lumen, since their activities were detected also in the MC samples. This has already been shown for aminopeptidases in *Lymantria dispar* (Lymantridae) and *Heliothis zea* (Noctuidae) [18,42]. On the other hand, carboxypeptidase activity in *H. zea* and *Erinnyis ello* (Sphingidae) was not found in the midgut tissue [42,43]. A question remains whether the molecules of the enzyme operate in the fluid layer adjacent to the epithelium or may also diffuse into the endoperitrophic space. Aminopeptidase

in Apollo midgut was similar to that stated in the gut of some other leaf-eating lepidopterans, in respect of both pH range (6.0–9.0 in *S. littoralis* [16] and 7.5–10.0 in *H. zea* [42]) and level of specific activity [18,42].

Carboxypeptidase A activity in Apollo larvae was very high in comparison with aminopeptidase activities. In comparative studies on larvae of 12 lepidopteran pest species, Christeller and co-workers found 10-fold higher carboxypeptidase activity in leaf-eaters than in flower-, fruit- and seed-eaters [19]. High activity of the enzyme in Apollo larvae fits well these findings. Hence, we conclude that in scarcity of available proteins, Apollo larvae probably use low-molecular-weight compounds, e.g., glycoproteins, to obtain amino acids. Our findings that in midgut tissue (but not in the gut contents), the enzyme activity was significantly correlated with activities of glucosidases and galactosidases, support this conclusion (Table 3). Supposed liberation of various aglycone parts with potentially adverse effects on larval metabolism is consistent with observation of limited larval feeding on a single food-plant [8]. It is then plausible that some chemical defence is induced in the Apollo larvae food-plant in response to feeding.

Acknowledgements

This work was supported by the Polish State Committee for Scientific Research (Project No. 6 P04F085 21). We express our thanks to Managing Director and Scientific Board of The Pieniny National Park for administrative support and Mr Tadeusz Oleś for his expertise and help in Apollo breeding.

