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Contribution of a tannase from *Atopobium parvulum* DSM 20469[T] in the oral processing of food tannins

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ABSTRACT

During oral passage, food tannins interact with the microbiota present in the oral cavity. *Atopobium parvulum* strains are inhabitants of the human oral cavity. A gene encoding a protein similar to bacterial tannases is present in *A. parvulum* strains. The *tanA*$_{Ap}$ (*apar_1020*) gene was cloned and expressed in *Escherichia coli* BL21 (DE3). The overproduced TanA$_{Ap}$ protein was purified to homogeneity. It exhibited optimal activity at pH 6.0 and broad temperature range, being these properties compatible with its action during food oral processing. However, purified TanA$_{Ap}$ protein presented the lowest specific activity among bacterial tannases (3.5 U/mg) and was unable to hydrolyze complex tannin, such as tannic acid. These biochemical properties discard a main role of TanA$_{Ap}$ in the breakdown of complex food tannins during oral processing.

Keywords:

Tannase, Food processing, Hydrolase, Gallic acid, Oral microbiome

40 1. Introduction

41

42 Vegetable tannins are abundant in plants utilized as human food. Tannins occur
43 widely on common foodstuffs, such as pomegranate, banana, strawberry, grape, cashew
44 nut, and hazelnut. Drinks like wine and tea also contain these phenolic compounds
45 (Shahidi & Naczk, 2003). The molar mass of tannins affects tannin characteristics directly,
46 and it has been suggested that small molecule tannins have more antioxidant activity
47 (Ordoudi & Tsimidou, 2006). To understand the biological effects of food tannins the
48 insight on the metabolic fate and bioavailability of these metabolites in the human body is
49 crucial, however this knowledge is currently scarce (Rechner, Kuhnle, Bremner, &
50 Hubbard, 2002; Moco, Martin, & Rezzi, 2012). The interaction of tannins with human
51 microbiota will determine in great extend the physiological effects of these polyphenols.
52 Although much work has been focused on factors that determine mechanical (e.g.
53 rheological and fracture) and sensory properties of foods, far less attention has been paid
54 to linking food transformations that occurs during oral processing with microbial action
55 (Chen, 2009). The human microbiome is a dynamic community changing in response to
56 natural perturbations such as diet (Turnbaugh, Hamady, Yatsunenko, Cantarel, Duncan,
57 Ley, et al., 2009; Wu, Chen, Hoffmann, Bittinger, Chen, Keilbaugh, et al., 2011; Spencer,
58 Hamp, Reid, Fishe, Zeisel, & Fodor, 2011; Zhang, Zhang, Wang, Han, Cao, Hua, et al.,
59 2010). The oral cavity of humans hosts several hundred taxa, with remarkable diversity
60 even among saliva, tongue, teeth, and other substrates (Segata, Haake, Mannon, Lemon,
61 Waldron, Gevers, et al., 2012; Dewhirst, Chen, Izard, Paster, Tanner, Yu, et al., 2010). All
62 of the surfaces of the mouth are covered in a bacterial biofilm (Wade, 2013). Studies on
63 the tongue biofilm have been relatively few in number, compared with the significant
64 number of investigations of dental plaque and the microbiota associated with periodontal

disease and dental caries. The tongue is known to harbour a very diverse microbiota at high cell density. Among the many bacteria present in the oral cavity, the species *Atopobium parvulum* is of interest because its members are frequently isolated from the human oral cavity, especially from the tongue (Riggio, Lennon, Rolph, Hodge, Donalson, Maxwell, et al., 2008; Copeland, Sikorski, Lapidus, Nolan, del Rio, Lucas, et al., 2009). The genome of *A. parvulum* type strain (IPP 1246^{T}) has been complete sequenced. An ORF (*apar_1020*) **encoding a "putative uncharacterized protein" had 40%** and 26% identity to $TanA_{Sl}$, a tannase from *Staphylococcus lugdunensis*, and $TanB_{Lp}$ (formerly called TanLp1), a tannase from *Lactobacillus plantarum*, respectively.

The enzyme tannase (or tannin acyl hydrolase EC 3.1.1.20) belongs to the serine esterases, catalyzing the hydrolysis of the ester bond (galloyl ester of an alcohol moiety) and depside bond (galloyl ester of gallic acid) in tannins to release gallic acid (Aguilar, Rodríguez, Gutierrez-Sánchez, Augur, Favela-Torres, Prado-Barragan, et al., 2007; Chávez-González, Rodríguez-Durán, Balagurusamy, Prado-Barragán, Rodríguez, Contreras, et al., 2012). Tannase activity contributes to the hydrolysis of natural tannins present in the diet. Although bacteria possessing tannase activity, such as *L. plantarum*, *Streptococcus gallolyticus*, and *Staphylococcus lugdunensis*, have been described in the human gastrointestinal tract (Abdulamir, Hafidh, Mahdi, Al-Jeboori, & Abubaker, 2009; Abdulamir, Hafidh, & Bakar, 2011; Noguchi, Ohashi, Shiratori, Narui, Hagiwara, Ko, et al., 2007; Rusniok, Couvé, de Cunha, El Gana, Zidane, Bouchier, et al., 2010), there are still many questions about the oral metabolism of food tannins. It will be interesting to know if during the short period of oral processing, tannin hydrolysis began in the mouth. The presence of a protein similar to bacterial tannases in *A. parvulum*, a species abundant in the oral cavity, will be important for predicting their contribution to food tannin breakdown. Therefore, the objective of this study was to find out the potential contribution

of $TanA_{Ap}$ (Apar_1020) protein from *A. parvulum* to tannin hydrolysis during food oral processing.

