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Development and application of assay for determining β -glucosidase activity in human saliva

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Abstract

Background: β -glucosidase is an enzyme important to flavour enhancement. It hydrolyzes glucosides to release aglycones—aroma precursors that are bound to a sugar molecule—thereby making them available to contribute to the flavour of foods and beverages. While there is strong interest within the food and beverage industry to optimizing flavour through the use of exogenous and endogenous glucosidase in production, little is known regarding the possible occurrence of these enzymes within the human oral cavity. This could be an important source of flavour release and/or account for some differences between individuals in flavour perception. In the present study, we determined whether β -glucosidase is present in human saliva. First, an existing spectrophotometric assay that uses p-nitrophenyl- β -D-glucopyranoside as a substrate was modified and optimized for use in human saliva. The following variables were evaluated and where necessary, optimized: linearity of the assay signal, possible matrix interference, the effect of heat inactivation of the saliva, absorbance wavelength maxima, substrate saturation concentration, maximum saliva volume and the inclusion of α -cyclodextrin. The modified assay was then used to screen for β -glucosidase activity in the saliva of 20 individuals. Of the 20 samples analyzed, four were tentatively identified as containing active β -glucosidase and were further investigated.

Results: Significant differences ($p < 0.05$) in absorbance values ($A_{400\text{nm}}$) between these saliva samples confirm low levels of β -glucosidase activity in approximately 20% of the population sampled.

Conclusions: Inter-individual variability exists in β -glucosidase activity within the oral cavity. The described method can be applied to rapidly assay a large population of individuals, and further elucidate the extent and significance of salivary β -glucosidase activity within the context of human flavour perception and enhancement.

Keywords: β -Glucosidase assay, Salivary hydrolysis, Flavour enhancement, Human flavour perception, Wine

Background

Flavour, a product of the integration of distinct physiologically defined sensory systems that combine taste, aroma and trigeminal sensations [1], is a key determining factor for the acceptance of food and beverages by consumers [2]. Flavour is a dynamic process; a series of events that involves the interaction of the product and the consumer [3]. Characteristic flavour and aroma profiles of food and beverages arise from the composition and concentration of the volatile

compounds present. Volatility refers to the ease of evaporation of a compound into the air, which allows for the volatile constituents to enter and move within the nasal or oral cavities where they can bind to olfactory receptors and elicit an olfactory response [4]. Also contributing to potential flavour are non-volatile compounds, some of which while not odour-active can be broken down to release volatile odorants. These non-volatile constituents are known as aromatic precursors, and their potential is realized when the flavour compound—the aglycone—is released from its glycosidically bound form [5]. A glycoside is a compound composed of both a carbohydrate (glycone) and non-carbohydrate (aglycone) residue within the same molecule. When the glycone portion of the glycoside is a glucose

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molecule, the compound is known as a glucoside [6]. The ratio of glycosidically bound aroma compounds to their free aroma compound counterparts is dependent on the matrix. For example, in Muscat and Riesling wine grape varieties, this ratio ranges between 1 and 5, whereas in the Gewürztraminer variety, bound compounds are up to 15 times more abundant than the free forms [7]. Thus, glycosidically bound compounds constitute a considerable reservoir of potential aromatic compounds that may enhance food and beverage flavour if their release can be realized. Glycosides can be separated from their sugar moieties through the use of heat, acid and enzyme hydrolysis [8], with the enzymes responsible for the latter known as glycoside hydrolases or glycosidases [9].

Juice and wine are two products that have been extensively studied with respect to glycosidically bound aroma precursors [10]. For instance, monoterpenes are important aglycone compounds that contribute to the flavour, aromatics and typicity of many grape varieties and their wines [5]. They exist in both a free volatile form, which can directly contribute to the aroma and flavour, and in a potentially volatile, glycosylated form, which are bound to either mono or disaccharides [5, 11]. Other examples of grape berry-derived aglycones that contribute to flavour include terpenoids, C13-norisoprenoids and benzene derivatives [12]. These examples elicit mostly pleasant aromas that contribute to the aroma profile and have a low perception threshold [13].