References

- [1] M. Nakonieczny, A. Kędziorski, Feeding preferences of the Apollo butterfly (*Parnassius apollo* ssp. *frankenbergeri*) larvae inhabiting the Pieniny Mts (southern Poland), C. R. Biologies 328 (2005) 235–242.
- [2] R. Żukowski, Rozważania nad pochodzeniem odmian *Parnassius apollo* L. (Lepidoptera, Papilionidae) w Polsce, Pol. Pismo Entomol. 29 (1959) 491–505.
- [3] P. Pekarsky, *Parnassius apollo* L. in den Karpaten, seine Geschichte und Formenbildung, Z. Wien. Entomol. Ges. 39 (1954) 137–356.
- [4] M. Dechamps-Cottin, M. Roux, H. Descimon, Valeur trophique des plantes nourricières et préférence de ponte chez *Parnassius apollo* L. (Lepidoptera, Papilionidae), C. R. Acad. Sci. Paris, Ser. III 320 (1997) 399–406.
- [5] M. Wolbiś, Badania związków polifenolowych niektórych gatunków rodzaju *Sedum* L. II. Flawonoidy *Sedum maximum* Sut., Acta Pol. Pharmacol. 44 (1987) 567–571.
- [6] A. Kędziorski, M. Nakonieczny, G. Rosiński, Relation between developmental and reproductive success of apollo butterfly (*Parnassius apollo* L.) and quality of its food-plant – implications for restitution program of the species in Pieniny National Park, Report from Project No. 6 P04F 085 21, 2004.
- [7] E.A. Bernays, S. Woodhead, Plant phenols utilized as nutrients by a phytophagous insect, Science 216 (1982) 201–202.
- [8] P. Adamski, K. Margielewska, Z. Witkowski, Compensation and induced defense in response to herbivory in *Sedum maximum*, Fragn. Florist. Geobot. 45 (2000) 193–202.
- [9] H.A. Moser, J.J. Oertli, Evidence of a biochemical interaction between insect and specific foodplant in the system *Parnassius apollo* – *Sedum album*, Rev. Suisse Zool. 87 (1980) 341–358.
- [10] F. Ekkehard, Breeding Butterflies and Moths. A Practical Handbook for British and European Species, Harley Books, England, 1986.
- [11] J.E. Baker, Isolation and properties of digestive carboxypeptidases from midgut of larvae of the Black carpet beetle, *Attagenus megatoma*, Insect Biochem. 11 (1981) 583–591.
- [12] R.M. Broadway, S.S. Duffey, The effect dietary protein on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*, J. Insect Physiol. 32 (1986) 673–680.
- [13] J.A.T. Dow, Extremely high pH in biological systems: a model for carbonate transport, Am. J. Physiol. 246 (1984) R633–R635.
- [14] I. Ishaaya, Nutritional and allelochemical insect–plant interactions relating to digestion and food intake: some examples, in: J.R. Miller, T.A. Miller (Eds.), Insect–plant interactions, Springer Verlag, New York–Berlin–Heidelberg–London–Paris–Tokyo, 1986, pp. 191–216.
- [15] R.M. Broadway, Characterization and ecological implications of midgut proteolytic activity in larval *Pieris rapae* and *Trichoplusia ni*, J. Chem. Ecol. 15 (1989) 2101–2113.
- [16] M. Lee, J.H. Anstee, Characterization of midgut exopeptidase activity from larval *Spodoptera littoralis*, Insect Biochem. Mol. Biol. 25 (1995) 63–71.
- [17] M. Lee, J.H. Anstee, Endoproteases from the midgut of larval *Spodoptera littoralis* include a chymotrypsin-like enzyme with an extended binding site, Insect Biochem. Mol. Biol. 25 (1995) 49–61.
- [18] A.P. Valaitis, Gypsy moth midgut proteinases: purification and characterization of luminal trypsin, elastase and the brush border membrane leucine aminopeptidases, Insect Biochem. Mol. Biol. 25 (1995) 139–149.
- [19] J.T. Christeller, W.A. Laing, N.P. Markwick, E.P.J. Burgess, Midgut protease activities in 12 phytophagous lepidopteran larvae: dietary and protease inhibitor interactions, Insect Biochem. Mol. Biol. 22 (1992) 735–746.
- [20] S. Marchetti, C. Chiabà, F. Chiesa, A. Bandiera, A. Pitotti, Isolation and partial purification of two trypsin-like enzymes from the larval midgut of *Spodoptera littoralis* (Boisduval), Insect Biochem. Mol. Biol. 28 (1998) 449–458.
- [21] M. Díaz-Mendoza, F. Ortego, M. García de Lacoba, C. Magaña, M. de la Poza, G.P. Fariños, P. Castañera, P. Hernández-Crespo, Diversity of trypsin-like enzymes in the Mediterranean corn borer *Sesamia nonagrioides* (Lepidoptera: Noctuidae), revealed by nucleic acid sequences and enzyme purification, Insect Biochem. Mol. Biol. 35 (2005) 1005–1020.
- [22] C. Novillo, P. Castañera, F. Ortego, Isolation and characterization of two digestive trypsin-like proteinases from larvae of the stalk corn borer, *Sesamia nonagrioides*, Insect Biochem. Mol. Biol. 29 (1999) 177–184.
- [23] M. Nakonieczny, K. Michalczyk, A. Kędziorski, Midgut glycosidase activities in monophagous larvae of Apollo butterfly, *Parnassius apollo* ssp. *frankenbergeri*, C. R. Biologies 329 (2006) 765–774.