2. Materials and methods

2.1. Strains, plasmids, and materials

A. parvulum DSM 20469^T (IPP 1246^T, ATCC 33793^T) used through this study was purchased from the DSM (German Collection of Microorganisms and Cell Cultures). *Escherichia coli* DH10B was used as host strain for all DNA manipulations. *E. coli* BL21 (DE3), providing a T7 RNA polymerase, was used for heterologous expression in the pURI3-Cter vector (Curiel, de las Rivas, Mancheño, & Muñoz, 2011). *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 ºC and shaking at 200 rpm. When required, ampicillin was added to the medium at a concentration of 100 μg/mL.

Plasmid DNA was extracted by a High Pure plasmid isolation kit (Roche). PCR product was purified with a QIAquick gel extraction kit (Quiagen). Oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany). *Dpn*I and Prime STAR HS DNA polymerase were obtained from Takara. His-tagged protein was purified by a Talon Superflow resin (Clontech). The compounds assayed in the study were methyl gallate (Fluka), ethyl gallate (Aldrich), propyl gallate (Sigma), lauryl gallate (Aldrich), ethyl protocatechuate (ethyl 3,4-dihydroxybenzoate) (Aldrich), and tannic acid (Sigma).

2.2. Cloning of $TanA_{Ap}$

The gene encoding for a putative tannase (*apar_1020*, or *tanA*$_{Ap}$) in *A. parvulum* DSM20469^{T} (accession YP_003180040) was amplified by PCR by using the primers 1394 (5´–*ACTTTAAGAAGGAGATATACATatg*tctgataatacgaatcaacctgca) and 787 (5´–*GCTATTAATGATGATGATGATGATG*agcagacgcacacgagacaatcca) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the *tanA*$_{Ap}$ gene sequence are written in lowercase letters). As a peptide signal was predicted in the TanA$_{Ap}$ sequence, oligonucleotides 1394 and 787 were used to clone TanA$_{Ap}$ lacking the 23–amino acid peptide signal sequence. Prime Star HS DNA polymerase (TaKaRa) was used for the PCR amplification. The 1.7–kb purified PCR product was inserted into the pURI3–Cter vector using a restriction enzyme– and ligation–free cloning strategy (Curiel et al., 2011). The vector produces recombinant proteins having a six–histidine affinity tag in their C–termini. *E. coli* DH10B cells were transformed, recombinant plasmids were isolated, and those containing the correct insert were identified by size, verified by DNA sequencing, and then transformed into *E. coli* BL21 (DE3) cells for expression.

2.3. Enzyme production and purification

E. coli BL21(DE3) harbouring the recombinant plasmid pURI3–Cter–TanA$_{Ap}$ was grown in LB medium containing100 μg/mL ampicillin on a rotary shaker (200 rpm) at 37 ºC until an optical density (OD) at 600 nm of 0.4 was reached. Isopropyl–β–D–thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM and protein induction was continued at 22 ºC during 18 h.

The induced cells were harvested by centrifugation (8,000 *g*, 15 min, 4 ºC), resuspended in phosphate buffer (50 mM, pH 6.5) and disrupted by French Press passages

139 (three times at 1,100 psi). The insoluble fraction of the lysate was removed by

140 centrifugation at 47,000 *g* for 30 min at 4 °C, and the supernatant was filtered through a

141 0.2 µm pore-size filter and then loaded onto a Talon Superflow resin (Clontech)

142 equilibrated in phosphate buffer (50 mM, pH 6.5) containing 300 mM NaCl and 10 mM

143 imidazole to improve the interaction specificity in the affinity chromatography step. The

144 bound enzyme was eluted using 150 mM imidazole in the same buffer. The purity of the

145 enzyme was determined by SDS-PAGE in Tris-glycine buffer. Fractions containing the

146 His6-tagged protein were pooled and analyzed for tannase activity.

147

148 *2.4. Enzyme activity assay*

149

150 Tannase activity was determined using a colorimetic assay using rhodanine, specific

151 for gallic acid (Inoue & Hagerman, 1988). Rhodanine reacts only with gallic acid and not

152 with galloyl esters or other phenolics. Gallic acid analysis in the reactions was determined

153 using the following colorimetric assay. Tannase enzyme (100 µg) in 700 µl of 50 mM

154 phosphate buffer pH 6.5 was incubated with 40 µl of 25 mM methyl gallate (1 mM final

155 concentration) during 5 min at 37 ºC. After this incubation, 150 µl of a methanolic

156 rhodanine solution (0.667% w/v rhodanine in 100% methanol) was added to the mixture.

157 After 5 min incubation at 30 °C, 100 µl of 500 mM KOH was added. After an additional

158 incubation of 5–10 min, the absorbance at 520 nm was measured on a spectrophotometer.

159 A standard curve using gallic acid concentration ranging from 0.125 to 1mM was

160 prepared. One unit of tannase activity was defined as the amount of enzyme required to

161 release 1 µmol of gallic acid per minute under standard reaction condition.

