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Tannin-binding salivary proteins in three captive rhinoceros species

Marcus Clauss[a,*], Janin Gehrke[b], Jean-Michel Hatt[c], Ellen S. Dierenfeld[d,1], Edmund J. Flach[e],
Robert Hermes[b], Johanna Castell[a], W. Juergen Streich[b], Joerns Fickel[b]

[a] *Institute of Animal Physiology, Physiological Chemistry and Animal Nutrition, Schönleutnerstr. 8, 85764 Oberschleissheim, Germany*
[b] *Institute of Zoo and Wildlife Research (IZW) Berlin, Germany*
[c] *Division of Zoo Animals and Exotic Pets, Zurich, Switzerland*
[d] *International Rhinoceros Foundation, USA*
[e] *Institute of Zoology, Whipsnade Wild Animal Park, Dunstable, UK*

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Abstract

Tannin-binding salivary proteins (TBSP) are considered to be counter-defences acquired in the course of evolution by animals whose natural forage contains such tannins. As tannins mostly occur in browse material but not in grasses, it is assumed that grazers do not have a need for TBSP. Whereas it has been shown in several non-ungulate species that TBSP can be induced by dietary tannins, their presence or absence in ungulates has, so far, been shown to be a species-specific characteristic independent of dietary manipulations. We investigated saliva from three rhinoceros species from zoological gardens fed comparable, conventional zoo diets. As expected, saliva from white rhinoceroses (*Ceratotherum simum*, grazer) had lower tannin-binding capacities than that from black rhinoceroses (*Diceros bicornis*, browser). Surprisingly, however, Indian rhinoceroses (*Rhinoceros unicornis*), commonly regarded as grazers as well, displayed the highest tannin-binding capacities of the three species investigated. It is speculated that this discrepancy might be a result of an evolutionarily recent switch to a grass-dominated diet in Indian rhinoceroses, and that the black rhinoceros, which is closer related to the white rhinoceros than the Indian species, has evolved an inducible mechanism of TBSP production. In separate trials during which the tannin content of the diets of black rhinoceroses was increased by the addition of either tannic acid or quebracho, the tannin-binding capacity of black rhinoceros saliva was increased to levels within the same range as that of Indian rhinoceroses on the conventional diets. While induction trials in white and Indian rhinoceroses remain to be performed for a full understanding of salivary anti-tannin defence in rhinoceroses, these results are the first report of an induced salivary response to increased dietary tannin levels in an ungulate species.

Keywords: *Ceratotherum simum*; *Diceros unicornis*; *Rhinoceros unicornis*; Saliva; Secondary plant compounds; Tannin; Condensed tannins; Hydrolysable tannins; Salivary tannin-binding proteins; Nutrition; Feeding habits; Feeding types; Browser; Grazer

1. Introduction

The presence of salivary tannin-binding proteins (TBPs) has been demonstrated in different herbivorous and omnivorous mammalian species—in marsupials (McArthur et al., 1995), rodents and lagomorphs (Mehansho et al., 1983, 1985; Spielman and Bennick, 1989; Mole et al., 1990; McArthur et al., 1995; Juntheikki et al., 1996), bears (Hagerman and Robbins, 1993), primates (Oppenheim et al., 1985, Ann and Lin, 1993), humans (Bennick and Connell, 1971, Bacon and Rhoades, 1998), and among ungulates in camels (Schmidt-Witty et al., 1994) and ruminants (Robbins et al., 1987; Austin et al., 1989; Juntheikki, 1996; Fickel et al., 1998). Most of these studies link the occurrence of TBPs to the presence of tannins in the natural diet. In contrast, animals with a low tannin content in their natural forage have little or no salivary TBPs (Austin et

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* Corresponding author. Tel.: +49 89 2180 78705; fax: +49 89 2180 78702.

E-mail address: clauss@tiph.vetmed.uni-muenchen.de (M. Clauss).

[1] Current address: St. Louis Zoo, St. Louis, MO, USA.

