

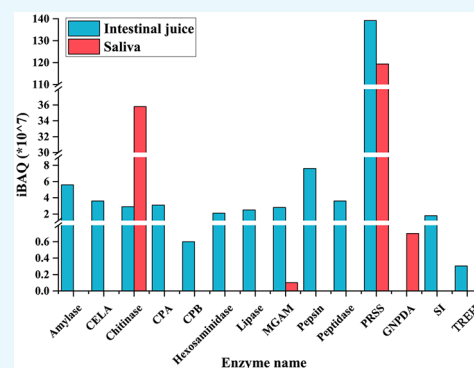
# Expression Profile of the Digestive Enzymes of *Manis javanica* Reveals Its Adaptation to Diet Specialization

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## Supporting Information

**ABSTRACT:** The expression of animal digestive enzymes reflects important dietary adaptations. The pangolin, also known as scaly anteater, is a specialized myrmecophage that consumes mainly ants and termites, but its digestive enzymes have not been fully investigated. Therefore, in this study, we used shotgun proteomic analysis to examine the protein components of the saliva and intestinal juice of a Sunda pangolin (*Manis javanica*) that died shortly after being rescued. The intestinal juice contained greater variety of digestive enzymes, including  $\alpha$ -amylase, maltase-glucoamylase,  $\alpha,\alpha$ -trehalase, sucrase-isomaltase, pepsin A, trypsin, pancreatic endopeptidase E, carboxypeptidase A1, carboxypeptidase B, dipeptidyl-peptidase 4, and pancreatic triacylglycerol lipase. The digestive enzymes identified in the saliva were maltase-glucoamylase and trypsin, and chitinase which was also found in the intestinal juice. Compared with other animals, the Sunda pangolin has less intestinal protease diversity and lacks key digestive enzymes, such as chymotrypsin and pancreatic elastase. The expression profile of the digestive enzymes of the Sunda pangolin reveals animal's adaptation to a diet consisting mainly of ants and termites. Our results will facilitate the preparation of artificial food for rescued pangolins and for those in captivity for conservation breeding efforts.



## INTRODUCTION

The extant mammals, under the powerful selective pressure exerted by the need for successful acquisition of food resources, have evolved a wide variety of dietary niches, ranging from broadly generalized to highly specialized.<sup>1</sup> However, food choices are constrained by the capability of an animal's digestive system to extract nutrients effectively.<sup>2</sup> The mammalian digestive system has evolved to include several anatomical structures, a complex microbial flora,<sup>3</sup> and digestive enzymes adapted to feeding habits.<sup>4,5</sup>

Pangolins are specialized myrmecophagous mammals that feed mainly on ants and termites<sup>6–12</sup> and have a unique external armor of overlapping scales; hence, they are often called scaly anteaters. Like some other myrmecophagous mammals, such as anteaters, pangolins have powerful front claws that allow them to dig into ant and termite nests. They also have a pointed snout, toothless mouth, and long, flexible, slimy tongue adapted to preying on ants and termites.<sup>13,14</sup> In addition to adaptation for predation, the efficient extraction of nutrients from food is another important challenge for pangolins. The polysaccharide chitin, a major exoskeleton component, makes up 5–20% of the dry weight of ants and termites;<sup>15</sup> thus, most of the protein value of ants and termites is locked in the exoskeleton,<sup>16</sup> and chitin is recalcitrant to digestion. How pangolins get energy and nutrients efficiently from ants and termites is not well understood. There is little