Of the three methods by which glycosides can be separated, enzymatic hydrolysis is regarded as the most efficient, and considerable research has focused on its application to flavour enhancement [14]. Acid hydrolysis of glycosides occurs at a slow rate and may induce terpenol rearrangements [13, 15]. Thermal hydrolysis has the potential to increase the breakdown of glycosides by 33%; however, high temperatures can also rearrange the natural structure of the glycosides [16]. Generally, glycosidases are best characterized as the group of enzymes that act on disaccharides, oligosaccharides and polysaccharides [17] to specifically cleave the glycosidic bond present between the aglycone and the glycone of the glycoside structure [6, 9, 18]. They are able to catalyze the hydrolysis of both *S*- and *O*-glycosidic linkages.

There is some evidence that human saliva may be involved with the release of non-volatile aroma compounds from glycoside structures. Saliva is a complex, heterogeneous, dilute aqueous solution that contains secretions from both the major and minor glands, including numerous inorganic salts, microorganisms, crevicular fluid, sloughed cells/food debris and a diverse selection of organic compounds such as proteolytic enzymes and mucous glycoproteins [19, 20]. In the mouth, flavour release from food and beverages is a dynamic

process influenced by factors such as release rates of odorants, matrix compounds, breathing, mastication and saliva flow [2, 20]. For non-volatile constituents, including aroma precursors, saliva acts as a carrier of the compounds to the taste buds located throughout the oral cavity, which is necessary to elicit a taste response [4]. Further, odour perception can be influenced by the length of time odour compounds persist after food or beverages are swallowed, by remaining in the oral cavity. Saliva plays a role in the intensity and duration of this aftertaste; it acts as a transport system by extracting odorants from materials that are not swallowed [21].

Most research to date on salivary enzymes, including β -glucosidase, has focused on their role in the diagnosis of periodontal disease [22] and responses to its treatment [23]. Few studies appear on the possible activity and prevalence of β -glucosidase in human saliva. Menguy et al. [24] suggested that approximately one in five people possess β -glucosidase in their saliva; however, the sample size of their study was quite small. Later, Arnaldos et al. [25] modified a spectrophotometric assay for strawberry callus protein for potential application in determining β -glucosidase in human saliva.

The optimal pH for β -glucosidase activity is approximately 5.5, with an activity range of 4.3–7.0 and an optimal temperature of 45 °C [26]. Varying activity of β -glucosidase within human saliva could represent a potential source of individual variation in the perception of flavour. Individual differences in flavour perception have been strongly linked to variation in food/beverage preferences, as well as habitual diet-related nutrition and health outcomes [27–31]. β -Glucosidase activity may increase the concentration of free volatile compounds available in the oral cavity through the hydrolysis of glycosidically bound non-volatiles, potentially enhancing flavour and overall perception of the food or beverage consumed.

Existing analytical methods for measuring β -glucosidase activity often require expensive, specialized equipment such as that used for high performance liquid chromatography (HPLC) [32]. Experimental methods and data analysis can be time-consuming with such techniques and require highly trained personnel. Further, current enzymatic approaches require extended incubation times and are highly temperature dependent [26]. The first objective of this study is to optimize an enzymatic method for measuring human salivary β -glucosidase activity that is rapid, simple and uses ubiquitous laboratory equipment (a spectrophotometer). A secondary objective is to apply this method to determine the prevalence of salivary β -glucosidase activity in humans. This research informs wider goals of fully eliciting the determinants of human flavour perception and sources of individual differences.

Methods

Assay development and validation

A method to measure β -glucosidase activity from solubilized strawberry callus protein developed by Arnaldos et al. [25] was evaluated and modified for use in human saliva. The reagents were 6 mM p-nitrophenyl- β -D-glucopyranoside (Sigma Aldrich, St. Louis, MO, USA), 6 mM α -cyclodextrin (Sigma Aldrich, St. Louis, MO, USA), 0.025 units of almond β -glucosidase (Sigma Aldrich, St. Louis, MO, USA) and 0.1 M phosphate buffer pH of 6.5, used to correct to a total volume of 2 mL. α -Cyclodextrin was included as it is reported to increase the sensitivity of the assay [25]. One enzyme unit is defined as the protein that hydrolyzes 1 μ mol of salicin per minute at pH 5 and 37 °C. The absorbance of the released p-nitrophenol from the substrate was monitored at 405 nm over the course of 5 min. The assay was evaluated and modified to measure β -glucosidase activity in human saliva with foci on (i) the time over which the assay gave a linear response; (ii) interference from human saliva; (iii) the absorbance wavelength maxima of p-nitrophenol; (iv) the substrate concentration required for saturation of the enzyme; (v) the maximum volume of saliva that could be used without causing interference and (vi) the effectiveness of α -cyclodextrin in enhancing the absorbance signal. Thus, the experimental plan

