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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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21 ABSTRACT

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

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37 *Keywords:*

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

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1. Introduction

Vegetable tannins are abundant in plants utilized as human food. Tannins occur widely on common foodstuffs, such as pomegranate, banana, strawberry, grape, cashew nut, and hazelnut. Drinks like wine and tea also contain these phenolic compounds (Shahidi & Naczki, 2003). The molar mass of tannins affects tannin characteristics directly, and it has been suggested that small molecule tannins have more antioxidant activity (Ordoudi & Tsimidou, 2006). To understand the biological effects of food tannins the insight on the metabolic fate and bioavailability of these metabolites in the human body is crucial, however this knowledge is currently scarce (Rechner, Kuhnle, Bremner, & Hubbard, 2002; Moco, Martin, & Rezzi, 2012). The interaction of tannins with human microbiota will determine in great extent the physiological effects of these polyphenols.

Although much work has been focused on factors that determine mechanical (e.g. rheological and fracture) and sensory properties of foods, far less attention has been paid to linking food transformations that occurs during oral processing with microbial action (Chen, 2009). The human microbiome is a dynamic community changing in response to natural perturbations such as diet (Turnbaugh, Hamady, Yatsunenko, Cantarel, Duncan, Ley, et al., 2009; Wu, Chen, Hoffmann, Bittinger, Chen, Keilbaugh, et al., 2011; Spencer, Hamp, Reid, Fishe, Zeisel, & Fodor, 2011; Zhang, Zhang, Wang, Han, Cao, Hua, et al., 2010). The oral cavity of humans hosts several hundred taxa, with remarkable diversity even among saliva, tongue, teeth, and other substrates (Segata, Haake, Mannon, Lemon, Waldron, Gevers, et al., 2012; Dewhirst, Chen, Izard, Paster, Tanner, Yu, et al., 2010). All of the surfaces of the mouth are covered in a bacterial biofilm (Wade, 2013). Studies on the tongue biofilm have been relatively few in number, compared with the significant 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_{SI}, 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 (Abdulmir, Hafidh, Mahdi, Al-Jeboori, & Abubaker, 2009; Abdulmir, 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 °C 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). *DpnI* 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′–*ACTTTAAGAAGGAGATATACATatgtctgataatacgaatcaacctgca*) and 787 (5′–*GCTATTAATGATGATGATGATGATGagcagacgcacacgagacaatcca*) (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 containing 100 µg/mL ampicillin on a rotary shaker (200 rpm) at 37 °C 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 °C during 18 h.

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

(three times at 1,100 psi). The insoluble fraction of the lysate was removed by centrifugation at 47,000 *g* for 30 min at 4 °C, and the supernatant was filtered through a 0.2 µm pore-size filter and then loaded onto a Talon Superflow resin (Clontech) equilibrated in phosphate buffer (50 mM, pH 6.5) containing 300 mM NaCl and 10 mM imidazole to improve the interaction specificity in the affinity chromatography step. The bound enzyme was eluted using 150 mM imidazole in the same buffer. The purity of the enzyme was determined by SDS-PAGE in Tris-glycine buffer. Fractions containing the His6-tagged protein were pooled and analyzed for tannase activity.

2.4. Enzyme activity assay

Tannase activity was determined using a colorimetric assay using rhodanine, specific for gallic acid (Inoue & Hagerman, 1988). Rhodanine reacts only with gallic acid and not with galloyl esters or other phenolics. Gallic acid analysis in the reactions was determined using the following colorimetric assay. Tannase enzyme (100 µg) in 700 µl of 50 mM phosphate buffer pH 6.5 was incubated with 40 µl of 25 mM methyl gallate (1 mM final concentration) during 5 min at 37 °C. After this incubation, 150 µl of a methanolic rhodanine solution (0.667% w/v rhodanine in 100% methanol) was added to the mixture. After 5 min incubation at 30 °C, 100 µl of 500 mM KOH was added. After an additional incubation of 5–10 min, the absorbance at 520 nm was measured on a spectrophotometer. A standard curve using gallic acid concentration ranging from 0.125 to 1 mM was prepared. One unit of tannase activity was defined as the amount of enzyme required to release 1 µmol of gallic acid per minute under standard reaction condition.

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 °C (4, 22, 30, 37, 45, 55 and 65 °C). To study the thermal stability of TanA_{Ap}, tannase was incubated at temperatures over the range of 22–65 °C (22, 30, 37, 45, 55 and 65 °C) 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 (Mg²⁺, K⁺, Ca²⁺, Hg²⁺, and Zn²⁺), 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 °C for 1h. After incubation, the residual activity was measured in

triplicate under the standard assay conditions. The relative activities were calculated with respect to the control where the reaction was carried out in the absence of additives (100%).

2.7. HPLC-DAD analysis of substrate specificity

The substrate specificity of TanA_{Ap} was determined using five commercial hydroxybenzoic esters (methyl gallate, ethyl gallate, propyl gallate, lauryl gallate, and ethyl protocatechuate), and a hydrolyzable tannin (tannic acid). The standard enzyme assay was modified by using 200 µg of TanA_{Ap}, and 1 mM substrate, in the reaction mixture and incubated at 37 °C during 10 min. As controls, phosphate buffer containing the reagents but the enzyme were incubated in the same conditions.