research on the pangolin digestive system; published studies have focused on its morphology and structure. The inner gastric surface of the Sunda pangolin (*Manis javanica*) has a thick layer of keratinized epithelial cells.<sup>17</sup> Like Chinese pangolin (*Manis pentadactyla*), the gastric fundus of Sunda pangolins has many mucosal folds to expand the gastric surface area,<sup>17–19</sup> and the pylorus has hard spines called “pyloric teeth”,<sup>17,19</sup> which are similar to the stomachs of other ant-eating animals, such as Indian pangolins (*Manis crassicaudata*),<sup>20</sup> grasshopper mouse (*Onychomys torridus*),<sup>21</sup> armadillo, and echidna<sup>22</sup> and is thought to be suitable for its ant-eating habit and toothless characteristics.<sup>17,22,23</sup> However, no horny ridges are found in the stomachs of the giant pangolin (*Smutsia gigantea*), long-tailed pangolin (*Phataginus tetradactyla*), or tree pangolin (*Phataginus tricuspis*), and this is thought to be a possible difference between Asian and African pangolins.<sup>19</sup> Neither the Chinese pangolin nor the Sunda pangolin has a caecum, and there is no obvious boundary between the large and small intestines.<sup>24</sup> The small intestine of Chinese pangolin is about nine times its body length, longer than that of a carnivore, and it is thought to take longer time to digest ants and termites.<sup>24</sup>

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Although digestive enzymes and gut microbes play an important role in animal digestion, there is only one report on the digestive enzymes and gut microbes of pangolins. The intestinal microbial community of the Sunda pangolin is similar to that of herbivores but different from that of ant-eaters and other ant-eating animals.<sup>25</sup> It does not conform to the theory that gut microbiomes evolved convergently in the mammals sharing the same feeding habits.<sup>26</sup> Another paper verified the presence of chitinase in the stomach of the Sunda pangolin.<sup>27</sup> However, there are no reports about the characteristics of digestive enzyme expression in saliva and small intestine. The small intestine, in particular, is the most important site for food digestion. What is more, the pangolins have a higher incidence of gastrointestinal diseases in captivity, and it is speculated to be related to the lack of understanding of pangolins' digestive physiology, and the artificial food provided is not well digested and absorbed. Hence, we hypothesize that pangolin has formed specialized digestive physiology, which lacks the expression of some proteases, but expresses more certain digestive enzymes, such as chitinase, to adapt to the specialized ant-eating habits.

In recent years, the development of proteomics has allowed investigation of the expression profiles of proteins in specific tissues or cells, and thus determination of the cellular metabolic potential.<sup>28,29</sup> The use of proteomics for the large-scale detection of proteins in animal digestive juices can provide insight into animal digestive physiology in an evolutionary environment.<sup>30</sup> Digestive enzymes and variation in the digestive tract play key roles in the decomposition of large food molecules into small absorbable compounds and are extremely important in dietary adaptation. Therefore, to understand the digestive function of the Sunda pangolin, this study used shotgun liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the first time to detect the variety and relative abundance of digestive enzymes in saliva and intestinal juice of the Sunda pangolin and to construct an expression profile. Our results provide insight into the mechanisms underlying the digestive adaptation of pangolins and a basis for the selection of artificial food ingredients in the ex situ conservation of these animals.

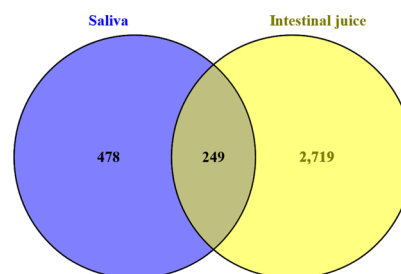
## RESULTS

**Identification of Proteins in the Saliva and Intestinal Juice of the Sunda Pangolin.** In the tested Sunda pangolin, shotgun LC-MS/MS and MaxQuant software analysis revealed significant differences in the variety of proteins in the two digestive fluids, with 727 distinct proteins in saliva and 2968 in the intestinal juice (Table 1). There were 296 and 1030 proteins with  $\geq 2$  unique peptides, respectively (Table 1). Of these identified proteins, 91 and 25 proteins could not be quantified. Myoglobin, keratin, and actin were the most abundant proteins in saliva and intestinal juice, with intensity-based absolute protein quantification (iBAQ) values of 7 351 000 000 and 9 135 400 000, respectively.

**Table 1. Proteins Identified in Two Digestive Juices from Sunda Pangolin HS-04**

sample	protein group	unique peptide			iBAQ = 0
		=0	=1	$\geq 2$	
saliva	727	45	386	296	91
intestinal juice	2968	224	1714	1030	25

Fewer specific proteins were found in saliva ( $n = 478$ ) than in intestinal juice ( $n = 2719$ ), whereas the two digestive juices had 249 proteins in common (Figure 1).