consisted of six factors assessed in two trials, in order to develop an assay to detect β -glucosidase activity in human saliva. All assays were performed in duplicate using a Spectronic Genesys 2 spectrophotometer and results presented as averages \pm standard deviation (SD), unless otherwise indicated. Table 1 summarizes the steps in developing and validating the assay.

Saliva collection and processing

Trial 1

A population of 20 healthy individuals (13 females, 7 males; 16 Caucasian, 4 non-Caucasian; 29.2 ± 9.7 years, range 21–53 years) free from oral disease participated in this trial by donating saliva samples. On the day of collection, participants were instructed to follow their normal morning routine (example: eating breakfast and brushing their teeth) and were asked to abstain from food or beverage for 2–3 h before sample collection. All β -glucosidase activities were assayed from fresh saliva specimens. We followed the protocols of Soderling [33] for the collection and processing of the saliva samples. Specifically, individuals were asked to spit into a 50-mL falcon tube over the course of approximately 5 min until a minimum volume of 5 mL of saliva was collected. Samples were then centrifuged at 8000 rpm in an SS34 rotor in a Sorval RC5C Plus at 4 °C. The supernatant

Table 1 Experimental design for assay development

Experiment	Factors	Treatments	Outcome
i Validate linearity of the assay signal	7.75 mM p-nitrophenyl- β -D-glucosidase + 0.0025 units of almond β -glucosidase, corrected to final volume of 2 mL with 100 mM phosphate buffer (pH 6.5); wavelength 400 nm over 10 min; no α -cyclodextrin	Absorbance measured every minute for 10 min after the addition of almond β -glucosidase	Linearity of the assay is confirmed
ii Assess interference from saliva	See (i) above	Absorbance values of buffer compared with and without 25 μ L heat-inactivated ^a human saliva	Heat-inactivated saliva does not interfere with the matrix
iii Determine absorbance maxima of p-nitrophenyl	See (i) above	Assay measured at 400 and 405 nm	400 nm selected
iv Determine substrate saturation concentration	Varying substrate concentrations + 0.0025 units of almond β -glucosidase, corrected to final volume of 2 mL with 100 mM phosphate buffer (pH 6.5); wavelength 400 nm over 10 min	Substrate concentration varied between 0 and 10 mM	7.75 mM selected
v Determine maximum saliva volume	7.75 mM p-nitrophenyl- β -D-glucosidase + 0.0025 units of almond β -glucosidase + varying volumes of heat-inactivated saliva, corrected to final volume of 2 mL with 100 mM phosphate buffer (pH 6.5); wavelength 400 nm over 10 min	Different amounts of heat-inactivated saliva evaluated: 25, 50, 100, 150 and 200 μ L	200 μ L selected
vi Evaluate efficacy of α -cyclodextrin	7.75 mM p-nitrophenyl- β -D-glucosidase + 0.0025 units of almond β -glucosidase + 200 μ L of heat-inactivated saliva, corrected to final volume of 2 mL with 100 mM phosphate buffer (pH 6.5); wavelength 400 nm over 10 min	Assays performed with and without 6 mM α -cyclodextrin	Inclusion of 6 mM α -cyclodextrin in assay

^aSaliva was heat-inactivated by boiling for 10 min in a water bath, then centrifuged at 8000 rpm for 10 min using a SS-34 rotor (Sorval RC5C Plus centrifuge)

was transferred into 1.5-mL Eppendorf tubes, placed on ice and assayed within 2 h of sample collection.