162

163 *2.5. Determination of pH and temperature effects on tannase activity*

The optimum pH value of $TanA_{Ap}$ was determined by measuring its activity at different pH values (3.0–10.0). The following buffers all at 100 mM were used for the assay: acetic acid–sodium acetate (pH 3.0–5.0), citric acid–sodium citrate (pH 6), sodium phosphate (pH 7), Tris–HCl (pH 8), glycine–NaOH (pH 9), and sodium carbonate–bicarbonate (pH 10). The rhodanine assay was used for the optimal pH characterization of tannase. Since the rhodanine–gallic acid complex forms only in basic conditions, after the completion of the enzymatic degradation of methyl gallate, KOH was added to the reaction mixture to ensure that the same pH value (pH 11) was achieved in all samples assayed. Determinations were done in triplicate.

The optimum temperature of $TanA_{Ap}$ was assayed by incubating the purified protein in 25 mM phosphate buffer (pH 6.5) at seven different temperatures in the range of 4–65 ºC (4, 22, 30, 37, 45, 55 and 65 ºC). To study the thermal stability of $TanA_{Ap}$, tannase was incubated at temperatures over the range of 22–65 ºC (22, 30, 37, 45, 55 and 65 ºC) for 30 min and 2, 4, 6, and 18 h. Aliquots were withdrawn in triplicate at these incubation times to test the remaining activity at standard conditions. The non–heated enzyme was considered as control (100%).

2.6. Effect of metal ions, reagents, and inhibitors on tannase activity

The effect of various metal ions (Mg^{2+}, K^{+}, Ca^{2+}, Hg^{2+}, and Zn^{2+}), metal chelator EDTA, surfactants (SDS, Tween 80, and Triton–X–100), and other reagents (DMSO, and β–mercaptoethanol) on tannase activity was investigated by the rhodanine assay using methyl gallate as substrate. Purified $TanA_{Ap}$ was incubated with additives (1 mM final concentration) at 30 ºC for 1h. After incubation, the residual activity was measured in

189 triplicate under the standard assay conditions. The relative activities were calculated with

190 respect to the control where the reaction was carried out in the absence of additives

191 (100%).

192

193 *2.7. HPLC–DAD analysis of substrate specificity*

194

195 The substrate specificity of $TanA_{Ap}$ was determined using five commercial

196 hydroxybenzoic esters (methyl gallate, ethyl gallate, propyl gallate, lauryl gallate, and

197 ethyl protocatechuate), and a hydrolyzable tannin (tannic acid). The standard enzyme

198 assay was modified by using 200 μg of $TanA_{Ap}$, and 1 mM substrate, in the reaction

199 mixture and incubated at 37 °C during 10 min. As controls, phosphate buffer containing

200 the reagents but the enzyme were incubated in the same conditions.

201 The reaction products were extracted twice with ethyl acetate (Lab–Scan, Ireland)

202 and analyzed hy HPLC–DAD. A Thermo (Thermo Electron Corporation, Waltham,

203 Massachussetts, USA) chromatograph equipped with a P400 SpectraSystem pump, and

204 AS3000 autosampler, and a UV6000LP photodiode array detector were used. A gradient

205 of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid,

206 78:20:2, v/v/v) was applied to a reverse–phase Nova–pack C_{18} (25 cm × 4.0 mm i.d.) 4.6

207 μm particle size, cartridge at room temperature as follows: 0–55 min, 80% B linear, 1.1

208 ml/min; 55–57 min, 90% B linear, 1.2 ml/min; 57–70 min, 90% B isocratic, 1.2 ml/min;

209 70–80 min, 95% B linear, 1.2 ml/min; 80–90 min, 100% linear, 1.2 ml/min; 100–120 min,

210 washing 1.0 ml/min, and reequilibration of the column under initial gradient conditions.

211 Detection was performed by scanning from 220 to 380 nm. Samples were injected onto the

212 cartridge after being filtered through a 0.45 μm PVDF filter. The identification of

213 degradation compounds was carried out by comparing the retention times and spectral data
214 of each peak with those of standards from commercial suppliers
215
216 3. Results and discussion
217
218 *3.1. Production and characterization of purified* $TanA_{Ap}$
219
220 Tannases are capable of hydrolyzing complex tannins, which represent an
221 important chemical group occurring in food plants. Studies related to tannase-producing
222 strain isolation have been conducted (Aguilar et al., 2007; Chávez-González et al., 2012).
223 Among the many bacteria present in the oral cavity, *Atopobium parvulum* strains are of
224 interest because they posses a gene putatively encoding a tannase. The *apar_1020* ($tanA_{Ap}$)
225 gene predicted to encode a 607 amino acid protein 39.5% identical to $TanA_{Sl}$, a tannase
226 from *Staphylococcus lugdunensis,* and 26% identical to $TanB_{Lp}$, a tannase from
227 *Lactobacillus plantarum*, the only two bacterial tannases genetically described so far (data
228 not shown). Noteworthy, $TanA_{Sl}$ and $TanB_{Lp}$ are only 27% identical among them
229 (Iwamoto et al., 2008). In $TanA_{Ap}$ a signal peptide was predicted with a cleavage site at
230 residue 23. Therefore, processed mature $TanA_{Ap}$ protein has 584 amino acid residues, with
231 a predicted molecular mass of 63.8 kDa, and an isoelectric point of 4.62. As $TanA_{Ap}$
232 showed high amino acid identity to bacterial tannases, the tannase activity of $TanA_{Ap}$
233 needs to be assayed.
234 The $tanA_{Ap}$ gene was cloned into the pURI3-Cter expression vector by a ligation -
235 free cloning strategy described previously (Curiel et al., 2011). The vector incorporates the
236 DNA sequence encoding hexa-histidine to create a His-tagged fusion enzyme for further
237 purification step. The integrity of the construct was confirmed by DNA sequencing. The

