Water-soluble proteinase activity of gastric fluids in the European whip snake, *Hierophis viridiflavus*: an experimental preliminary survey

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Abstract. Understanding the mechanism of metabolic digestion and of food assimilation in wild snake species is of fundamental importance to compare trophic niches, their adaptive significance and evolutionary patterns of diet selection for different snake species. From two adult *Hierophis viridiflavus* males in fasting condition, we sampled gastric fluids using fiberoptic endoscopy. We analyzed the proteolytic activities (optimal pH and temperature, response to inhibitors) of snake samples by zymography techniques. The two major protease activity bands were always detected at every tested pH. Activity bands were also detectable over 37 °C; in particular both bands showed the highest activity ranging from 55 °C to 60 °C. Ranging from 65 °C to 85 °C, one band remained visible while the other band disappeared over 60 °C. The pattern of proteolytic enzymes activity of sampled gastric fluids highlighted a scenario composed of few active proteases, reflecting the feeding status (e.g., fasting) of studied snakes. Detection of proteolytic activity until 85 °C, as in prokaryotic organisms, supported the hypothesis about the presence of proteases of exogenous source.

Keywords. European whip snake, *Hierophis viridiflavus*, water-soluble proteinase, stomach.

Food (e.g., prey type, prey abundance and feeding frequency) acts as a primary influencing factor in all biological processes of an organism life. Energy metabolism, food consumption (i.e., energy gain), and digestive performance have been measured quite rarely in reptiles in the wild (Congdon et al., 1982; Wikelsky et al., 1993; Henen et al., 1998), while most effort has been done on wild snakes under laboratory conditions (e.g., Secor and Nagy, 1994; Secor et al., 1994; Hopkins et al., 2004; Zuffi et al., 2010). Nevertheless, recent research indicates that vicariance may cause differences of feeding patterns in snakes (Zuffi, 2001; Luiselli et al., 2005), or influences the digestive performance in lizards (Pafilis et al., 2007). The relative patterns of growth that are dependent on the quality of food (i.e., different prey types, different frequencies) have been analyzed more in depth (Bonnet et al., 2001; Cox and Secor, 2007; Secor et al., 2007; Zuffi, 2007). It has been emphasized how newborn and juve-

nile snakes can grow to different sizes and show different phenotypes according to frequency and quality of food supply (Bonnet et al., 2001). Evidence of such a pattern is widely documented in the literature (see Shine, 1993 for a comprehensive review): snakes species may show deep inter-population body size differences due to area effect [i.e., insular gigantism or dwarfism (Boback, 2003)], to different food resource allocation (Shine, 1993; Rugiero et al., 1995; Pleguezuelos et al., 2007) or to both effects (Fornasiero et al., 2007).

Analysis of biochemical and physiological patterns of digestion in reptiles and in snakes could allow further comparisons of metabolic demands among species with different food regimes (Bradshaw et al., 1987; Luiselli, 2006), from highly specialized (Houston and Shine, 1994; Luiselli et al., 2001, 2007), to broadly generalist (Rugiero and Luiselli, 1995; Pleguezuelos and Fahd, 2004; Filippi et al., 2005) snake species. In snakes, different diet regimes

and food preferences could lead to a different pattern of digestion (e.g., efficiency; Zuffi et al., 2010) or to different enzymatic activity processes (Secor, 2008).

As model species we used the European Whip snake, *Hierophis viridiflavus*. This oviparous colubrid snake has a very wide prey spectrum (Heimes, 1993; Capula et al., 1997; Zuffi, 2001, 2007), and also displays an ontogenetic shift in diet composition (Rugiero and Luiselli, 1995; Zuffi, 2007), as typically observed in several other snake species (Luiselli et al., 1996, 2007; Shine et al., 2002). It can reach up to 200 cm total length, with an average body length of around 130 to 140 cm, while insular populations are significantly smaller than continental ones (Fornasiero et al., 2007; Zuffi, 2007). Nevertheless, most of the available knowledge on food preference or food ecology of the European Whip snake refer to some localities only (i.e., Capula et al., 1997), thus limiting comparison of feeding rate and food preference patterns among populations. Therefore, analyzing and understanding the mechanism of metabolic digestion (i.e. duration, sexual variation) and of food assimilation in wild species may be of fundamental importance to understand and compare i) trophic niches in different snake species and or between sexes, ii) the adaptive significance of selecting a different diet and evolutionary patterns of diet selection for snake species, characterized by different food regimes.