Trial 2

Four saliva samples with high rates of enzymatic activity were re-evaluated by processing the saliva with two additional treatments. For the first treatment, the saliva was re-centrifuged for 10 min at 8000 rpm (SS-34 rotor in a Sorval RC5C Plus centrifuge). The clarified supernatant was placed on ice and assayed within 2 h of processing. The second treatment used the saliva collection and centrifugation method outlined in Trial 1, then the saliva was heat-inactivated at 100 °C for 10 min. The heat-inactivated samples were then placed on ice and assayed within 2 h.

Statistical analysis

Differences between variables were determined using the XLSTAT statistical software package (Addinsoft, Version 7.1; New York, NY, USA). Statistical methods used were analysis of variance (ANOVA) with mean separation by Tukey's honestly significant difference (HSD) test ($p < 0.05$) and linear regression analysis.

Results

Assay development and validation

The hydrolysis reaction of the p-nitrophenyl- β -D-glucopyranoside by almond β -glucosidase is linear for the first 10 min of the reaction (linear regression analysis, R^2 value of 0.999; data not shown). Both the 0.1 M phosphate buffer and the 0.1 M phosphate buffer containing 25 μ L of heat-inactivated saliva spiked with 0.025 units of almond β -glucosidase show no significant difference in their change in absorbance over the first 10 min. This assay was also performed with both heat-inactivated and non-heat-inactivated human saliva without the presence

of almond β -glucosidase, and no significant change in absorbance was observed (Fig. 1).

Due to different reported values for the p-nitrophenol wavelength maxima (405 nm, [25]; 400 nm, [34]), we measured almond β -glucosidase activity using 0.1 M phosphate buffer as well as 0.1 M phosphate buffer containing 25 μ L of heat-inactivated human saliva at both 400 and 405 nm. There was no significant difference between the reaction rates observed for each wavelength for either matrix (Fig. 2). Therefore, for the measurement of β -glucosidase activity in human saliva, we used 400 nm as the p-nitrophenol wavelength maxima.

To examine the substrate saturation concentration, we varied the concentration of p-nitrophenyl- β -D-glucopyranoside in 25 μ L of heat-inactivated human saliva between 0 and 10 mM. Figure 3 shows the change in absorbance at 400 nm plotted against the time frame of the reaction. The plateau (representing substrate saturation) occurs at 6–8 mM of p-nitrophenyl- β -D-glucopyranoside, indicating that a concentration of substrate between 6 and 8 mM in the β -glucosidase assay will allow for the maximum rate of hydrolysis.

An assay was performed using five different volumes of heat-inactivated human saliva (25, 50, 100, 150 and 200 μ L) each spiked with 0.025 units of almond β -glucosidase to determine maximum saliva concentrations. The reaction rates did not differ between volumes ($F = 2.3$ (4,5); $p = 0.19$) (data not shown). Therefore, to maximize the level of β -glucosidase activity (predicted to be low) that could be detected in human saliva, the maximum volume of 200 μ L for each human saliva sample was used.

We also assessed the effectiveness of α -cyclodextrin in enhancing the absorbance signal of the p-nitrophenol- α -cyclodextrin chromophore by measuring absorbance in the presence and absence of 6 mM of α -cyclodextrin.