238 recombinant plasmid was transformed into *E. coli* BL21 (DE3) and expressed under the
239 control of an inducible IPTG promoter. Cell extracts were used to detect the presence of
240 overproduced proteins. SDS–PAGE analysis showed that there was one major band of
241 protein, approximately 66 kDa, in the intracellular soluble fraction of the pURI3–Cter–
242 $TanA_{Ap}$ cells, which was absent in the control pURI3–Cter cells (Fig.1). The molecular
243 weight of the overproduced protein was consistent with the calculated molecular weight of
244 $TanA_{Ap}$. Since the cloning strategy would yield a His–tagged protein variant, *A. parvulum*
245 pURI3–Cter–$TanA_{Ap}$ could be purified on an immobilized metal affinity chromatography
246 (IMAC) resin. The recombinant protein was observed as single band on SDS–PAGE (Fig.
247 1). Routinely about 12 mg of purified protein from 1–liter culture was obtained.

248 $TanA_{Ap}$ protein purified by the affinity resin was biochemically characterized. A
249 method specific for the detection of gallic acid could be used for a reliable quantification
250 of tannase activity. Since tannase catalyzes the hydrolysis of the galloyl ester linkage
251 liberating gallic acid, the activity of tannase could be measured by estimating the gallic
252 acid formed due to enzyme action (Mueller–Harvey, 2001). Inoue & Hagerman (1988)
253 described a rhodanine assay specific for determining free gallic acid. Rhodanine reacts
254 with gallic acid to give a red complex with a maximum absorbance at 520 nm. Rhodanine
255 assay was used to determine the specific activity of $TanA_{Ap}$, simultaneously, the activity
256 of the previously described $TanB_{Lp}$ tannase from *L. plantarum* was also determined as
257 reference. Using methyl gallate as substrate, the specific activity of $TanA_{Ap}$ purified
258 enzyme was 3.5 U/mg, 116 times lower than that of $TanB_{Lp}$ (408 U/mg). This low specific
259 activity could indicate that even though tannase action from *A. parvulum* could begin
260 almost immediately after food ingestion, its contribution to tannin breakdown might not be
261 relevant.

In relation to the biochemical properties of the enzyme, $TanA_{Ap}$ showed optimal activity at pH 6 (Fig. 2A), slightly more acidic than the optimal pH for $TanB_{Lp}$ (pH 7). During food oral processing, saliva provides buffering effects. It was indicated that the pH of saliva rises during the first 5 min after the intake of most foods, and falls to around 6, or lower, approximately15 min after food consumption (Humphrey & Williamson, 2001). Therefore, $TanA_{Ap}$ could found an adequate pH for activity during food oral processing.

Despite the optimum temperature of $TanA_{Ap}$ was 55 ºC, at 37 ºC, the physiological temperature for humans, 80% of the maximal activity was found. Similarly, more that 80% maximal activity was obtained at 20, 42, and 65 ºC (Fig. 2B). Tannase $TanB_{Lp}$ from *L. plantarum* showed maximal activity at 40 ºC, having only 50% of the maximal activity at 30 or 60 ºC. The thermal stability profile for $TanA_{Ap}$ is shown in Fig. 2C. According to the thermal stability profile, $TanA_{Ap}$ was most stable at temperatures between 37 and 65 ºC, and more than 60% enzyme activity remained after 18 h at 45 ºC (Fig. 2C). Tannase $TanB_{Lp}$ from *L. plantarum* kept less than 20% of the maximal activity after incubation at 37 ºC during 20 h. The data for $TanA_{Ap}$ demonstrated that the enzyme exhibited high thermal stability under prolonged incubation up to 45 ºC. Despite the specific activity of $TanA_{Ap}$ is remarkably lower than the activity of $TanB_{Lp}$, $TanA_{Ap}$ is more thermostable, and therefore it is able to resist thermal unfolding in the absence of its substrate.

The effects of several ions and additives are shown in Table 1. Contrarily to $TanB_{Lp}$, $CaCl_2$ did not activate $TanA_{Ap}$ (Curiel, Rodríguez, Acebrón, Mancheño, de las Rivas, & Muñoz, 2009). Tannase activity of $TanA_{Ap}$ was not increased by any of the additives assayed. Similarly to $TanB_{Lp}$, activity was greatly inhibited by β–mercaptoethanol and by the metal ion Hg^{2+} (Curiel et al., 2009). $ZnCl_2$ significantly inhibited $TanA_{Ap}$ activity (relative activity 24%). The other metal ions and additives assayed partially affected tannase activity (relative activity 74–96%). The different

additive effect observed would suggest that there are notable structural differences among both bacterial tannases, $TanA_{Ap}$ from *A. parvulum*, and $TanB_{Lp}$ from *L. plantarum*.