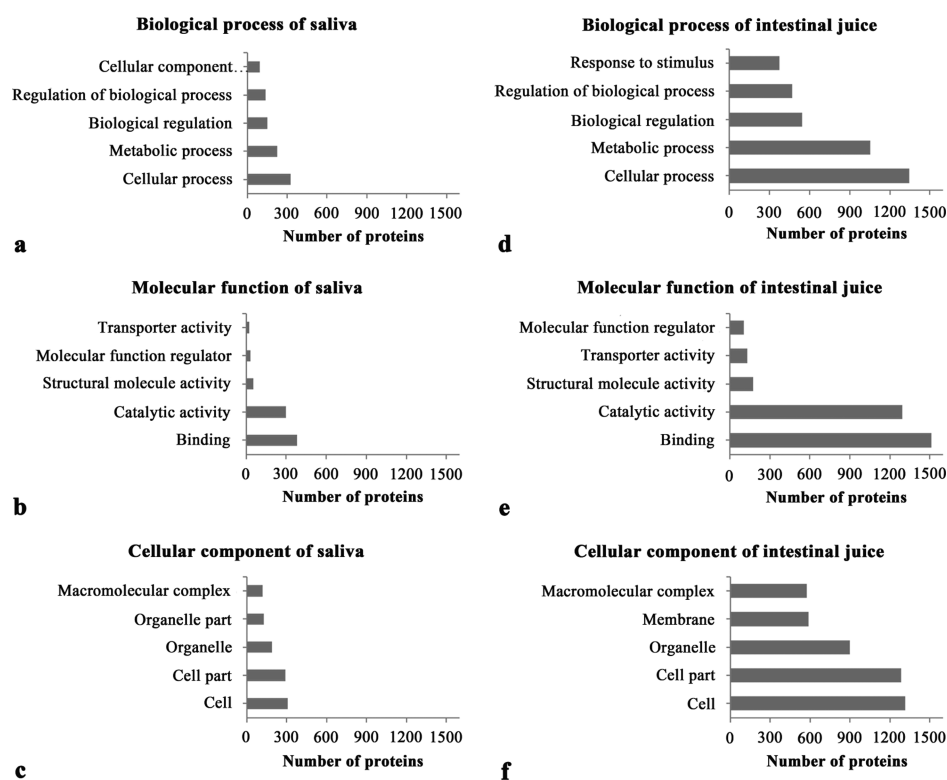
**Figure 1.** Venn diagram of the common and unique proteins among the total proteins in the saliva and intestinal juice of the Sunda pangolin HS-04.

**Functional Predictions for the Proteins Identified in Saliva and Intestinal Juice.** Protein functional annotation provides insight into the physiological function of a sample. Gene ontology (GO) annotation was performed for 719 and 2957 proteins in saliva and intestinal juice of Sunda pangolin HS-04, respectively, of which 8 and 11, respectively, could not be matched because of a lack of annotation. According to prediction analyses, most of the proteins in the two digestive juices were involved in binding and catalytic activity in the molecular function: 383 and 300 proteins in saliva (Figure 2b) and 1513 and 1295 proteins in intestinal juice (Figure 2e). Further analysis assigned most of the proteins from the two digestive fluids to cellular and metabolic processes in the biological process and to cell and cell parts in cellular components (Figure 2).

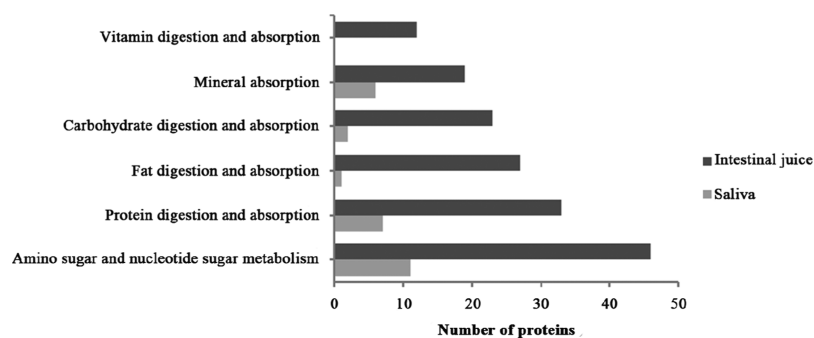
**Digestive Enzyme Expression Analysis.** In the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, 316 and 356 pathways were enriched in saliva and intestinal juice of Sunda pangolin HS-04, respectively (Tables S1 and S2). In accordance with the purpose of this study, we focused only on the key pathways associated with digestion and absorption. These five pathways were related to carbohydrate digestion and absorption, protein digestion and absorption, fat digestion and absorption, vitamin digestion and absorption, and mineral absorption. All of these pathways were annotated in the intestinal juices, whereas the vitamin digestion and absorption pathway was not annotated in saliva.