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al., 1989; Hagerman and Robbins, 1993). Two main hypotheses are being discussed regarding the functional role of TBPs. These hypotheses propose both maintenance of oral homeostasis and counteracting dietary tannins as the main functions of PRPs but differ in their assumption as to which function is the ancestral and the derived one, respectively. The current state of knowledge is summarized by McArthur et al. (1995) and favours an ancesteral function of PRPs in oral homeostasis based on the fact that PRPs are effective in oral homeostasis at low secretion levels, whereas counteracting tannin requires high secretion levels. From carnivores through omnivores to exclusively plant-eaters, the dietary nitrogen level is progressively reduced, whereas plant allelo-chemical intake, including tannins, increases. During omnivory evolution, selective pressure from a low nitrogen but high tannin-diet became sufficiently strong to favour both an increased secretion level and diversification of PRPs for dealing with tannins. If correct, then carnivores should secrete low levels of PRPs for oral homeostasis, whereas omnivores dieting a higher proportion of tannin-containing foliage or fruit should generally be able to produce high levels of salivary PRPs. Browsers and frugivores should also secrete high levels of PRPs, but grazers may have reduced secretion rates depending on the antiquity of their dietary habit (McArthur et al., 1995).

In some rodents, salivary TBPs are induced by dietary tannins (Mehansho et al., 1983, 1985; Asquith et al., 1985; Mehansho, 1992), whereas in grazing or strictly browsing ruminants, their secretion remained unaffected by dietary tannin levels (Makkar and Becker, 1998; Austin et al., 1989; Clauss et al., 2003). The fundamental difference in the coping mechanism of herbivorous mammals in this respect indicates that both the constitutive and induced production of TBPs are metabolically viable options. Because the production of TBPs infers metabolic costs for the animal, a feedback mechanism that adapts the amount of TBPs produced to the tannin content of the forage helps to keep these costs from exceeding the benefit derived from the TBPs. Under this assumption, animals whose natural diet varies in tannin content (e.g. seasonally) would benefit from an inducible TBP production. However, such tuning mechanism might not have evolved in species whose diet contained no tannins or had tannin concentrations below an inductive threshold, or might have become lost in species consuming constant, high dietary tannin concentrations in their natural forage.

Among the extant rhinoceros species, dietary choices of free-ranging animals differ significantly between species. The Black (*Diceros bicornis*) and the Sumatran rhinoceros (*Dicerorhinus sumatrensis*) are reported to be exclusive browsers (Ritchie, 1963; Goddard, 1968; Schenkel and Schenkel-Hullinger, 1969; Goddard, 1970; Mukinya, 1977; Hall-Martin et al., 1982; Van Strien, 1986; Loutit et al., 1987; Oloo et al., 1994; Muya and Oguge, 2000), whereas the White rhinoceros (*Ceratotherium simum*) is a grazer (Player and Feely, 1960; Owen-Smith, 1973; Owen-Smith, 1988; Pienaar, 1994; Kiefer et al., 2003). The Indian rhinoceros (*Rhinoceros unicornis*) consumes mainly grasses (Groves, 1967; Brahmachary et al., 1971, 1974; Laurie, 1982; Dinerstein, 1989; Dinerstein and Price, 1991). Depending on season, grasses account for 70–89% of the diet (Laurie, 1982). Thus, the Indian rhino is generally regarded as a “mixed feeder” with a high proportion of grass in its diet (Owen-Smith, 1988).

Although explicit tannin analyses of black rhinoceros forages have not yet been performed on a larger scope (but cf. Loutit et al., 1987; Atkinson, 1995; Wright, 1998; Muya and Oguge, 2000, and the tannin analyses of Furstenburg and Van Hoven, 1994 and Dube et al., 2001 which include forage species that are included in the natural diet records of black rhinoceroses), it is beyond reasonable doubt that this strict browser ingests significant amounts of tannins in its natural habitat. In contrast, monocots such as grasses are generally low in tannin content and hydrolyzable tannins are notably absent from monocots (Ellis et al., 1983). Thus, based on the likely tannin content of the natural forages, it is hypothesized that the Black rhinoceros produces higher levels of salivary TBPs than the White rhinoceros, and that the Indian rhinoceros has intermediate levels. Of all rhinoceroses, only the saliva of captive black rhino has so far been investigated for the presence of such proteins (Nieper, 1998). In that study, salivary proteins were incubated with tannins prior to their electrophoretic separation, and a subsequent detection of darkened protein bands was interpreted as indicative for the presence of TBPs, leading to the conclusion that black rhinos were adapted to tannin-containing forages. Conventional zoo diets for captive rhinoceros species consist mainly of hay, pelleted concentrates and produce (Dierenfeld, 1995), and can therefore be regarded as either tannin-free or low in tannins.