Despite the low specific activity showed by $TanA_{Ap}$, this protein posses biochemical properties compatible with its action during food oral passage, since its pH and temperature for activity are provided by the human saliva during food processing. Oral processing occurs during a short time period, however, it has been described that during the short period of oral processing, about 50% of bread and 25% of pasta starch are hydrolyzed and transformed into smaller molecules by the amylase enzyme present in the human saliva (Hoebler, Karinthi, Devaux, Guillon, Gallant, Bouchet, et al., 1998; Hoebler, Devaux, Karinthi, Belleville, & Barry, 2000). The interaction of amylase enzyme with starch ingredients produces almost an immediate effect on hydrolysis, and thus making the food intake much easily mixable and digestible in the stomach. A similar situation could be envisaged for the action of $TanA_{Ap}$ on the tannins present on the diet.

3.2. Contribution of $TanA_{Ap}$ to the hydrolysis of tannins from the diet

Tannins are natural polyphenolic compounds present in food plants. They are characterized by their ability to form strong complexes with different minerals and macromolecules, such as proteins, cellulose, starch, etc, causing astringency and precipitation effects (Mingshu, Kai, Quiang, & Dongying, 2006; Serrano, Puupponen–Pimiä, Dauer, Aura, & Saura–Calixto, 2009). As a result, tannins are considered antinutritional. Tannases catalyzes the hydrolysis reaction of ester bonds present in the gallotannins, complex tannins, and gallic acid and protocatechuic acid esters (Aguilar et al., 2007; Curiel et al., 2009; Chávez–González et al., 2012). In order to know the substrate specificity of $TanA_{Ap}$, several gallate and protocatechuate esters were assayed. As showed

312 in Fig. 3, none of the esters assayed were significantly hydrolyzed. Methyl, ethyl, and
313 propyl gallate and ethyl protocatecuate were minimally hydrolyzed. Lauryl gallate,
314 possessing a long aliphatic alcohol chain, was not hydrolyzed at all (data not shown).
315 Contrarily to these results, *L. plantarum* tannase ($TanB_{Lp}$) was able to fully hydrolyze
316 gallic esters even those having an alcohol substituent as longer as lauryl (C12) (Curiel et
317 al, 2009). Structural differences among both bacterial proteins will be responsible of the
318 different spatial requirements observed for tannase activity.

319 It is noteworthy to mention that the colorimetric rhodanine assay used for the
320 detection and quantification of tannase activity is much more sensitive that the analysis of
321 the reaction products by HPLC. By using methyl gallate as substrate, the rhodanine assay
322 allowed to determine properly the biochemical properties of $TanA_{Ap}$ assayed; however,
323 this characterization would not be possible by the HPLC analysis.

324 In order to evaluate the contribution of $TanA_{Ap}$ action during oral processing of
325 diet tannins, a complex and natural tannin, tannic acid, was incubated in the presence of
326 $TanA_{Ap}$. Tannic acid is almost exclusively formed by poly-galloyl glucose derivatives
327 whose nature and complexity vary with the plant source. When $TanA_{Ap}$ was incubated on
328 tannic acid, an hydrolysis profile identical to the control without enzyme was observed
329 (data not shown). This was an expected result considering the minimal degradation on
330 simple gallic acid esters observed after $TanA_{Ap}$ action. As $TanA_{Ap}$ did not show activity
331 on tannic acid, it could be possible that the natural tannin substrate for this enzyme will be
332 different and still remained unknown. In addition, specific reaction conditions or the
333 presence of an unknown cofactor will be required to increase $TanA_{Ap}$ activity during food
334 processing. Further research will be need to known the physiological role of $TanA_{Ap}$ in *A.*
335 *parvulum* metabolism.

The above results indicated that, even though *A. parvulum* tannase action could begin almost immediately after food ingestion, its contribution to tannin breakdown would not be relevant. Most of the tannin digestion could result from bacterial intestinal tannases rather than from oral tannase. In the microbiome of the major site of food tannin hydrolysis, the intestinal tract, at least three tannase-producing bacteria have been isolated, *L. plantarum, S. lugdunensis* or *Streptococcus gallolyticus* (Iwamoto, Tsuruta, Nishitani, & Osawa, 2008; Noguchi et al., 2007; Sly, Cahill, Osawa, & Fujisawa, 1997; Rusniok et al., 2010). From these intestinal bacteria, only the biochemical properties of TanB_{Lp} from *L. plantarum* have been studied. TanB_{Lp} posses adequate properties for intestinal tannin degradation. However, further testing would be required to define the metabolism of these phenolic compounds comprehensively. In particular, the activity of the complex communities of microorganisms present in all parts of the human digestive tract would need to be examined.

4. Conclusions

In the present study, a novel bacterial tannase namely TanA_{Ap} from *Atopobium parvulum*, an inhabitant of the human oral cavity, was purified. TanA_{Ap} was biochemically characterized by using a sensitive colorimetric method. Among bacterial tannases, TanA_{Ap} possessed low specific activity and was unable to hydrolyze complex tannins. These biochemical properties are not favourable for the breakdown of complex food tannins during oral processing.