The number of varieties of proteins related to digestion and absorption pathways enriched in saliva and intestinal juice is shown in Figure 3. Among the two digestive fluids of this pangolin, the larger variety of proteins involved in the five digestion and absorption pathways was annotated in intestinal juice, in which the number of types of annotated digestive enzymes was also greater and included  $\alpha$ -amylase, maltase-glucoamylase,  $\alpha, \alpha$ -trehalase, sucrase-isomaltase, pepsin A, trypsin, pancreatic endopeptidase E, carboxypeptidase A1, carboxypeptidase B, dipeptidyl-peptidase 4, angiotensin-converting enzyme 2, neprilysin, pancreatic triacylglycerol lipase, secretory phospholipase A2, and pancreatic lipase-related protein 1 (Table 2). Only two digestive enzymes, maltase-glucoamylase and trypsin, were annotated in the saliva of the pangolin.

We also considered the amino sugar and nucleotide sugar metabolism pathway as it is related to chitin degradation and



**Figure 2.** Functional classification of the biological processes, molecular functions, and cellular components of saliva (a–c) and intestinal juice (d–f) for Sunda pangolin HS-04 based on GO annotation.



**Figure 3.** Five digestion and absorption pathways and the amino sugar and nucleotide sugar metabolism pathways in saliva and intestinal juice of Sunda pangolin HS-04 according to KEGG annotation.

the pangolin diet contains a considerable amount of chitin. This pathway was enriched in two digestive juices of this tested pangolin, with the larger variety of related proteins present in the intestinal juice (Figure 3, Table S2). In addition to the chitinolytic enzyme chitinase and hexosaminidase (Table 2), these proteins included multiple enzymes associated with amino sugar metabolism (Figure 4). In the saliva, chitinase and several other enzymes associated with amino sugar metabolism, such as glucosamine-6-phosphate deaminase, were detected (Table 3 and Figure 5). The iBAQ values for these important digestive enzymes identified in the saliva and intestinal juice ranged from  $10^6$  to  $10^9$  (Figure 6).

## DISCUSSION

The enzymes in the digestive system of animals are one of the key factors related to their digestive function. Pangolin is a mammal with specialized food, that is, ants and termites. In this study, we speculated that the expression of digestive

enzymes in pangolin's digestive system had formed an adaptation to its specialized food and analyzed the types of digestive enzymes in saliva and intestinal fluid of a Sunda pangolin. Unfortunately, we were unable to detect the types of digestive enzymes in gastric fluid because of problems in sample preservation. The higher diversity of proteins and digestive enzymes in the Sunda pangolin was found in intestinal juice than saliva. Trypsin was detected in two digestive fluids and was the most abundant digestive enzyme (Figure 6). In addition to protein digestion, trypsin plays a central role in regulating the activities of other digestive enzymes.<sup>31</sup> This may explain why trypsin was present in the saliva of the pangolin and in its small intestine.