We compared the tannin binding capacities of salivary proteins of captive Black, White and Indian rhinoceros on conventional zoo diets. Based on their natural feeding strategies, we expected the saliva of Black rhinoceroses to have a high and that of White rhinoceroses to have a low capacity to bind tannins, with the saliva of Indian rhinoceros in between. Second, we also experimentally increased the dietary tannin intake of several Black rhinoceroses, in order to investigate potential adaptational responses to increased dietary tannin concentrations.

2. Methods

For the first aim of this study, saliva was sampled from 9 White (*Ceratotherium simum*), 10 Black (*D. bicornis*) and 8 Indian (*R. unicornis*) rhinoceroses from a total of 12 zoological institutions. Saliva was obtained with a cotton swab from the oral cavity of conscious animals or animals that were immobilized for medical treatment

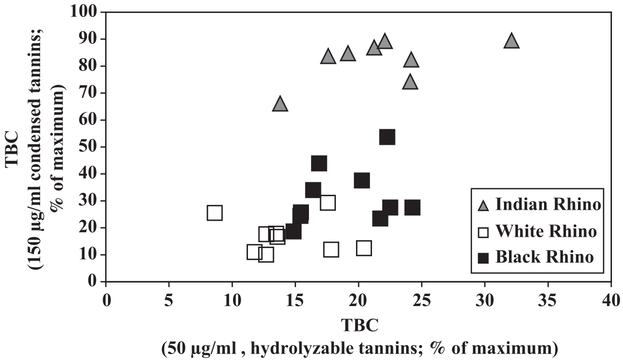

Fig. 1. Tannin-binding capacity (TBC) of salivary proteins of captive individuals of three rhinoceros species on regular zoo diets for tannic acid (a hydrolysable tannin) and quebracho (a source of condensed tannins).

(number of samples from conscious/immobilized animals: White rhinoceros 2/7, Black rhinoceros 10/0, Indian rhinoceros 4/4). The diets of the animals consisted of regular zoo diets with hay as the staple item and additional produce and concentrates. Saliva was separated from the cotton by centrifugation in salivettes (Sarstedt, Nümbrecht, Germany) at 5500×*g* for 20 min at 8 °C and desalted using Centricon YM 3 (Millipore, Eschborn, Germany). Filters had a molecular mass cut off of 3 kDa. Protein concentration of the total saliva was determined by the BCA-method of Smith et al. (1985) which was adapted to microtitre plates. Dilution series of bovine serum albumin (BSA) were used as standards. For the assay, 10 µL of either BSA (0–2.5 mg/mL) or desalted salivary proteins was mixed with 150 µL of the Smith Standard Working Reagent and incubated for 30 min at 37 °C. Absorbance was measured at 492 nm using a microtitre plate-reader (SLT Laborinstrumente, Crailsheim, Germany). Tannin binding capacity was measured by a tannin binding assay (Fickel et al., 1998, 1999). Samples were analysed on four plates. Tannic acid (Merck, Darmstadt, Germany) and quebracho (Tannin Corporation, MS, USA; non-purified, estimated condensed tannin content 75% according to Robbins et al., 1991) were used as standards for hydrolyzable and condensed tannins, respectively.

For the second aim, 7 out of the 10 Black rhinoceroses were additionally exposed to two different feeding periods each as part of another study on the influence of dietary tannins on digestive parameters in Black rhinoceroses. The animals first received their regular zoo diet with an addition of 5% tannic acid (hydrolysable tannin) to the pelleted ingredient of their diet for three months. This period was followed by a second 3-month period during which the dietary tannic acid was replaced by 5% quebracho (condensed tannin). This resulted in an average addition of 11 g tannic acid or quebracho per kg ingested dry matter (range: 5–15 g). Saliva collection from conscious animals and analytical procedures were performed as described above.

For statistics, one-way ANOVA and post hoc tests were performed in order to compare the species' tannin binding capacities for regular diet. The Black rhinoceroses' tannin binding capacities for different diets were compared using paired *t*-tests. Unpaired *t*-tests were applied for comparisons between Black and Indian rhinoceroses fed different diets. The significance level was generally set to $\alpha=0.05$. All statistical calculations were performed with the SPSS 11.0 statistical package (SPSS, Chicago, IL, USA).

