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RESEARCH ARTICLE

First Identification of Tannin-Binding Proteins in Saliva of *Papio hamadryas* Using MS/MS Mass Spectrometry

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Hamadryas baboons possess salivary proline-rich proteins (PRP), as indicated by the presence of pink-staining protein bands using 1D SDS gel electrophoresis and Coomassie R250 staining. The ability of these protein bands to interact with tannic acid was further examined. In a tannin-binding assay using 5 μg tannic acid mixed with hamadryas whole saliva, we recently found four distinct protein bands of apparently 72, 55, 20, and 15 kDa that were precipitated during the experiments. In this work, we were able to identify these protein bands in a follow-up analysis using MS/MS mass spectrometry after excising such bands out of air-dried gels. Albumin and α-amylase were present in the tannic acid–protein complexes, with albumin already known to nonspecifically interact with a great diversity of chemical compounds. More interesting, we also identified a basic PRP and a cystatin precursor protein. This was the first successful attempt to identify a PRP from precipitated tannin–protein complexes in hamadryas baboons using MS/MS mass spectrometry. On the other hand, the role of cystatins in tannin binding is not yet well understood. However, there are recent reports on cystatin expression in saliva of rats responding to astringent dietary compounds. In conclusion, the follow-up data on tannin-binding proteins present in salivary secretions from hamadryas baboons adds important knowledge to primate physiology and feeding ecology, in order to shed light on the establishment and development of food adaptations in primates. It also demonstrates that tannin binding is characteristic for PRP, but might not be restricted to this particular group of proteins in primate species. Am. J. Primatol. 73:896–902, 2011.

Key words: cystatins; mass spectrometry; hamadryas baboons; proline-rich proteins (PRP); saliva; tannic acid

INTRODUCTION

Salivary glands and their products play a major role in the adaptation of animals to their nutritional environments [Tabak & Kuska, 2004]. Particularly, salivary proteins are crucial variables enabling animals to adapt very rapidly to new diets.

Many dicotyledonous plants produce secondary metabolites that deter herbivorous animals from feeding on them [Harborne, 1991; Iason & Van Wieren, 1999]. One such class of secondary compounds are tannins—a group of astringent polyphenols [Bernays et al., 1989; Shimada, 2006]. Tannins are antinutritional factors for many animals, by conferring an unpleasant bitter taste and reducing food digestibility in the intestine [Robbins et al., 1987; Shimada, 2006]. However, some animals have evolved counter-adaptations, such as salivary proline-rich proteins (PRP) with tannin-binding properties to cope with tannin's antinutritional effects [e.g. Clauss et al., 2005; McArthur et al., 1995; Mehansho et al., 1983, 1985; for review: Shimada, 2006].

Widely distributed across the Horn of Africa and the southwestern Arabian Peninsula, sacred or hamadryas baboons (*Papio hamadryas*) are adapted to life in semi-arid harsh habitats [Swedell, 2002]. Hamadryas baboons consume varied omnivorous diets ranging from grasses, roots, and berries to small vertebrates and insects [Swedell, 2002]. At Erer-Gota, Ethiopia, for instance, it has been described that hamadryas baboons consumed 45% fruit, 28% leaves, 22% flowers, and 2% underground food items [Kummer, 1968]. Fruit (>47%) was also

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the primary component in the diet of hamadryas baboons at Filoha, Ethiopia, though leaves, flowers, and underground food items; noticeably, small mammals, birds, and invertebrates are also an important feature of their diet [Swedell et al., 2008]. At zoos, hamadryas diets tend to differ significantly from the wild. However, although the exact tannin content of the diet is unknown, it is likely that hamadryas baboons, especially in the wild, consume considerable amounts of tannic acid with fruits and leaves. Therefore, it may be inferred that hamadryas baboons possess a significant ability to bind and precipitate tannic acid with the help of tannin-binding proteins, as previously demonstrated [Mau et al., 2009]. In fact, in recent tannin-binding assays using the saliva of captive *P. hamadryas*, we detected for the first time a number of yet unidentified proteins together with the characteristic pink staining of a PRP with a molecular mass of approximately 23 kDa [Mau et al., 2009]. This specific staining method uses Coomassie Brilliant Blue R250 to stain PRP [Beeley et al., 1991]. Interestingly, only PRP are considered to stain pink, whereas all other salivary proteins are stained in a light blue color. The described protein was within the reported molecular mass range of PRP: between 5 and 25 kDa [Bennick, 2002; McArthur et al., 1995]. Furthermore, this result was consistent with previous reports of PRP being present in saliva of other omnivorous primates, including *Macaca fascicularis* and *Homo sapiens* [Oppenheim et al., 1971, 1979].