A diet consisting of insects, especially ants and termites, contains considerable amounts of the protein value and other nutrients locked in chitin.<sup>16</sup> Although chitin is as an excellent source of carbon, energy, and nitrogen, it must first be broken down by chitinase into *n*-acetylglucosamine residues that can

**Table 2. Variety of Digestive Enzymes Identified in the Intestinal Juice of Sunda Pangolin HS-04**

no.	protein ID	name	definition
1	G9LIC1	amylase	$\alpha$ -amylase [EC 3.2.1.1]
2	A0A287A042	MGAM	maltase-glucoamylase [EC 3.2.1.20, 3.2.1.3]
3	M3WU26	MGAM	maltase-glucoamylase [EC 3.2.1.20, 3.2.1.3]
4	D2HHN0	MGAM	maltase-glucoamylase [EC 3.2.1.20, 3.2.1.3]
5	F7DGG1	MGAM	maltase-glucoamylase [EC 3.2.1.20, 3.2.1.3]
6	G1PWG9	MGAM	maltase-glucoamylase [EC 3.2.1.20, 3.2.1.3]
7	U6D7J0	TREH	$\alpha,\alpha$ -trehalase [EC 3.2.1.28]
8	A0A172ZB06	SI	sucrase-isomaltase [EC 3.2.1.48, 3.2.1.10]
9	S7PEU0	SI	sucrase-isomaltase [EC 3.2.1.48, 3.2.1.10]
10	E1BGH5	SI	sucrase-isomaltase [EC 3.2.1.48, 3.2.1.10]
11	M3YE08	SI	sucrase-isomaltase [EC 3.2.1.48, 3.2.1.10]
12	W5NU90	SI	sucrase-isomaltase [EC 3.2.1.48, 3.2.1.10]
13	A0A1S2ZY73	pepsin	pepsin A [EC 3.4.23.1]
14	P00761	PRSS	trypsin [EC 3.4.21.4]
15	L5LNG8	PRSS	trypsin [EC 3.4.21.4]
16	M3WP64	PRSS	trypsin [EC 3.4.21.4]
17	F1PI75	CELA	pancreatic endopeptidase E [EC 3.4.21.70]
18	M3WKB7	CPA	carboxypeptidase A1 [EC 3.4.17.1]
19	L5KB43	CPB	carboxypeptidase B [EC 3.4.17.2]
20	A0A212C1I6	peptidase	dipeptidyl-peptidase 4 [EC 3.4.14.5]
21	L5JZX8	peptidase	dipeptidyl-peptidase 4 [EC 3.4.14.5]
22	E2DHI6	peptidase	angiotensin-converting enzyme 2 [EC 3.4.17.23]
23	L8IHS5	peptidase	nepriysin [EC 3.4.24.11]
24	F5C3N2	peptidase	nepriysin [EC 3.4.24.11]
25	P00591	lipase	pancreatic triacylglycerol lipase [EC 3.1.1.3]
26	U6DM40	lipase	secretory phospholipase A2 [EC 3.1.1.4]
27	D2H719	lipase	pancreatic lipase-related protein 1 [EC 3.1.1.3]
28	A0A1S2ZFC8	lipase	pancreatic lipase-related protein 1 [EC 3.1.1.3]
29	A0A212CG51	3.2.1.14	chitinase [EC 3.2.1.14]
30	G1LGG0	3.2.1.14	chitinase [EC 3.2.1.14]
31	G9K475	3.2.1.52	hexosaminidase [EC 3.2.1.52]

be transported into the cell.<sup>32</sup> In this study, it was found that chitinase was expressed in the saliva and intestinal juice of Sunda pangolin HS-04, and hexosaminidase was also expressed in the intestinal juice. A diet rich in certain nutrients exerts selective pressure on the evolution of genes encoding more specific digestive enzymes in different parts of the digestive system.<sup>4,5</sup> Chitinase is commonly found in the stomachs of mammals as acidic mammalian chitinase.<sup>15,33–35</sup> For better digestion of chitin in food, in addition to the stomach, chitinase is found in the intestines and saliva of some other insect-eating animals, such as bats and rodents.<sup>32,34</sup> It was also detected in the saliva and intestinal juice of the Sunda pangolin, in amounts similar to those of most other digestive enzymes (Figure 6). This implies the existence of strong evolutionary pressure for Sunda pangolins to digest chitin effectively. The expression of chitinase in the mouth, stomach,

and small intestine allows efficient nutrient extraction. Moreover, compared to those of carnivores, pangolins have longer intestinal tracts,<sup>24</sup> which may allow chitinase more time for chitin digestion in the intestine. Together with the chitinase expressed in its saliva and stomach,<sup>27</sup> these constitute an adaptation that allows the utilization of ants and termites as primary food sources.