- [24] B. Dahlmann, K.-D. Jany, G. Pfeleiderer, The midgut endopeptidases of the honey bee (*Apis mellifica*): comparison of the enzymes in different ontogenetic stages, *Insect Biochem.* 8 (1978) 203–211.
- [25] E. Baumann, Isolation and partial characterization of chymotrypsin-like endoprotease from cockroach intestinal system, *Insect Biochem.* 20 (1990) 761–768.
- [26] B.F. Erlanger, N. Kokowesky, W. Cohen, The preparation and properties of two new chromogenic substrates of trypsin, *Arch. Biochem. Biophys.* 95 (1961) 271–278.
- [27] B.F. Erlanger, F. Edel, A.G. Cooper, The action of chymotrypsin on two new chromogenic substrates, *Arch. Biochem. Biophys.* 115 (1966) 206–210.
- [28] J.E. Baker, Resolution and partial characterization of the digestive proteinases from larvae of the Black carpet beetle, in: G. Bhaskaran, S. Friedman, J.G. Rodriguez (Eds.), *Current Topics in Insect Endocrinology and Nutrition*, Plenum Publ. Corp., New York, 1981, pp. 283–315.
- [29] E.D. Wachsmuth, I. Fritze, G. Pfeleiderer, An aminopeptidase occurring in pig kidney. I. An improved method of preparation. Physical and enzymic properties, *Biochemistry* 5 (1966) 169–174.
- [30] Y. Narahashi, M. Yanagita, Studies on proteolytic enzymes (pronase) of *Streptomyces griseus* K1. I. Nature and properties of the proteolytic enzyme system, *J. Biochem. (Tokyo)* 62 (1967) 633–641.
- [31] C.W. Ward, Resolution of proteases in the keratinolytic larvae of the webbing clothes moth, *Aust. J. Biol. Sci.* 28 (1975) 1–23.
- [32] S. Moore, W. Stein, Photometric ninhydrin method for use in the chromatography of amino acids, *J. Biol. Chem.* 176 (1948) 367–388.
- [33] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye-protein binding, *Anal. Biochem.* 72 (1976) 248–254.
- [34] W.R. Terra, C. Ferreira, Biochemistry of digestion, in: L.I. Gilbert, K. Iatrou, S.S. Gill (Eds.), *Comprehensive Molecular Insect Science*, Elsevier BV, Oxford, 2005, pp. 171–224.
- [35] W.R. Terra, C. Ferreira, Insect digestive enzymes: properties, compartmentalization and function, *Comp. Biochem. Physiol.* 109 B (1994) 1–62.
- [36] J.G. Houseman, B.J.R. Philogène, A.E.R. Downe, Partial characterization of proteinase activity in the larval midgut of the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae), *Can. J. Zool.* 67 (1989) 864–868.
- [37] D.W. Pritchett, S.Y. Young, C.R. Geren, Proteolytic activity in the digestive fluid of larvae *Trichoplusia ni*, *Insect Biochem.* 11 (1981) 523–526.
- [38] I.-H. Tsai, K.-L. Chuang, J.L. Chuang, Chymotrypsins in digestive tracts of crustacean decapods (shrimps), *Comp. Biochem. Physiol.* 85 B (1986) 235–239.
- [39] R.V. Barbehenn, Digestion of uncrushed leaf tissues by leaf-snipping larval Lepidoptera, *Oecologia* 89 (1992) 229–235.
- [40] J.M. Scriber, F. Slansky Jr., Food consumption and utilization, in: G.A. Kerkut, L.I. Gilbert (Eds.), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, Pergamon Press, Oxford–New York, 1985, pp. 87–163.
- [41] A. Kędziorski, M. Nakonieczny, K. Pyrak, J. Bembenek, G. Rościński, Energy metabolism in the Apollo butterfly (*Parnassius apollo* L.; Lepidoptera, Papilionidae), in: *Proc. 2nd International Conference on Insects: Chemical, Physiological and Environmental Aspects*, 1998, pp. 114–120.
- [42] C.J. Lenz, J. Kang, W.C. Rice, A.H. McIntosh, G.M. Chippendale, K.R. Schubert, Digestive proteinases of larvae of the corn earworm, *Heliothis zea*: characterization, distribution and dietary relationship, *Arch. Insect Biochem. Physiol.* 16 (1991) 201–212.
- [43] C.D. Santos, W.R. Terra, Distribution and characterization of oligomeric digestive enzymes from *Erinnyis ello* larvae and interferences concerning secretory mechanisms and the permeability of the peritrophic membrane, *Insect Biochem.* 16 (1986) 691–700.