3. Results

On the regular zoo diets, tannin binding capacity was lowest in the White rhinoceroses and highest in the Indian rhinoceroses (Fig. 1). Black rhinoceros saliva had higher tannin binding capacities than White rhinoceros saliva for both tannic acid (19.0%±3.6 vs. 14.3%±3.6, $p=0.069$) and quebracho (31.6%±10.7 vs. 16.9%±6.7, $p=0.004$); the difference, however, was only significant for the binding capacity for quebracho. Whereas tannin binding capacity of black rhinoceros saliva was similar to that of Indian rhinoceroses for tannic acid (21.8%±5.4, $p=0.532$), Indian rhinoceros saliva had a 2.5 times higher capacity to bind quebracho (82.1%±8.1, $p<0.001$).

Compared to the regular zoo diets, feeding diets with additionally supplemented tannic acid led to a significant increase in tannic acid-binding capacity of Black rhinoceros saliva (18.4%±3.2 vs. 26.2%±5.6, $p=0.026$), and a significant increase in quebracho-binding capacity (34.3%±12.0 vs. 77.3%±12.0, $p<0.001$). Feeding the diets with supplemented quebracho led to no increase in tannic acid-binding capacity (18.5%±8.2, $p=0.866$) as compared to the regular zoo diet, but again to a significant increase in quebracho-binding capacity (60.8%±25.1, $p=0.023$) (Fig. 2). After supplementation of both tannic acid or quebracho, the quebracho-binding capacity of Black rhinoceros saliva did not differ from the one measured in Indian rhinoceroses

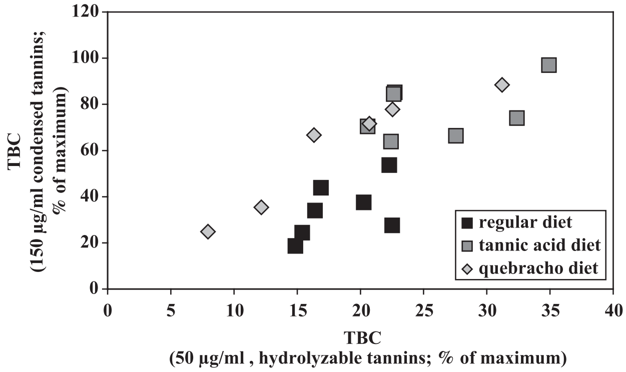

Fig. 2. Tannin-binding capacity (TBC) of salivary proteins of individual captive black rhinos on their regular zoo diet and on diets supplemented either with tannic acid or quebracho. TBC for tannic acid (a hydrolysable tannin) and quebracho (a source of condensed tannins).

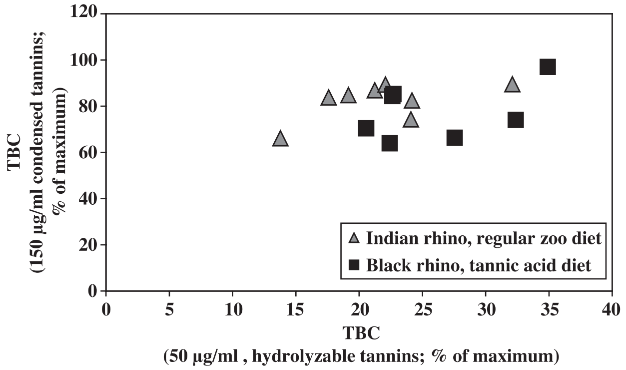

Fig. 3. Combined data from Fig. 1 for Indian rhinos on the regular zoo diet and from Fig. 2 for Black rhinos on a tannic-acid supplemented diet.

on the regular zoo diets (p=0.373 and p=0.093, respectively, Fig. 3).