However, although we found evidence for tannin-binding PRP in hamadryas saliva, their identities as well as the identities of the other non-PRP proteins that were additionally precipitated by tannic acid were still unknown. Therefore, the goal of this follow-up study was (i) to identify the chemical class of the present PRP and (ii) to identify the additionally precipitated proteins in saliva of hamadryas baboons using MS/MS mass spectrometry. This would give a first clue of the identity of classical and potential nonclassical tannin-binding proteins in the saliva of hamadryas baboons.

METHODS

In the previous study, saliva samples of eight adult male hamadryas baboons (*P. hamadryas*), born in captivity, were provided by the Zoological Garden Cologne, Germany, using a previously described sampling method [Mau et al., 2009]. The research complied with the ASP Ethical Treatment of Nonhuman Primates, and all research protocols reported in the manuscript were reviewed and approved by the Institute of Animal Science, Bonn, following German and European Union directives on animal experimentation.

For PRP staining, saliva samples were mixed with calculated volumes of loading buffer (K929.1; Roth, Hamburg, Germany) to reach a total protein concentration of 3 μg/μl. Subsequently, 30 μg protein extract were separated by SDS PAGE for 2 hr at 125 V on a 12% acrylamide resolving gels, as described [Mau et al., 2009]. Gels were stained overnight in Coomassie-Brilliant-Blue-R250. To visualize PRP, gels were destained according to Beeley et al. [1991]. After destaining, Coomassie gels were packed in cellophane foil (Anamed, Groß-Bieberau, Germany), air-dried for 7 days, and stored until further MS analysis.

Saliva was probed for tannin-binding proteins according to Austin et al. [1989]. Crude saliva (each with 120 μg of total protein) was mixed with tannic acid dissolved in 50% methanol (10 μl solution containing 5 μg of tannic acid; 48811; Sigma-Aldrich, Taufkirchen, Germany). The mixture based on saliva of one individual was incubated initially for 2 hr and compared with 8 hr incubation at 4°C in a refrigerator with continuous shaking (Thermomixer MHR-11, HA04.1; Roth). Later, additional samples were incubated for 8 hr only. Control samples contained 50% methanol without tannic acid. The samples were centrifuged for 10 min at $800 \times g$ and 4°C and the supernatant was separated from the pellet. Protein pellets were subsequently used for gel electrophoresis, as described above. Gels were then stained for the presence of PRP, dried, and stored until MS/MS mass spectrometry analysis.

Bands of interest (Fig. 1) were excised from two different individuals of *P. hamadryas*, were subsequently destained, and trypsin digested using methodology thoroughly described [Almeida et al., 2010]. Briefly, spots were destained in water and 50% (v/v) acetonitrile, reduced with dithiothreitol, alkylated with iodoacetamide, and dried in a SPD 121 speedvac (Thermo scientific, Waltham, MA). Gel pieces were rehydrated with digestion buffer (50 mM NH_4HCO_3) containing trypsin (6.7 ng/μl) (Promega, Madison, WI) and incubated overnight at 37°C. The buffered peptides were acidified with formic acid, desalted, and concentrated with C8 microcolumns (POROS R2, Applied Biosystems, Foster City, CA). The peptides were eluted with matrix solution that contained 10 mg/ml α-cyano-4-hydroxycinnamic acid dissolved in 70% (v/v) acetonitrile/0.1 % (v/v) trifluoroacetic acid. The mixture was then allowed to air dry.

Protein identification was done by MALDI-TOF-TOF with an Applied Biosystem 4800 Proteomics Analyser (Applied Biosystems, Foster City, CA) in MS and MS/MS mode. Each MS spectrum was obtained in an independent acquisition mode with 800 laser shots per spectra and a fixed laser intensity of 3,500 V. Spectra were externally calibrated using des-Arg-Bradykinin (904.468 Da), angiotensin 1 (1296.685 Da), Glu-Fibrinopeptide B (1570.677 Da), ACTH (1–17) (2093.087 Da), and ACTH (18–39) (2465.199) (Calibration Mix from Applied Biosystems).