The reaction products were extracted twice with ethyl acetate (Lab-Scan, Ireland) and analyzed by HPLC-DAD. A Thermo (Thermo Electron Corporation, Waltham, Massachusetts, USA) chromatograph equipped with a P400 SpectraSystem pump, and AS3000 autosampler, and a UV6000LP photodiode array detector were used. A gradient of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 78:20:2, v/v/v) was applied to a reverse-phase Nova-pack C₁₈ (25 cm × 4.0 mm i.d.) 4.6 µm particle size, cartridge at room temperature as follows: 0–55 min, 80% B linear, 1.1 ml/min; 55–57 min, 90% B linear, 1.2 ml/min; 57–70 min, 90% B isocratic, 1.2 ml/min; 70–80 min, 95% B linear, 1.2 ml/min; 80–90 min, 100% linear, 1.2 ml/min; 100–120 min, washing 1.0 ml/min, and reequilibration of the column under initial gradient conditions. Detection was performed by scanning from 220 to 380 nm. Samples were injected onto the cartridge after being filtered through a 0.45 µm PVDF filter. The identification of

degradation compounds was carried out by comparing the retention times and spectral data of each peak with those of standards from commercial suppliers

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3. Results and discussion

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3.1. Production and characterization of purified TanA_{Ap}

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Tannases are capable of hydrolyzing complex tannins, which represent an important chemical group occurring in food plants. Studies related to tannase-producing strain isolation have been conducted (Aguilar et al., 2007; Chávez-González et al., 2012). Among the many bacteria present in the oral cavity, *Atopobium parvulum* strains are of interest because they possess a gene putatively encoding a tannase. The *apar_1020* (*tanA_{Ap}*) gene predicted to encode a 607 amino acid protein 39.5% identical to TanA_{SI}, a tannase from *Staphylococcus lugdunensis*, and 26% identical to TanB_{Lp}, a tannase from *Lactobacillus plantarum*, the only two bacterial tannases genetically described so far (data not shown). Noteworthy, TanA_{SI} and TanB_{Lp} are only 27% identical among them (Iwamoto et al., 2008). In TanA_{Ap} a signal peptide was predicted with a cleavage site at residue 23. Therefore, processed mature TanA_{Ap} protein has 584 amino acid residues, with a predicted molecular mass of 63.8 kDa, and an isoelectric point of 4.62. As TanA_{Ap} showed high amino acid identity to bacterial tannases, the tannase activity of TanA_{Ap} needs to be assayed.

The *tanA_{Ap}* gene was cloned into the pURI3-Cter expression vector by a ligation – free cloning strategy described previously (Curiel et al., 2011). The vector incorporates the DNA sequence encoding hexa-histidine to create a His-tagged fusion enzyme for further purification step. The integrity of the construct was confirmed by DNA sequencing. The

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

TanA_{Ap} protein purified by the affinity resin was biochemically characterized. A method specific for the detection of gallic acid could be used for a reliable quantification of tannase activity. Since tannase catalyzes the hydrolysis of the galloyl ester linkage liberating gallic acid, the activity of tannase could be measured by estimating the gallic acid formed due to enzyme action (Mueller-Harvey, 2001). Inoue & Hagerman (1988) described a rhodanine assay specific for determining free gallic acid. Rhodanine reacts with gallic acid to give a red complex with a maximum absorbance at 520 nm. Rhodanine assay was used to determine the specific activity of TanA_{Ap}, simultaneously, the activity of the previously described TanB_{Lp} tannase from *L. plantarum* was also determined as reference. Using methyl gallate as substrate, the specific activity of TanA_{Ap} purified enzyme was 3.5 U/mg, 116 times lower than that of TanB_{Lp} (408 U/mg). This low specific activity could indicate that even though tannase action from *A. parvulum* could begin almost immediately after food ingestion, its contribution to tannin breakdown might not be 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, approximately 15 min after food consumption (Humphrey & Williamson, 2001). Therefore, TanA_{Ap} could find an adequate pH for activity during food oral processing.

Despite the optimum temperature of TanA_{Ap} was 55 °C, at 37 °C, the physiological temperature for humans, 80% of the maximal activity was found. Similarly, more than 80% maximal activity was obtained at 20, 42, and 65 °C (Fig. 2B). Tannase TanB_{Lp} from *L. plantarum* showed maximal activity at 40 °C, having only 50% of the maximal activity at 30 or 60 °C. 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 °C, and more than 60% enzyme activity remained after 18 h at 45 °C (Fig. 2C). Tannase TanB_{Lp} from *L. plantarum* kept less than 20% of the maximal activity after incubation at 37 °C during 20 h. The data for TanA_{Ap} demonstrated that the enzyme exhibited high thermal stability under prolonged incubation up to 45 °C. 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}, CaCl₂ 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 Hg²⁺ (Curiel et al., 2009). ZnCl₂ 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 possesses 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, Karinthy, Devaux, Guillon, Gallant, Bouchet, et al., 1998; Hoebler, Devaux, Karinthy, 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 catalyze 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

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

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

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

Acknowledgements

This work was financially supported by grants AGL2011-22745, S2009/AGR-1469 (ALIBIRD) (Comunidad de Madrid), and RM2012-00004 (INIA). We are grateful to J.M. Barcenilla and M. V. Santamaría. N. Jiménez and L. Santamaría are recipients of FPI fellowships from the MINECO. M. Esteban-Torres is a recipient of a JAE predoctoral fellowship from the CSIC.

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Figure captions

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

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

Figure 3

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