4. Discussion

It is highly unlikely that the differences in salivary tannin-binding capacities are the result of different dietary regimes in the different zoological institutions. In such case, a more variate scatter of data points is expected and not the quite distinct cluster according to species. The results are as expected for White and Black rhinoceroses and reflect their respective dietary adaptations. Another measure correlated with the detoxification capacity of an animal—regarding hydrolysable tannins and other secondary plant metabolites—is its relative liver size, as most detoxification processes occur in the liver. Hofmann (1989) stated that browsing ruminants have larger livers than grazing ruminants of comparable size. A similar fact was observed comparing the browsing Black rhinoceros with the grazing White rhinoceros (Kock and Garnier, 1993). The binding capacity of Indian rhinoceros saliva to both tannins, however, and especially the high affinity to condensed tannins, is surprising. Two potential explanations are conceivable:

1. The diet of free-ranging Indian rhinos contains higher amounts of condensed tannins than presumed. On one hand, this could mean a higher dietary proportion of browse material than reported (Laurie, 1982). To test this hypothesis, more information on foraging behaviour and diet composition of free-ranging Indian rhinos is required. On the other hand, it could also mean that the tannic secondary plant compounds in the gramineous species usually consumed by Indian rhinoceroses is higher than assumed. Grasses can uniquely produce ent-epicatechin, a monomer found in condensed tannins (Ellis et al., 1983). In this respect, it would be interesting to compare the secondary plant compounds in the natural forage of White vs. Indian rhinoceroses.
2. The high amount of TBPs in Indian rhino saliva could be an ancestral trait from evolutionary older times when the ancestors of the Indian rhino relied more heavily on browse forage; the Indian rhino might simply not have lost these TBPs, maybe due to a continuous stimulation by the low but consistent percentage browse in its natural diet (Laurie, 1982). Based on the analyses of perissodactyl mitochondrial gene sequences, Asian and African rhinoceros lineages were estimated to have diverged at about 26 million years ago (Norman and Ashley, 2000; Tougard et al., 2001). This event predates the miocenic radiation of gramineae (23.8–5.3 my) leading to the conclusion that the ancestral rhinoceroses had to be browsers. If Indian and Black rhinos still had the same ancestrally inherited mechanism to deal with dietary tannins, one would expect similar levels of TBPs in Indian and Black rhinoceroses. However, the two species are phylogenetically more distant than the Black and the White rhinoceros (Morales and Melnick, 1994; Norman and Ashley, 2000; Tougard et al., 2001). Because divergence time between the latter two species was already sufficiently long to allow for well distinguished feeding strategies to evolve (browser vs. grazer), the even larger time scale since the divergence of Indian and Black rhinoceroses from their last common ancestor allows also for an independent evolution of either new or further developed mechanisms to deal with dietary tannins. Thus, in Black rhinoceros, a mechanism for TBP-production could have evolved that responds to the dietary tannin content (induceable TBPs) but produces only comparatively low tannin binding capacities on a tannin-free conventional zoo diet.

In order to test this latter hypothesis, saliva was tested from Black rhinoceroses that received different tannin supplementations to their regular captive diet as part of another digestion study. The results indicate that the Black rhinos investigated increased their production of salivary TBP in response to increased levels of dietary tannins. The levels of TBPs attained by Black rhinoceroses after the dietary tannin supplementation were similar to the ones attained by Indian rhinoceroses on un-supplemented diets (Fig. 3). To our knowledge, this is the first report of an induceable TBP production in an ungulate species. The fact that TBPs capable to bind hydrolyzable tannin were more responsive to dietary stimulation could indicate that hydrolyzable tannins play a greater role in the Black rhino's natural environment than condensed tannins. On the other hand, the lack of response to quebracho tannins could either indicate that there was no selective pressure on Black rhinos to adapt to condensed tannins or simply that the used quebracho tannins do not resemble the condensed tannins these animals had to adapt to in their natural environment (Hagerman et al., 1992). Taken into account that the 5% tannin supplement in the pelleted feed resulted only in app.

1% tannin supplement in the totally consumed dietary dry matter, the response indicates that the feedback mechanism of Black rhinos can detect comparatively small amounts of tannic substances and react to them. Although the black rhinoceros is a strict browser and tannins have been found in its natural diet (Wright, 1998), potential seasonal or regional shifts in dietary tannin concentrations have not been investigated to date. If such shifts are hypothesized, these animals would benefit from an adjustable mechanism to produce TBP.

The data indicate the feasibility of induction trials with rhinoceroses. The reaction of other rhinoceros species to a dietary tannin supplementation would be particularly interesting in comparison; given the data from our study, one can only speculate that both White and Indian rhinoceroses might not react to dietary tannin supplementation at all.

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