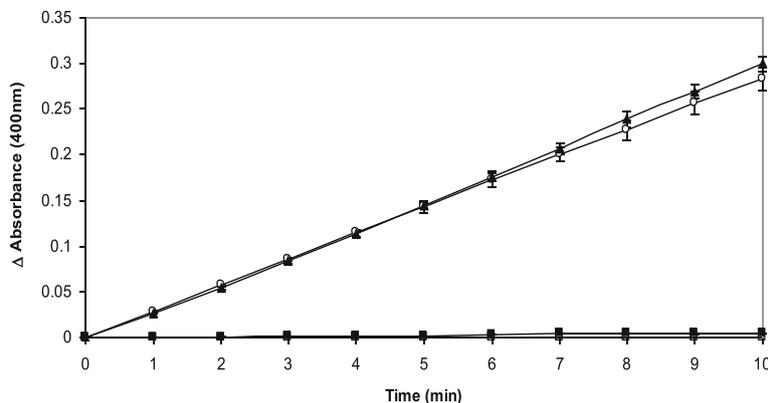
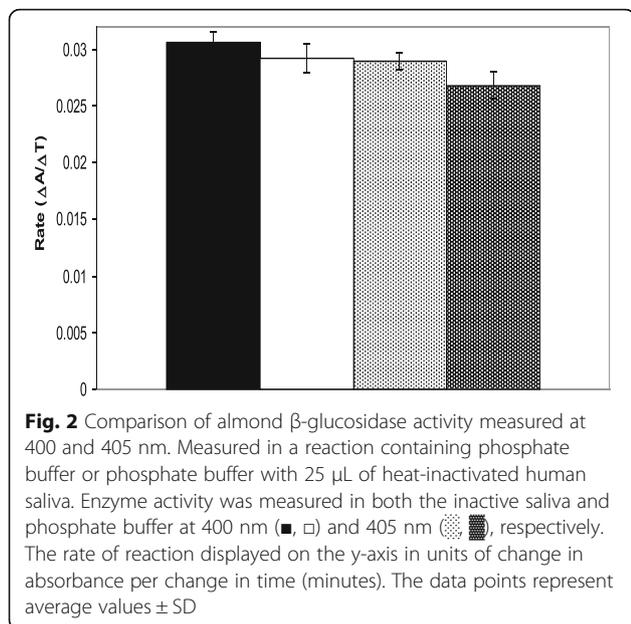


Fig. 1 Almond β -glucosidase activity in the presence and absence of heat-inactivated human saliva. Almond β -glucosidase was measured in a reaction containing phosphate buffer (○) and phosphate buffer with 25 μ L of heat-inactivated human saliva (▲). The background activity of the heat inactivated saliva (■) and phosphate buffer both without almond β -glucosidase (□) were also measured. Each treatment and time point was measured in duplicate. The data points represent average values \pm SD



Absorbance was increased two-fold when α -cyclodextrin is included in the β -glucosidase assay at a concentration of 6 mM (0.056 ± 0.001 versus 0.028 ± 0.000) (data not shown). This is further demonstrated by the change in molar absorption coefficients of p-nitrophenol (7.75 mM) and the α -cyclodextrin (6 mM)/p-nitrophenol (7.75 mM) complex measured in heat-inactivated human saliva and calculated using the Beer-Lambert Law ($A = \epsilon cl$): p-nitrophenol absorbance, 0.0277 ($=3.57 \text{ Lmol}^{-1} \text{ cm}^{-1}$), α -cyclodextrin/p-nitrophenol complex absorbance, 0.0563 ($=7.26 \text{ Lmol}^{-1} \text{ cm}^{-1}$).

Given these findings, the optimized method for determining β -glucosidase activity in human saliva was as follows: 7.75 mM of p-nitrophenyl- β -D-glucopyranoside (Sigma Aldrich, St. Louis, MO, USA), 6 mM of α -cyclodextrin (Sigma Aldrich, St. Louis, MO, USA), 200 μ L of human saliva, 0.1 M phosphate buffer at pH

6.5 (used to correct to a total volume of 2 mL) and absorbance measured at wavelength of 400 nm.