Endoscopic examination. All experiments were performed with no lethal methods and the snakes survived at the end of the experiment; they were released immediately after the experiment. Two adult males (970 and 1010 mm snout to vent length, Tombolo di Pisa, Pisa, central Tuscany, northern Italy; examined the day after capture; maintained at 27°C two hours before examination, 12:12 light-darkness cycle) were examined with flexible fiberscope GIF Q10 Olympus 100 cm, ø 10mm and a xenon light source CLE 10 Olympus, without anesthesia, restraining them manually in a dorsal position on a thermal pad at 38°C. The endoscope tip was gently introduced in the mouth and in both cases it was spontaneously swallowed. Gastrointestinal fluids were collected by a catheter injecting before a 100 mM Tris-HCl buffer (pH 8.0, 38 °C) that was aspirated after 30 seconds.

Handling and experimentation were conducted under the approval of the University of Pisa (Italy) Animal Care and Use Committee and "Ministero dell'Ambiente e della Tutela del Territorio e del Mare. Direzione per la Protezione della Natura" (permissions # N DPN/IIDIV/2006/7167 to MAL Zuffi).

Zymography. Protein concentration was measured according to Bradford's method (Bradford, 1976). SDS-

PAGE was performed according to Laemmli (Laemmli, 1970). Proteins from the gastric fluid sample underwent proteolytic activity-staining electrophoresis (Heussen and Dowdle, 1980), using 0.5% gelatine as precast protein substrate in a discontinuous 12% T Laemmli's gel in mini vertical unit SE260 electrophoresis system (Amersham Bioscience) under semi-denaturing conditions (before loading, samples were 1:2 diluted with 2% SDS without boiling). No β-mercaptoethanol was added. Each clarified extracts (~5 µg of proteins) were loaded in each lane. Electrophoresis run at 20mA at 15°C for 3 hours (Felicioli et al., 2004; Piccolomini et al., 2006). 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 2 hours, then stained with 0.1% Blue R250 Coomassie and de-stained with 40% methanol and 10% acetic acid. For the pH activity assay (Jousson et al., 2007), electrophoretic slabs were incubated in 2% triton X-100 for 30 min, then at 37 °C for 2 h in a 0.1M buffer. The buffer was Tris-acetate at pH 3, 4, 5 and 6; Tris–HCl at pH 7-8 and; and borate at pH 9-10. For the thermal stability assay, 10 lanes of gel corresponding to 10 aliquots of the crude extract of the sample after electrophoretic run were separately incubated for 2 hours in 100 mM Tris-HCl buffer, pH 8.0 at 4 °C, 15 °C, 22 °C, 37 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C and 85 °C. All the replicates have been scanned with an Epson Expression 1680 Pro scanner and analyzed using Quantity One 4.2.3 version Bio-Rad Laboratories (Milano, Italy). Phenylmethylsulfonylfluoride (PMSF) and Pepstatin A (Sigma-Aldrich) were used as inhibitors according to Barrett (1994). Two major activity bands were chosen for optimal pH activity, thermal stability, molecular mass estimation, and inhibition experiments.

Proteolytic pattern variations. The gastric area (Fig. 1) was correctly identified by endoscopic examination and gastric fluids were sampled.. Gastric fluid samples (crude extracts, pH 7.2) showed 0.66 mg/ml of total proteins.

Figure 2 shows the zymography on a porcine gelatine precast gel of crude extracts from the gastric fluid of fasting *H. viridiflavus*. Four major activity bands of different intensities were found (lane 2), showing estimated molecular weights of 93 kDa (A), 64 kDa (B), 43 kDa (C) and 38 kDa (D), respectively. Within the major activity bands, the 43 kDa and 38 kDa bands showed the highest intensity. No activity bands were detected at 23.4 kDa [lane 1, porcine pancreatic trypsin standard (T)], revealing the absence of trypsin-like protease. For subsequent experi-
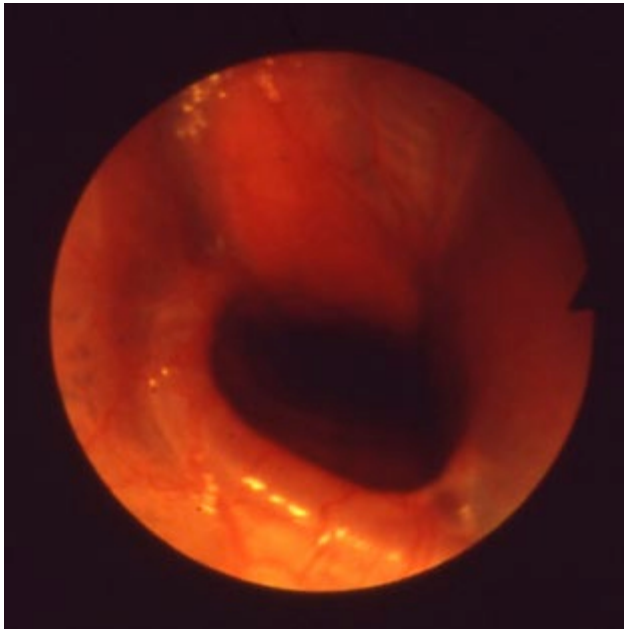
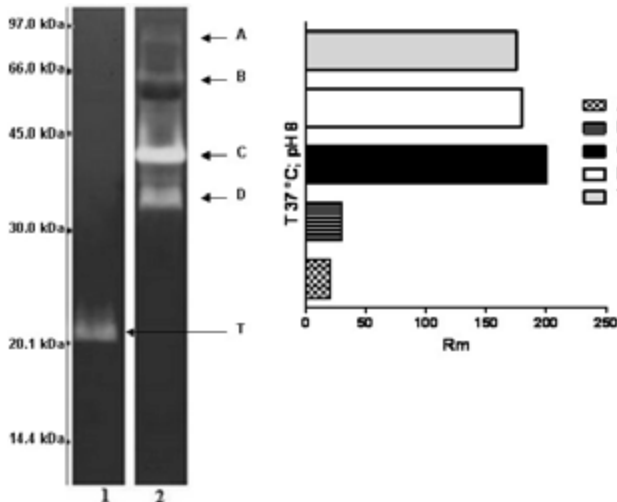**Fig. 1.** Endoscopic image of gastric area