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366 References

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368 Abdulamir, A. S., Hafidh, R. R., Mahdi, L. K., Al–Jeboori, T., & Abubaker, F. (2009).
369 Investigation into the controversial association of *Streptococcus gallolyticus* with
370 colorrectal cancer and adenoma. *BMC Cancer*, 9, 403.
371 Abdulamir, A. S., Hafidh, R. R., & Bakar, A. (2011). The association of *Streptococcus*
372 *bovis/gallolyticus* with colorectal tumors: the nature and the underlying
373 mechanisms of its etiological role. *Journal of Experimental and Clinical Cancer*
374 *Research*, 30, 11.
375 Aguilar, C.N., Rodríguez, R., Gutiérrez–Sánchez, G., Augur, C., Favela–Torres, E., Prado–
376 Barragan, L.A., Ramirez–Coronel, A., & Contreras–Esquivel, J.C. (2007).
377 Microbial tannases: advances and perspectives. *Applied Microbiology and*
378 *Biotechnology*, 76, 47–59.
379 Chávez–González, M., Rodríguez–Durán, L.V., Balagurusamy, N., Prado–Barragán, A.,
380 Rodríguez, R., Contreras, J.C., & Aguilar, C.N. (2012). Biotechnological advances
381 and challenges of tannase: an overview. *Food Bioprocess Technology*, 5, 445–459.
382 Chen, J. (2009). Food oral processing–A review. *Food Hydrocolloid*, 23, 1–25.
383 Copeland, A., Sikorski, J., Lapidus, a., Nolan, M., del Rio, T. G., Lucas, S., Chen, F.,
384 Tice, H., Pitluck, S., Cheng, J.–F., Pukall, R., Chertkov, O., Brettin, T., Han, C.,
385 Kuske, C., Bruce, D., Goodwin, L., Ivanova, N., Mavromatis, K., Mikhailova, .,

Chen, A., Palaniappan, K., Chain, P., Rohde, M., Göker, M., Bristol, J., Eisen, J. A., Markowitz, V., Hugenholtz, P., Kyrpides, N. C., Klenk, H.-P., & Setter, J. C. (2009). Complete genome sequence of *Atopobium parvulum* type strain (IPP 12456^{T}). *Standards in Genomic Sciences*, 1, 166–173.

Curiel, J.A., Rodríguez, H., Acebrón, I., Mancheño, J.M., de las Rivas, B., & Muñoz, R. (2009). Production and physicochemical properties of recombinant *Lactobacillus plantarum* tannase. *Journal of Agricultural and Food Chemistry*, 57, 6224–6230.

Curiel, J.A., de las Rivas, B., Mancheño, J.M., & Muñoz, R. (2011). The pURI family of expression vectors: a versatile set of ligation independent cloning plasmids for producing recombinant His-fusion proteins. *Protein Expression and Purification*, 76, 44–53.

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C., Yu, W. H., Lakshmann, A., & Wade, W. G. (2010). The human oral microbiome. *Journal of Bacteriology*, 192, 5002–5017.

Hoebler, C., Devaux, M.-F., Karinthi, C., Belleville, C., & Barry, J.-L. (2000). Particle size of solid food after human mastication and in vitro simulation of oral breakdown. *International Journal of Food Science and Nutrition*, 51, 353–366.

Hoebler, C., Karinthi, A., Devaux, M.-F., Guillon, F., Gallant, D. J. G., Bouchet B., Melegari, C., & Barry, J.-L. (1998). Physical and chemical transformation of cereal food during oral digestion in human subjects. *British Journal of Nutrition*, 80, 429–436.

Humphrey, S. P., & Williamson, R. T. (2001). A review of saliva: Normal composition, flow, and function. *Journal of Prosthetic Dentistry*, 85, 162–169.

Inoue, K. H., & Hagerman, A. E. (1988). Determination of gallotannins with rhodanine. *Analytical Biochemistry*, 169, 363–369.

Iwamoto, K., Tsuruta, H., Nishitaini, Y., & Osawa, R. (2008). Identification and cloning of a gene encoding tannase (tannin acylhydrolase) from *Lactobacillus plantarum* ATCC 14917^{T}. *Systematic and Applied Microbiology*, 31, 269–277.

Mingshu, L., Kai, Y., Quiang, H., & Dongying, J. (2006). Biodegradation of gallotannins and ellagitannins. *Journal of Basic Microbiology,* 46, 68–84.

Moco, S., Martin F.–P. J., & Rezzi, S. (2012). Metabolomics view on gut microbiome modulation by polyphenol–rich foods. *Journal of Proteome Research,* 11, 4781–4790.

Mueller–Harvey, I.(2001). Analysis of hydrolysable tannins. *Animal Feed Science and Technology*, 91, 3–20.

Noguchi, N., Ohashi, T., Shiratori, T., Narui, K., Hagiwara, T., Ko, M., Watanabe, K., Miyahara, T., Taira, S., Moriyasu, F., & Sasatsu, M. (2007). Association of tannase–producing *Staphylococcus lugdunensis* with colon cancer and characterization of a novel tannase gene. J. Gastroenterol. 42, 346–351.