Application: prevalence of β -glucosidase in human saliva

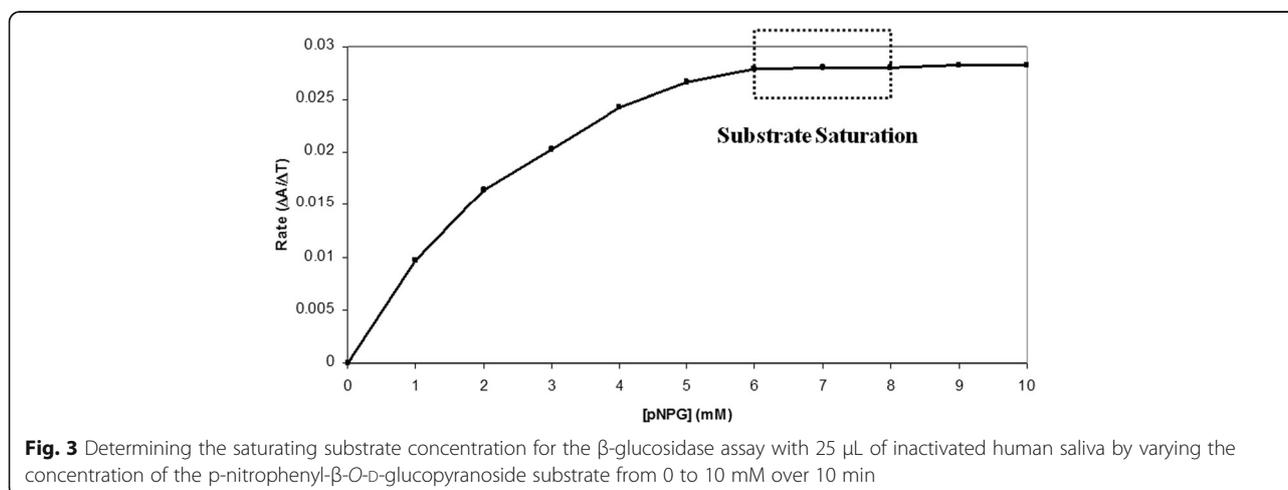
The optimized and validated method described above was used to examine β -glucosidase activity in the saliva of 20 individuals. The results are presented in Fig. 4. As can be noted, there was a large and statistically significant variation in activity between individual saliva samples ($p < 0.05$). Some activity is suggested in 19 of the 20 samples, with four in particular showing relatively high values.

As participants 8, 9, 11 and 12 show significantly higher reaction rates than others, we conducted further testing to ensure that other components of the saliva matrix were not interfering, that β -glucosidase was in fact being measured, and to determine whether or not the samples were following a typical pattern of enzyme activity. These samples were evaluated with additional treatments to confirm that the change in absorbance was due to the hydrolysis of the p-nitrophenyl- β -D-glucopyranoside. The reaction time frame was extended to 30 min to observe the enzyme activity pattern. Treatments (1×10 min of centrifugation, 2×10 min of centrifugation and 1×10 min of centrifugation plus 10 min in a 100°C water bath) and results are shown in Fig. 5.

Figure 5 illustrates that the change in absorbance does not increase linearly after approximately 10 min, representative of a classic enzyme curve. Individuals 8, 9 and 12 display similar levels of β -glucosidase activity as in Trial 1, whereas absorbance values for individual 11 show an approximate two-fold decrease between the two trials.

Discussion

The first phase of this study sought to adapt and validate the assay developed by Arnaldo et al. [25] for measuring β -glucosidase activity in a non-saliva matrix to whole



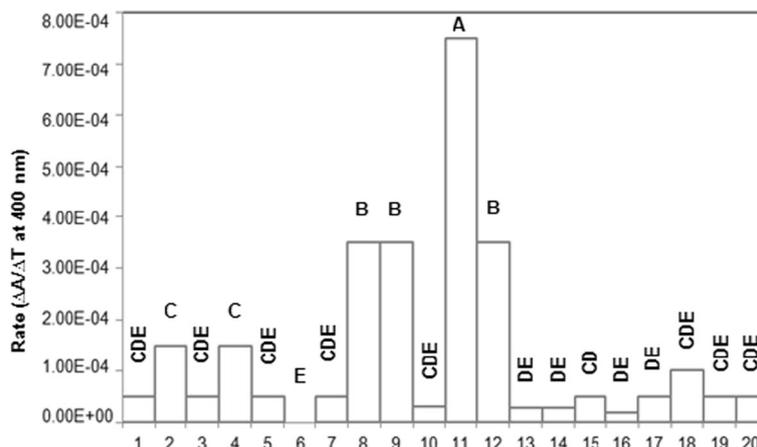


Fig. 4 β -glucosidase activity in 20 human saliva samples. Two control assays were performed using 0.1 M phosphate buffer with and without 25 μ L almond β -glucosidase. The rate of reaction displayed on the y-axis is in units of change in absorbance per change in time (minutes). The samples are labelled 1–20. Samples with different letters (A–E) have significantly different rates (Tukey HSD_{0.05})