Fig. 2. Gelatine-precast zymography PAGE of crude extract of *H. viridiflavus* gastric fluid. Lane 1: porcine pancreas trypsin; lane 2: *H. viridiflavus* gastric fluid. Molecular weights are shown on the left side. Under zymography figure, densitometric results of main bands (expressed as Rm, relative mobility) are also shown.

Fig. 3. Gelatine-precast pH-activity zymography PAGE of crude extract of *H. viridiflavus* gastric fluid. Each lane corresponds to a specific pH incubation. Molecular weights are shown on the left side. Under pH activity figure, densitometric results of main bands (expressed as Rm, relative mobility) are also shown (represented values are expressed as mean of two independent experiments).

Fig. 4. Gelatine-precast thermal activity zymography PAGE of crude extract of *H. viridiflavus* gastric fluid. Each lane corresponds to a specific temperature (°C) incubation. Molecular weights are shown on the left side. Under thermal activity figure, densitometric results of main bands (expressed as Rm, relative mobility) are also shown (represented values are expressed as mean of two independent experiments).

ments, we focused attention only on C and D bands. Figure 3 shows the pH activity of crude extracts from the gastric fluid of fasting *H. viridiflavus*. The two major bands (C and D) were always detected at every tested pH. Densitometric results showed very low activity levels of band D until pH 7, while band C exhibited higher activity levels than band C (3-4-fold at pH 7, pH 8 and pH 9).
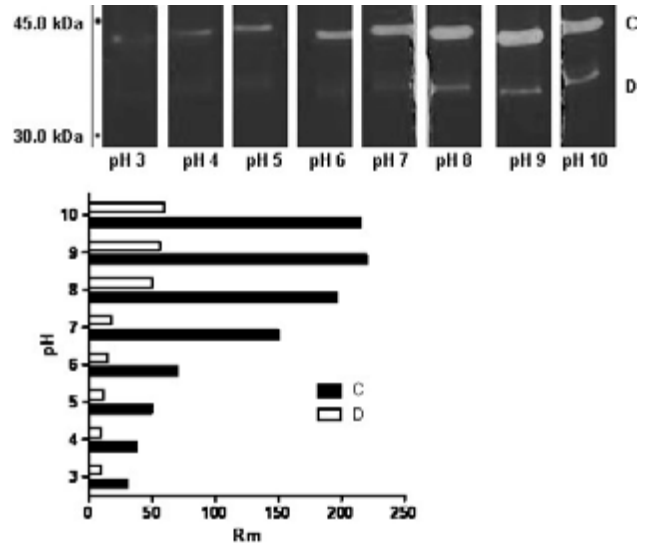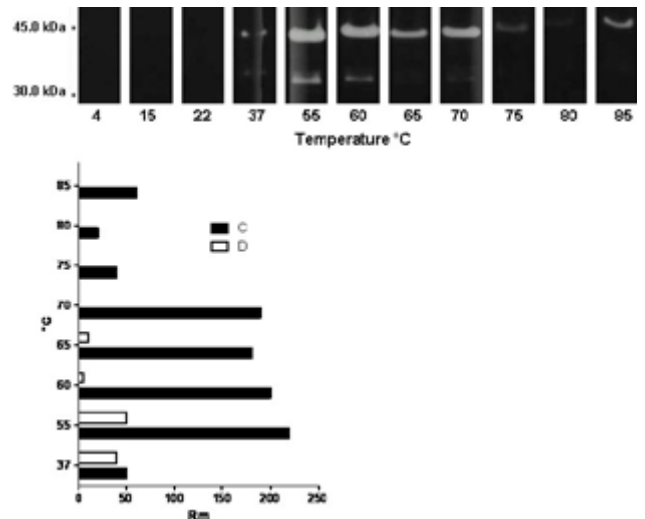
Figure 4 shows the thermal activity of crude extracts from the gastric fluid of fasting *H. viridiflavus*. Activity bands were detectable over 37 °C; in particular both bands showed the highest activity ranging from 55 °C to

60 °C. Ranging from 65 °C to 85 °C the C band remained visible while the D band disappeared over 60 °C.