Ordoudi, S. A., & Tsimidou, M. Z. (2006). Crocin bleaching assay (CBA) in structure–radical scavenging activity studies of selected phenolic compounds. *Journal of Agricultural and Food Chemistry*, 54, 9347–9356.

Rechner, A. R., Kuhnle, G., Bremner, P., & Hubbard, G. P. (2002). The metabolic fate of dietary polyphenols in humans. *Free Radical Biology and Medecine*, 220.235.

Riggio, M. P., Lennon, A., Rolph, H. J., Hodge, P. J., Donaldson, A., Maxwell, A. J., & Bagg, J. (2008). Molecular identification of bacteria on the tongue dorsum of subjects with and without halitosis. *Oral Diseases,* 14, 251–258.

Rusniok C., Couvé, E., da Cunha, V., El Gana, R., Zidane, N., Bouchier, C., Poyart, C., Leclercq, R., Trieu–Cuot, P., & Glaser, P. (2010). Genome sequence of

435 *Streptococcus gallolyticus*: insights into its adaptation to the bovine rumen and its ability to cause endocarditis. *Journal of Bacteriology*, 192, 2266–2276.

437 Segata, N., Haake, S. K., Mannon, P., Lemon, K. P., Waldron, L., Gevers, D., Huttenhower, C., & Izard, J. (2012). Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat, and stool samples. *Genome Biology 13, R42 (doi:10.1186/gb–2012–13–6–r42).*

441 Serrano, J., Puupponen–Pimiä, R., Dauer, A., Aura, A.–M., & Saura–Calixto, F. (2009). Tannins: Current knowledge of food sources, intake, bioavailability and biological effects. *Molecular Nutrition and Food Research*, 53, S310–S329.

444 Shahidi, F., & Naczk, M. (2003). Phenolics in food and nutraceuticals. CRC Pres, London, United Kingdom.

446 Sly, L.I., Cahill, .M., Osawa, R., & Fujisawa, T. (1997). The tannin–degrading species *Streptococcus gallolyticus* and *Streptococcus caprinus* are subjective synonyms. *International Journal of Systematic Bacteriology*, 47, 893–894.

449 Spencer, M. D., Hamp, T. J., Reid, R. W., Fischer, L. M., Zeisel, S. H., & Fodor, A. A. (2011). Association between composition of the human gastrointestinal microbiome and development of fatty liver with choline deficiency. *Gastroenterology*, 140, 976–986.

453 Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., Sogin, M. L., Jones, W. J., Roe, B. A., Affourtit, J. P., Egholm, M., Henrissat, B., Heath, A. C., Knight, R., & Gordon, J. I. (2009). A core gut microbiome in obese and lean twins. *Nature*, 457, 480–484.

457 Wade, W. G., 2013. Characterization of the human oral microbiome. *Journal of Oral Biosciences*, 55, 143–148.

459 Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y. Y., Keilbaugh, S. A.,Bewtra,
460 M., Knights, D., Walters, W. A., Knight, R., Sinha, R., Gilroy, E., Gupta, K.,
461 Baldassano, R., Nessei, L., Li, H., Bushman, F. D., & Lewis, J. D. (2011). Linking
462 long–term dietary patterns with gut microbial enterocytes. *Science*, 334, 105–108.
463 Zhang, C., Zhang, M., Wang, S., Han, R., Cao, Y., Hua, W., Mao, Y., Zhang, X., Pang,
464 X., Wei, C., Zhao, G., Chen, Y., & Zhao, L. (2010). Interactions between gut
465 microbiota, host genetics and diet relevant to development of metabolic syndromes
466 in mice. *ISME Journal*, 4, 232–241.

467

468 Figure captions

469

470 Fig. 1. Purification of $TanA_{Ap}$ tannase from *A. parvulum*. Analysis by SDS–PAGE of
471 soluble cell extracts of IPTG–induced *E. coli* BL21(DE3) (pURI3–Cter) (1) or *E. coli*
472 BL21(DE3) (pURI3–Cter–$TanA_{Ap}$) (2), flowtrough (3), or fractions eluted after His affinity
473 resin (4–6). The gel was stained with Coomassie blue. Molecular mass markers are located
474 at the left (SDS–PAGE Standards, Bio–Rad).

475

476 Fig. 2. Biochemical properties of $TanA_{Ap}$ protein. (A) pH–activity profile of $TanA_{Ap}$. (B)
477 Temperature–activity profile of $TanA_{Ap}$. (C) Thermal stability profile for $TanA_{Ap}$ after
478 preincubation at 22 ºC (filled diamond), 30 ºC (filled square), 37 ºC (filled triangle), 45 ºC
479 (cross), 55 ºC (star), and 65 ºC (filled circle) in phosphate buffer (50 mM, pH 6.5); at
480 indicated times, aliquots were withdrawn, and analyzed as described in the Materials and
481 Methods section. The experiments were done in triplicate. The mean value and the
482 standard error are showed. The percentage of residual activity was calculated by
483 comparing with unincubated enzyme.