The wide variety of proteins annotated in the amino sugar and nucleotide sugar metabolism pathway in the tested pangolin intestinal juice indicates not only an evolutionary adaptation for chitin digestion but also the importance of chitin in the pangolin diet. The role of chitin in the dietary nutrition of pangolins and giant anteaters (*Myrmecophaga tridactyla*) was initially thought to be as a source of dietary fiber.<sup>24,36</sup> However, the annotation of other enzymes involved in amino sugar and nucleotide sugar metabolism in the pangolin intestinal tract (besides chitinase and hexosaminidase; Figure 4) suggests that chitin is broken down into *n*-acetylglucosamine monomers, which are then converted to glucose and nucleotide sugars. Accordingly, chitin likely provides substantial nutrients for pangolins.

There is low protease diversity in the Sunda pangolin intestine, which lacks some protein digestive enzymes. Proteins are eventually digested by proteases into amino acids, which provide key nutrients for growth, development, repair, energy, and many other bodily functions. The significant overlap in functional protease subtypes (i.e., the multiple parallel and redundant systems) in the animal digestive tract ensures an adequate supply of all the key amino acids despite a diverse and inconsistent diet.<sup>31</sup> However, we found that several common intestinal proteases, such as chymotrypsin B1, chymotrypsin, chymotrypsin-like protease, pancreatic elastase 2 (EC 3.4.21.71), carboxypeptidase A2 (EC 3.4.17.15), and carboxypeptidase B2 (EC 3.4.17.20), were missing from the protein digestive system of Sunda pangolin HS-04. This is also the case in several primates, such as platyrrhine monkeys, which rely primarily on insect protein and thus have less pepsin diversity than do primates that depend on foliage protein.<sup>37,38</sup> Thus, insectivorous species may not need multiple pepsins to digest insect proteins, whereas chitinase is essential for the digestion of insect exoskeletons.<sup>2</sup> As the diversity and complexity of proteins in the diet of Sunda pangolins, which feed on ants and termites, are stable, a large number of parallel and redundant systems for dietary protein digestion would not be needed. The digestive enzymes in the animal gut represent the response to dietary diversity, and changes in their expression or type are essential to allow dietary adaptations, including those enabling the use of complex or otherwise indigestible foods.<sup>2</sup> Sunda pangolins can digest artificial food made from invertebrates well but not feed containing fish meal, eggs, or dairy products.<sup>32</sup> Because different proteases have different specific pockets where single peptide bonds are hydrolyzed,<sup>31,39,40</sup> the inability to digest some products may be related to the absence in the pangolin digestive system of chymotrypsin, elastase, and other proteases needed for the breakdown of these proteins. The digestive system of Sunda pangolins also lacks the enzymes needed to break down lactose, which explains why these animals are unable to digest dairy products.<sup>2,41</sup> Therefore, artificial food for pangolins should be designed to be compatible with the physiological characteristics of pangolin digestion, including the spectrum of enzymes in its digestive system.



**Table 3. Variety of Digestive Enzymes Identified in the Saliva of Sunda Pangolin HS-04**

no.	protein ID	name	definition
1	M3X654	MGAM	maltase-glucoamylase [EC 3.2.1.20, 3.2.1.3]
2	P00761	PRSS1_2_3	trypsin [EC 3.4.21.4]
3	G1LGG0	E3.2.1.14	chitinase [EC 3.2.1.14]
4	T0NRJ1	E3.2.1.14	chitinase [EC 3.2.1.14]

have led to a rapid global decline in the populations of these animals.<sup>43,44</sup> They are difficult to study because of their nocturnal habits, rarity, secretiveness, and solitary nature. They cannot be captured for research needs because they are critically endangered on the IUCN Red List of Threatened Species. Although the samples examined in this study were collected from a single dead Sunda pangolin, they provided basic data and an opportunity to elucidate more about the digestive physiology of this species. However, the results of this study only comes from one pangolin, we need to obtain more samples for in-depth studies to validate the results. Then, we will be able to better understand the digestive physiology of this species.