As presented in this research, it is evident how very large meals requiring compensatory adjustments in blood flow, acid secretion, and regulation of acid-base homeostasis (Barboza et al., 2010) are processed by carnivores, such as snakes (intermittently feeders). Consequently, the digestive system appears to be a suitable model, because of its phenotypic plasticity and its integration with other physiological systems, including reproduction, circulation or thermoregulation (Barboza et al., 2010).

Heat is the main energy that snakes (and all the other reptiles) use to perform metabolic activities. The maximum recorded temperature of voluntary activity is the temperature at which most of the physiological, metabolic, and biological processes occur in several reptile species (Bradshaw, 1986). Lelièvre et al., 2010 reported that the European Whip snake showed maximum thermal optimum at about 31-32 °C, with measured temperature of 28.7 ± 0.9 °C during digestion and 27.9 ± 1°C during post-prandial condition.

Digestion processes of large meal feeders could be driven by the quantitative and qualitative composition of gastric fluids. Since few information are available about in vivo gastric secretion in reptiles, including partial or complete characterization of gastric protease, actual knowledge of *in vivo* gastric enzymatic patterns could contribute to the understanding of selected feeding ecology issues. In the present study, the pattern of proteolytic enzymes activity from the extracted gastric fluids reported a scenario composed of few active proteases, reflecting the feeding status (fasting) of studied snakes. Zhalka and Bdolah (1987) reported low levels of gastric protease activity in *Natrix tessellata*. The observed pH value (7.2) of gastric fluid samples was consistent to those of other snakes (Cox and Secor, 2010; Bessler and Secor, 2012) in fasting condition. In addition, the absence of trypsin-like activity bands in our samples supports the hypothesis that endoscopy really sampled the gastric area, since trypsin (used as 23.4 kDa proteolytic standard) is a pancreatic protease secreted into the gut. Zymogram analysis revealed two main protease activity bands on which our attention was focused (band C and D).

Regarding pH-activity, band C revealed proteolytic activity all over the tested pH, with an optimal activity at basic pH, while band D appeared to be active only at basic pH. These observations, together with the inhibition experiments, supported the hypothesis that the observed proteases may belong to a class of alkaline proteases; in fact pepstatin A (90 μM), a typical acidic protease inhibitor, did not inhibited both the bands while PMSF (1.3 mM), a typical serine protease inhibitor, strongly inhibited both the bands (data not shown).

Regarding the thermo-activity, contrasting information are available about high thermo-stable protease in ectothermic vertebrates. De Souza Bezerra et al. (1999) observed in a tropical fish (*Colossoma macropum*) 35 °C as optimum temperature for stomach acid protease activity, whereas 65 °C was found as the optimum value for the pyloric caeca (gut) alkaline protease activity. Moreover, band C surprisingly revealed high activity until 85 °C supporting the hypothesis about the presence of an exogenous origin protease. In fact some prokaryotic organisms possess such thermostable protease: Kuzu et al. (2012) described the production of a thermostable chitinase by *Bacillus thuringiensis* subsp. *kurstaki* with high levels of activity at 110 °C into an alkaline pH range. Regarding reptile chitinases, a putative one was also identified in the stomach of a phrynosomatid lizard (Marsh et al., 2001), but in an acidic pH range. No information about temperature range are available to date.

In conclusion, we can summarise that: a) fiberoptic endoscopy could be a suitable non-lethal method for gastrointestinal sampling in snakes; b) the absence of trypsin-like protease in sampled fluids confirmed the correct gastric location of the endoscopic probe; c) four major proteolytic activity bands were detected in sampled fluids; d) fasting condition of snakes resulted in a not so various pattern of proteolytic enzymes; e) the two major proteolytic activity bands (C and D) seemed to belong to a class of alkaline proteases; f) the high temperature stability at alkaline pH of band C activity supported the hypothesis of a protease of exogenous source (e.g., bacteria); g) band D, with a thermal activity until 65 °C in an alkaline range, seemed to be a putative protease.

While several laboratory analyses were carried out on pet snakes (e.g., *Python* species; Ott and Secor, 2007; Cox and Secor, 2008; Secor, 2008), wild species were considered quite rarely (McCue, 2007; Chia-Wei et al., 2009; Secor et al., 2012), preventing in depth comparative analyses. Furthermore, because wild snake feeding behavioural physiology is virtually unknown, this study may lead to forthcoming research such as experiments on different prey regimes.

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