484

485 Fig. 3. Enzymatic activity of tannase from *A. parvulum* against gallic and protocatechuic

486 acid esters. Hydrolase activity of purified $TanA_{Ap}$ compared with control reactions on

487 which the enzyme was omitted. HPLC chromatograms of $TanA_{Ap}$ (200 μg) incubated in

488 50 mM phosphate buffer pH 6, and 1 mM of methyl gallate (A), ethyl gallate (B), propyl

489 gallate (C), and ethyl protocatechuate (D). The methyl gallate (MG), ethyl gallate (EG),

490 propyl gallate (PG), ethyl protocatechuate (EP), gallic acid (GA), and protocatechuic acid

491 (PA) detected are indicated. The chromatograms were recorded at 280 nm.

Figure 1

Figure 1

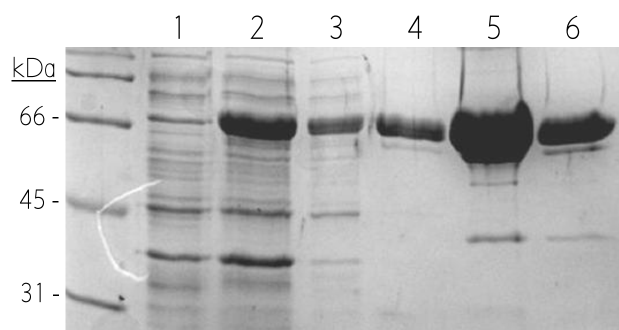


Figure 2

Figure 2

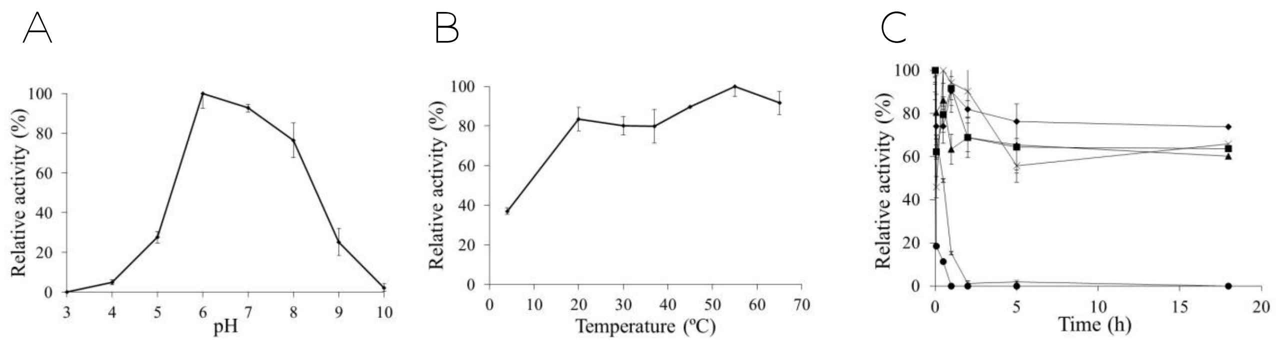
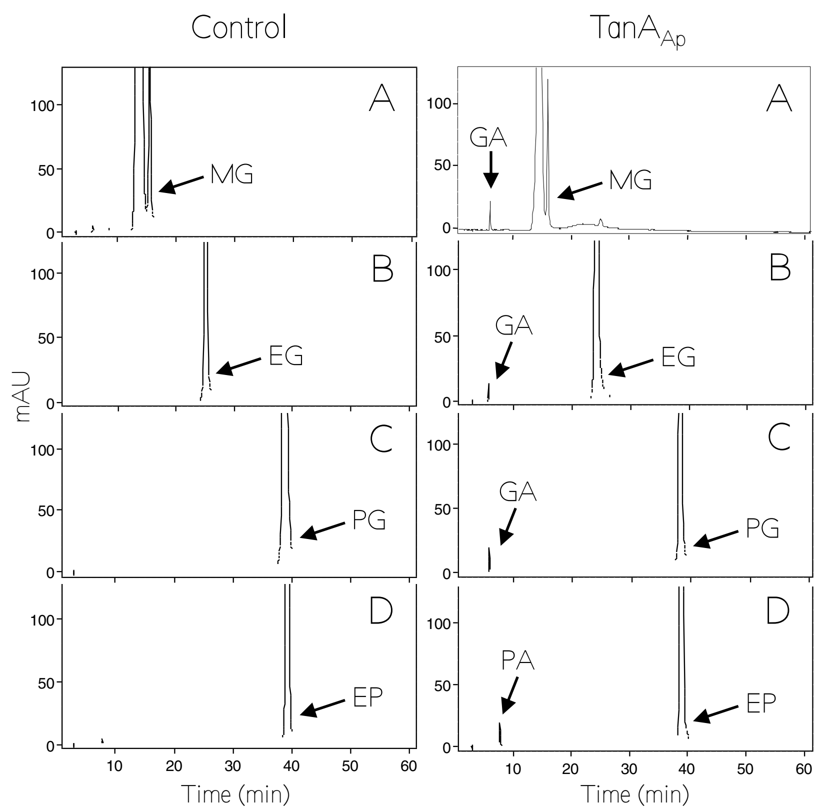


Figure 3



Table

Table 1

Table 1. Effect of additives on *A. parvulum* tannase activity

Additions (1 mM)	Relative activity (%)
Control	100
EDTA	74
KCl	80
$HgCl_2$	16
$CaCl_2$	97
$MgCl_2$	96
$ZnCl_2$	24
Triton X 100	83
DMSO	82
Tween 80	94
Urea	83
β-mercaptoethanol	15