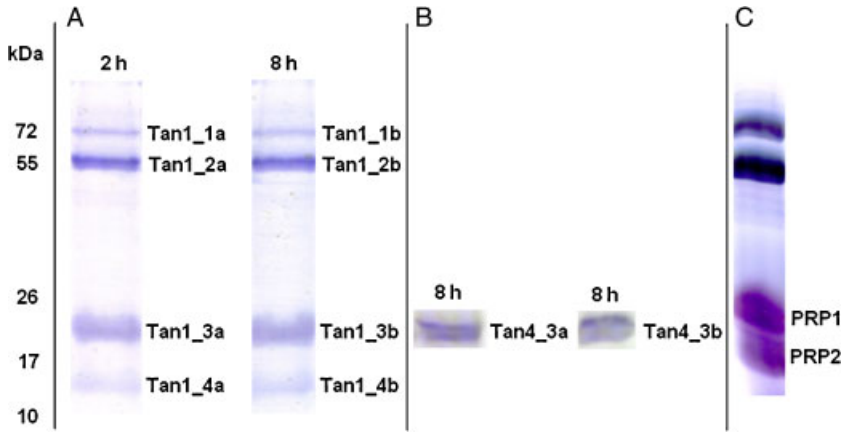

Fig. 1. Identification of tannin-binding proteins in saliva of *Papio hamadryas* precipitated with tannic acid using MS/MS mass spectrometry on one-dimensional SDS gels stained with Coomassie R250. (**A**) Hamadryas saliva obtained from one individual was incubated with tannic acid for either 2 or 8 hr. Precipitated bands (Tan1_1a–4a, Tan1_1b–4b) were cut out and analyzed using MS/MS mass spectrometry for identification. (**B**) Hamadryas saliva obtained from two different individuals was incubated with tannic acid for 8 hr. Proline-rich protein (PRP) bands from the precipitates (Tan4_3a; Tan4_3b) were analyzed. (**C**) Identification of PRP in untreated whole saliva of *Papio hamadryas* (V). Pink-staining protein bands named PRP1 and PRP2 were cut out and subjected to MS/MS mass spectrometry. kDa, molecular masses of the protein molecular mass marker.

Ten s/n best precursors from each MS spectrum were selected for MS/MS analysis. The MS/MS analyses were performed with Collision Induced Dissociation, using a collision energy of 1 kV and a gas pressure of 1×10^6 torr. The MS/MS spectra were acquired with 2,000 laser shots and a laser intensity of 3,500 V.

The generated mass spectra were used to search the NCBI protein database with the algorithms Paragon, from Protein Pilot software v 2.0 (Applied Biosystems, MDS Sciex), and Mowse, from MASCOT-demon 2.1.0 Software (Matrix-Science). Protein score above 2.0 ($P < 0.01$) for Paragon and a threshold of 95% ($P < 0.05$) for Mowse were considered for confident protein identification. In the analysis using Protein Pilot, other parameters considered were: enzyme, trypsin; Cys alkylation, iodoacetamide; special factor, urea denaturation; species, none; and ID focus, biological modification. Regarding Mascot search, the analysis of results was performed in the GPS Explorer Software (Version 3.5, Applied Biosystems), using the following parameters: missed cleavage, one; peptide tolerance, 50 ppm; fragment mass tolerance, 0.25 Da; fixed modification, carbamidomethylation of cysteine; and variable modification, methionine oxidation. An identification was considered valid for protein scores above the defined thresholds and when at least one peptide was fragmented. In such cases, a minimum of three sequential b and y ions was necessary to validate the identification.

RESULTS

Because there was no available data on tannic acid binding in baboon saliva, we were unable to predict in advance the time of how long the tannin-binding proteins from baboon saliva would need to cause a detectable pellet after the addition of tannic acid. Therefore, it was decided to initially compare a short-term incubation (2 hr) with a long-term incubation (8 hr). Both incubation times led to the same result. However, to perform the experiments in accordance to the procedure of Austin et al. [1989], the long-term incubation of 8 hr was chosen later on. Proteins that interacted with tannic acid were identified as serum albumin (2492797, NCBI), amylase (157921585, NCBI), cystatin SA precursor (114681308, NCBI), and basic PRP (342283, NCBI; Fig. 1; Table I).

DISCUSSION

Tannin-binding proteins enable animals to use a broader range of food by switching from tannin-free grasses to tannin-rich leaves. As described earlier, *P. hamadryas* is more or less an omnivorous primate species, feeding on leaves, flowers, pods, and seeds of e.g. *Acacia* trees [Swedell, 2002]. Because dicotyledonous leaves often contain tannins, we expected higher levels of salivary PRP with tannin-binding activity to be present in hamadryas baboon [Mau et al., 2009]. In fact, in a recent study, we used a tannic acid assay on hamadryas saliva to show for the first time the presence of a group of proteins, including PRP binding to tannins [Mau et al., 2009]. This study now provides the first identification of these tannin-bound proteins from hamadryas saliva using MS/MS mass spectrometry, found to share a consistent homology with proteins from other primate species. Besides classical tannin-binding proteins, such as basic PRP, further proteins, such as serum albumin, salivary amylase, and cystatins, were present and were demonstrated