## CONCLUSIONS

The proteases expressed are simplified in the tested Sunda pangolin intestinal tract. This might indicate that species' adaptation to its diet is stable with respect to protein diversity and complexity because the pangolin feeds exclusively on ants and termites. Chitinase has been detected in its saliva and intestinal juice and allows the efficient digestion and utilization of ants and termites. This may reflect its adaptation to a chitin-rich diet. KEGG annotation of the proteins in the pangolin's intestinal juice also implied that chitin provides carbon, nitrogen, and energy, and is therefore a substantial nutrient for the Sunda pangolin. Although only one Sunda pangolin was available for our study, it provided us with a valuable opportunity to understand the digestive physiology and eating habits of this critically endangered species.

## MATERIALS AND METHODS

**Subject.** Samples were taken from a female Sunda pangolin (HS-04) at the Pangolin Research Base for Artificial Rescue and Conservation Breeding of South China Normal University (PRB-SCNU). The pangolin weighed 1.8 kg when it arrived at PRB-SCNU and died 1 day later because of unknown causes.

**Collection of Saliva and Intestinal Juice.** Immediately after its death, pangolin HS-04 was dissected. No obvious lesion was found in its oral cavity or intestines. The back of a clean scalpel was used to gently swab the saliva on the tongue surface and the inside of the pangolin's cheek. Intestinal contents were collected 3–5 cm past the opening of the duodenal bile duct. All collected fluids were placed in a cryopreservation tube and stored immediately in liquid nitrogen, and then shipped to the laboratory on dry ice. Although gastric juice of this pangolin was also collected during sampling, the sample was exposed to room temperature for a long time because of the damage of the packaging box of gastric juice during transportation, which seriously affected the quality of the sample, so it was not tested and analyzed.

The pangolin was housed and post-mortem sampling was conducted in accordance with procedures approved by the Ethics Committee for Animal Research at SCNU and the

Guidelines for Animal Care established by the National Institute of Health.

**Sample Preparation and Protein Separation by SDS-PAGE.** The samples were prepared following the methods of Wiśniewski et al.<sup>45</sup> After the addition of SDT buffer, the samples were boiled for 15 min and centrifuged at 14 000g for 40 min; the protein in the supernatant was then quantified using a BCA protein assay kit (Bio-Rad, Hercules, CA, USA). The samples were then stored at  $-80\text{ }^{\circ}\text{C}$ .

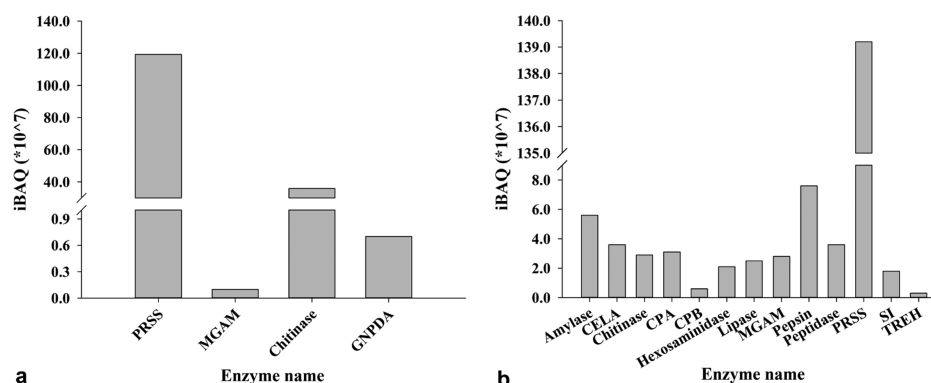
For sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 20  $\mu\text{g}$  of protein per sample was mixed with 5 $\times$  loading buffer and boiled for 5 min. The proteins were separated on a 12.5% polyacrylamide gel (constant 14 mA current, 90 min), and the resulting bands were visualized by Coomassie Blue R-250 staining.