human saliva. Reproducible and reliable performance was obtained using 0.1 M phosphate buffer, 0.025 units almond β -glucosidase, 7.75 mM p-nitrophenyl- β -D-glucopyranoside, 6 mM of α -cyclodextrin, 200 μ L saliva and a wavelength of 400 nm. Importantly, we show that the inclusion of 6 mM of α -cyclodextrin increases the sensitivity of the assay, and that inclusion of heat-inactivated human saliva is a necessary component of

the new method. As observed in Fig. 5, heat inactivation reduced the activity level in the saliva, indicating there is a background hydrolysis of the substrate that is not due to β -glucosidase activity. The difference in activity between the non-heat-treated saliva and the heat-treated sample represents the β -glucosidase activity in the saliva. This result shows the importance of subtracting out any contribution of substrate hydrolysis from a heat-

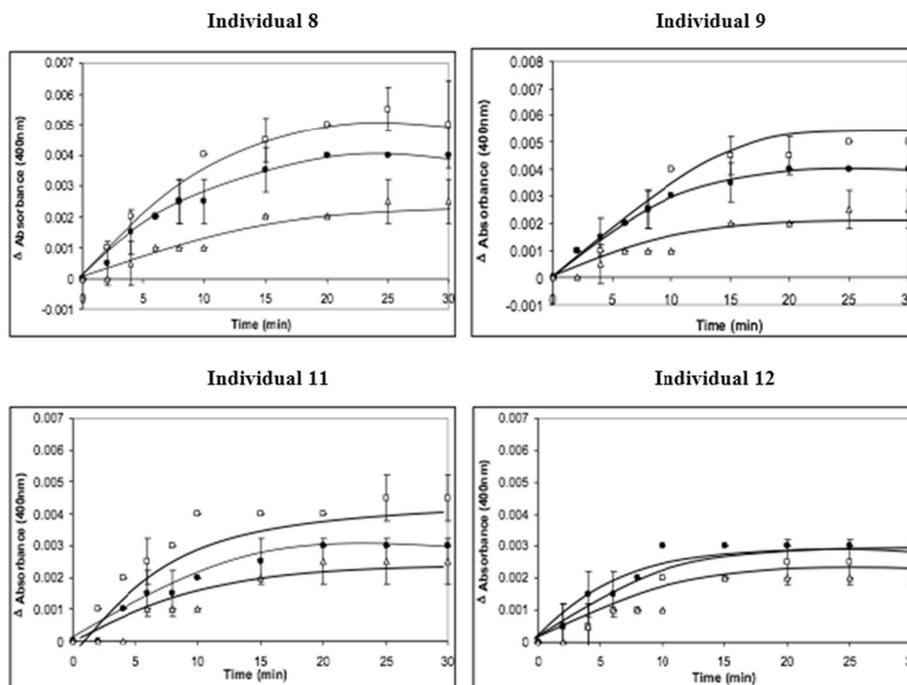


Fig. 5 Retesting of four saliva samples to evaluate β -glucosidase activity in saliva over a 30-min time period. After 10 min of centrifugation (\square), after 2 \times 10 min of centrifugation (\bullet) and after 10 min of centrifugation and 10 min of heat inactivation (Δ). The data points represent average values \pm SD

inactivated sample to ensure all background hydrolysis is accounted for and only β -glucosidase activity is being reported. An existing study conducted by Walle et al. [35] also evaluated human saliva for β -glucosidase activity via high performance liquid chromatography (HPLC), with a minimum detectable amount of $1 \mu\text{mol}/(\text{L saliva} \times \text{h})$. Their method measured the hydrolysis of the flavonoid glucoside genistin as a proxy for enzyme activity. Evidence from other studies [36, 37] suggests that HPLC and spectrophotometric analysis are equally accurate (based on recovery methods), but HPLC may be less variable than spectrophotometric measurements. However, given the rapid nature of our modified spectrophotometric assay, its ease of use, the time required to run analyses and the reduced associated costs, it offers an attractive option for analysis of β -glucosidase activity in human saliva.