TABLE I. Protein Identification Results for Salivary Proteins From *Papio hamadryas* That Precipitated With Tannic Acid

Gel band reference	Protein name	Matching species	Protein accession number (NCBI)	Total protein score[a]	Total ion score	Peptides matched in MS/MS	Theoretical molecular mass (kDa)	Matched peptides[b]		Sequence coverage (%)[c]
								MS	MS/MS	
Tan1_1a	Serum albumin	*Macaca mulatta*	2492797	641 (M)	578	LVNEVTEFAK YLYEVAR RHPDYSVMLLLR VFDEFQPLVEEPQNLVK QNCELFEQLGEYK FQNALLVR KVPQVSTPTLVEVSR MPCAEDYLSVVLNR RPCFSALELDEAYVPK	67.837	*25*	*7*	*42*
Tan1_2a	α-Amylase	*Colobus angolensis*	157921585	837 (M)	805	TSIVHLFEWR ALVFVDNHDNQR NWGEGWGFMPSDR GHGAGGASILTFWDAR MAVGFMLAHPYGFTR IAEYMNNLIDMGVAGFR NVVDGQPFTNWYDNG SNQVAFGR	57.569	*16*	*7*	*44*
Tan1_3a	α-Amylase 2A	*Macaca mulatta*	109011570	110 (M)	88	TSIVHLFEWR GHGAGGASILTFWDAR	57.556	*12*	*2*	*29*
Tan1_4a	Cystatin SA precursor	*Pan troglodytes*	114681308	88 (M)	74	ALHFAISEYNK	14.113	*5*	*1*	*42*
Tan1_1b	Serum albumin	*Macaca mulatta*	2492797	707 (M)	623	FKDLGEEHFK LVNEVTEFAK YLYEVAR VFDEFQPLVEEPQNLVK QNCELFEQLGEYK FQNALLVR KVPQVSTPTLVEVSR RPCFSALELDEAYVPK GVMDNFAAFVEK	67.837	*27*	*9*	*41*
Tan1_2b	α-Amylase	*Colobus angolensis*	157921585	818 (M)	777	TSIVHLFEWR ALVFVDNHDNQR NWGEGWGFMPSDR GHGAGGASILTFWDAR MAVGFMLAHPYGFTR IAEYMNNLIDMGVAGFR NVVDGQPFTNWYDNGS NQVAFGR	57.57	*17*	*7*	*44*
Tan1_3b	–	–	–	–	–	Not determined	–	–	–	–
Tan1_4b	Cystatin SA precursor	*Pan troglodytes*	114681308	98 (M)	73	ALHFAISEYNK	14.113	*5*	*1*	*50*

TABLE I. Continued

Gel band reference	Protein name	Matching species	Protein accession number (NCBI)	Total protein score[a]	Total ion score	Peptides matched in MS/MS	Theoretical molecular mass (kDa)	Matched peptides[b]		Sequence coverage (%)[c]
								MS	MS/MS	
Tan4_3a	Basic PRP	*Macaca fascicularis*	342283	2.03 (P)	–	KPQGPPPPGKPQGPPK KPQGPPPPGKPQGPPQ QQGQPQQGGNRPQGPP SPPGNAQGPPQQGGK	19.135	–	3	*50.5*
Tan4_3b	–		–	–	–	Not determined	–	–	–	–
PRP1	Basic PRP	*Macaca fascicularis*	342283	75 (M)	75	KPQGPPPPGKPQGPPK	19.124	*1*	*1*	*8*
PRP2	Basic PRP	*Macaca fascicularis*	342283	116 (M)	116	KPQGPPPPGKPQGPPK	19.124	*1*	*1*	*8*

[a]Identification scores obtained with the algorithms Paragon (P) and Mowse (M). A result is considered to be a significant identification when a score above 68 (M) and 2.00 (P) is attained respectively
[b]Number of peptides, matching the identified protein, whose sequence differs in at least one amino acid residue.
[c]Percentage of the identified protein sequence covered by the matched peptides.

to interact with tannic acid in saliva of hamadryas baboons.