**Filter-Aided Sample Preparation.** The proteins in the samples were digested using a filter-aided sample preparation method.<sup>45</sup> For each sample, 200  $\mu\text{g}$  of protein was mixed with 30  $\mu\text{L}$  of SDT buffer (4% SDS, 100 mM dithiothreitol (DTT), and 150 mM Tris-HCl; pH 8.0). The detergent, DTT, and other low-molecular-weight components were removed using UA buffer (8 M urea and 150 mM Tris-HCl; pH 8.0) by repeated ultrafiltration (Microcon units, 10 kDa). Reduced cysteine residues were blocked by the addition of 100  $\mu\text{L}$  of iodoacetamide (100 mM indole-3-acetic acid in UA buffer). The samples were then incubated for 30 min in the dark, washed three times with 100  $\mu\text{L}$  of UA buffer and twice with 100  $\mu\text{L}$  of 25 mM  $\text{NH}_4\text{HCO}_3$  buffer, and then incubated overnight at 37  $^{\circ}\text{C}$  with 4  $\mu\text{g}$  of trypsin (Promega, Madison, WI, USA) in 40  $\mu\text{L}$  of 25 mM  $\text{NH}_4\text{HCO}_3$  buffer. The resulting peptides were collected as a filtrate, desalted on C18 cartridges [Empore SPE C18 cartridges (standard density), bed I.D. 7 mm, volume 3 mL; Sigma, St. Louis, MO, USA], concentrated by vacuum centrifugation, and reconstituted in 40  $\mu\text{L}$  of 0.1% (v/v) formic acid. The peptide content was estimated based on the absorption at 280 nm using an extinction coefficient of 1.1 for a 0.1% (g/L) solution, calculated based on the frequencies of tryptophan and tyrosine residues in vertebrate proteins.

**Shotgun LC-MS/MS and Data Analysis.** Nano LC-MS/MS was performed on the products obtained from the enzymatic hydrolysis of 3 g of samples according to the quantitative results, and each sample was injected once. The peptide mixture was loaded onto a reverse-phase trap column (Thermo Scientific Acclaim PepMap100, 100  $\mu\text{m} \times 2\text{ cm}$ , nanoViper C18; Thermo Fisher Scientific, Waltham, MA, USA) connected to a C18 reversed-phase analytical column (Thermo Scientific EASY-Column, 10 cm long, 75  $\mu\text{m}$  inner diameter, 3  $\mu\text{m}$  resin; Thermo Fisher Scientific) in buffer A (0.1% formic acid) and separated with a linear gradient of buffer B (84% acetonitrile and 0.1% formic acid) at a flow rate of 300 nL/min, controlled by IntelliFlow technology. The linear gradient was as follows: 0–35% buffer B for 50 min, 35–1100% buffer B for 5 min, and holding in 100% buffer B for 5 min.

LC-MS/MS was performed on a Q Exactive mass spectrometer (Thermo Fisher Scientific) coupled to an EASY-nLC device [Proxeon Biosystems (Thermo Fisher Scientific)] for 60 min. The mass spectrometer was operated in the positive ion mode. MS data were acquired using a data-dependent top 10 method, with dynamic selection of the most abundant precursor ions from the survey scan (300–1800  $m/z$ ) for HCD fragmentation. The automatic gain control target was set to  $3 \times 10^6$ , and the maximum injection time to 50 ms.





**Figure 6.** iBAQ intensities of the digestive enzymes detected in saliva (a) and intestinal juice (b) of Sunda pangolin HS-04.

many)<sup>46</sup> and searched against uniprot\_Laurasiatheria\_553856\_20180403.fasta (553 856 total entries, downloaded 03/04/2018, <http://www.uniprot.org>). An initial search was set at a precursor mass window of 6 ppm. The search followed an enzymatic cleavage rule of trypsin/P and allowed a maximum of two missed cleavage sites and a mass tolerance of 20 ppm for fragment ions. It was designed as follows: enzyme = trypsin, missed cleavage = 2, fixed modification: carbamidomethyl (C), variable modification: oxidation (M), and decoy database pattern = reverse. The cutoff of the global false discovery rate for peptide and protein identification was set to 0.01.

No unexpected or unusually high safety hazards were encountered.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b02845.

Pathways and proteins enriched in the saliva of Sunda pangolin HS-04 and pathways and proteins enriched in the intestinal juice of Sunda pangolin HS-04 (PDF)

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### Notes

The authors declare no competing financial interest.

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