The second aim of this study was to assess the prevalence of salivary β -glucosidase activity in a sample of 20 individuals. The accuracy of the spectrophotometer is ± 0.003 absorbance units in the absorbance range being measured. Given this measurement uncertainty, only four of the 20 samples examined appear to contain β -glucosidase activity (absorbance values greater than 3×10^{-4} absorbance units). Fresh saliva samples were obtained and re-evaluated from these individuals, with the resultant absorbance: time responses consistent with classic enzyme activity curves, further suggesting there is active β -glucosidase in these samples.

This relatively low prevalence of human salivary β -glucosidase activity is consistent with the findings of Menguy et al. [24] (1 from 5 individuals) and Zambon et al. [23] (0 from 5 individuals). The aforementioned study by Walle et al. [35] measured the rates of enzymatic hydrolysis in 17 individuals; three individuals showed very high rates (defined by consumption of substrate), one individual showed no hydrolysis and the remainder fell within a range of barely detectable to moderate levels of hydrolysis. Given the similarity of these results to those obtain in the current study, the literature is now converging around an incidence of β -glucosidase activity in human saliva of approximately 20%.

Human salivary pH ranges from 6.2 to 7.4 [38], encompassing the upper limits of the β -glucosidase pH activity range (4.3–7.0; optima = 5.5) [26]. It is therefore possible that β -glucosidase activity and potentially flavour perception may be more pronounced in individuals with low relative salivary pH. However, this speculation should be considered in the context of the pH of the food or beverage containing the glucosides. For instance, the buffering capacity of wine has been shown in both *in vitro* and *in vivo* studies to be superior to that of saliva; the pH of wine/saliva mixtures in the mouth is essentially that of the wine (3.4–3.8) [39], presumably

negating any individual differences in initial salivary pH. Indeed, in the case of wine, this profound decrease in the pH of the saliva/wine mixture suggests that glucoside hydrolysis from β -glucosidase in this glucoside-rich beverage would be negligible.

In the present study, the absorbance readings for individual 11 varied approximate two-fold between the two evaluations, raising questions around the source of this day-to-day variability in activity and also why some individuals possess salivary β -glucosidases at all. Participants were instructed to brush their teeth and eat breakfast as usual the morning their saliva was collected. However, it is possible that those protocols were not followed, and information on the teeth brushing practices of participants was not collected. Bacterial flora in the oral cavity, bacteroides and shedded oral epithelial cells are potential sources of β -glucosidases, as shown by the effectiveness of antibacterial agents such as Listerine® in inhibiting salivary hydrolysis, even 24 h after use [22, 35]. This could have a significant effect on the prevalence and extent of β -glucosidase activity reported here. For instance, some participants may have increased concentration of bacterial flora due to not brushing, while others may have used mouthwash and/or tooth brushing prior to samples being collected. Similarly, differences between replicate sample activities from the same individual(s) may be due to changes in daily oral hygiene practices. It is also possible that inter-individual variation in activity could have a genetic origin with, for instance, some individuals more predisposed to periodontal disease than others [22]. Finally, future research might also consider the association of demographic factors and dietary habits with variation in oral β -glucosidase activity.

Conclusions

This study successfully measured low levels of β -glucosidase activity in human saliva, with the estimated prevalence of 20% in agreement with other studies. Further research should establish the ecological significance of this finding; that is, whether individuals with significant levels of salivary β -glucosidase activity gain any flavour advantage or perceive components of flavour differently. Time-intensity methodologies, including temporal dominance approaches, may be helpful in this regard. The rapid assay developed and described here offers an inexpensive alternative to HPLC methods for measuring β -glucosidase activity and can be applied to larger populations of individuals to further elucidate the extent and significance of human salivary β -glucosidase activity. This should provide additional insights into individual flavour perception and advance understanding of the role of flavour perception in food and beverage preferences and subsequent ingestion behaviours.

Abbreviations

ANOVA: Analysis of variance; HPLC: High performance liquid chromatography; HSD: Honestly significant difference; SD: Standard deviation

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Availability of data and materials

The data supporting the conclusions of this article will be available in the Brock University digital repository (<http://dr.library.brocku.ca/handle/10464/4>).

Authors' contributions

DI and GP conceived the experiment. LS collected and analyzed the data. LS and JK prepared the manuscript, with contribution from DI and GP. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

Ethics approval was obtained for this study through Brock University. Participants consented to participation in the study and saliva samples being taken.

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