In general, there are two major groups of classic tannin-binding proteins, namely PRP and histatins [Mehansho et al., 1983, 1985; Mole et al., 1990; Sugiyama & Ogata, 1993; Yan & Bennick, 1995; for review: Shimada, 2006]. Histatins are very small proteins (approximately 5 kDa), which were described in humans and *M. fascicularis*, and on the genetic level also recently in other nonhuman primates [Bennick, 2002; Padovan et al., 2010; Sabatini et al., 1989]. Likewise, PRPs were first detected in human saliva, but are expressed in various mammalian species, e.g. in roe deer (*Capreolus capreolus*) and *M. fascicularis* [Mandel et al., 1965; Shimada, 2006]. They are characterized by a high content of proline, which could make up to 20% of its amino acid sequence [Kauffman & Keller, 1979; Mole et al., 1990]. Basic PRP are mainly secreted by the parotid glands, and contain generally more proline and have much higher affinity to tannins than acidic PRPs [Shimada, 2006]. The main function is regarded as counteracting dietary tannic acid [Bacon & Rhodes, 2000; Chan & Bennick, 2001]. Interestingly, some species, such as koalas, cattle, and pigs, are known to express salivary proteins, which are characterized by a high content of proline but do not show higher affinity to tannins [Mole et al., 1990]. However, standardized tannins that are mostly used in vitro might enormously differ from the diverse group of tannins found in a species' diet [Fickel et al., 1999]. Furthermore, tannin binding by proteins is highly dependent on pH, protein characteristics, ions, and the tannin used [Fickel et al., 1999; Martin et al., 1985]. Thus, in in vitro experiments, underestimated binding of tannins is possible to occur when the chosen tannin is not naturally present in a species diet or producing only soluble complexes [Hagerman et al., 1992]. Therefore, additional experiments using different kinds of tannins or natural isolates should be run to identify tannin-binding proteins in animal saliva [Hagerman et al., 1992; Robbins et al., 1991].

Nevertheless, besides classical PRP and histatins, other types of tannin-binding proteins were described in several studies, but not identified or named yet [Shimada, 2006]. Some salivary proteins that originally have other functions are supposed to have also a high affinity to tannins, and consequently act as defence mechanisms against dietary tannins [Shimada, 2006]. In our study, we detected three different proteins (serum albumin, salivary α-amylase, cystatins) that precipitated in the presence of tannic acid, but are not referred to as classic PRP, and eventually interacted with tannins.

Serum albumin is a protein that is often used as a model in polyphenol/protein interaction studies [Soares et al., 2007]. Although very prominently expressed in saliva, its tannin-binding activity is

rather low compared with other tannin-binding proteins. Tannic acid, for example, showed a higher affinity to salivary α-amylase than to BSA [Soares et al., 2007]. As generally expected, amylase was demonstrated to decrease its enzymatic activity after the addition of tannic acid [Kandra et al., 2004; McDougall et al., 2005; Rawel et al., 2006]. Interestingly, in mice, an increase in the level of a salivary amylase isoform was observed after tannin consumption [da Costa et al., 2008]. However, this might be an unspecific reaction to dietary tannins and the result of the stimulation of sympathetic pathways and amylase inhibition [da Costa et al., 2008]. Nonetheless, further work is needed to finally unravel a role of amylase in tannin defence in animals.

A new and very interesting finding is the presence of cystatins in the precipitated fractions after tannic acid addition to the saliva of hamadryas baboons. The induction of cystatin SA was earlier shown in rat submandibular saliva and was suggested to be one line of protection of oral mucosa from toxic or irritating phytochemicals, such as capsaicin [Katsukawa & Ninomiya, 1999; Katsukawa et al., 2002]. However, our study is the first to demonstrate an interaction of cystatin SA with tannins in a primate species. Nonetheless, whether this effect originates from specific interaction of cystatins with tannins or is caused by unspecific protein precipitation remains to be further studied. Because normal primate diet, and also that of hamadryas baboons, consists mainly of fruits, seeds, and leaves, there is also a high potential for exposure to papain- and legumain-like enzymes from plants [Dickinson, 2002]. It has been shown earlier that human cystatins SN and SA inhibit papain and related enzymes [Baron et al., 1999]. Therefore, cystatins are considered mainly to block the noxious effects of dietary cysteine proteases and to protect salivary proteins from degradation [Dickinson, 2002]. A constitutive expression of salivary cystatins has been reported from humans and might be characteristic for primates [Dickinson, 2002].

In conclusion, besides classical basic PRP, other salivary proteins, particularly amylase and cystatins, could help baboons to detoxify tannic acid and other polyphenols from their diets, thus reducing their risk of digestive disorders. Therefore, in future studies, it would be important not only to refer to classic PRP and their tannin-binding activity, but also to the nonclassic tannin-binding proteins in saliva that might hold a significant benefit in species feeding on tannin-rich diets, namely leaves or fruits.

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