nature protocols

SUPPLEMENTARY INFORMATION

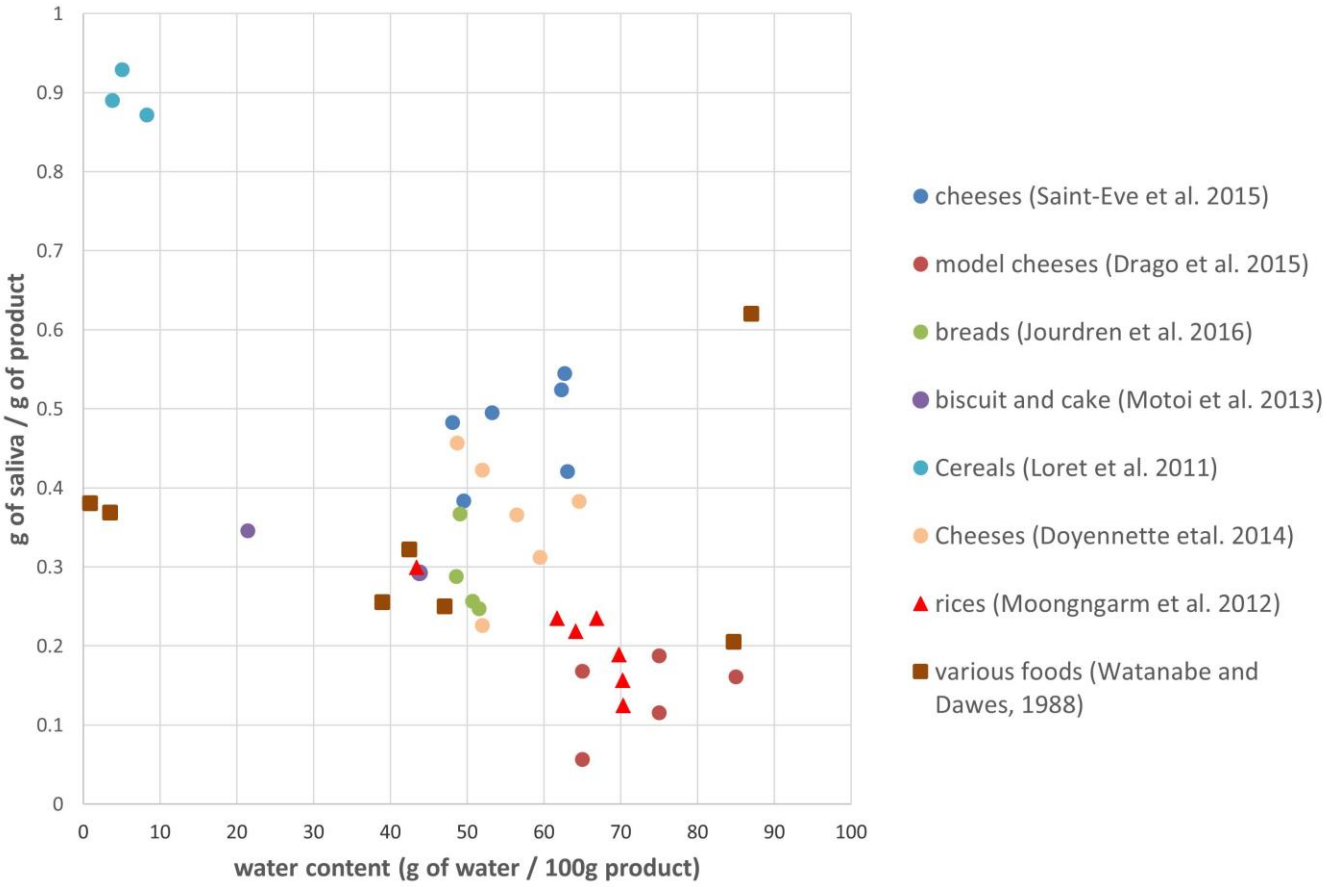
https://doi.org/10.1038/s41596-018-0119-1

In the format provided by the authors and unedited.

INFOGEST static in vitro simulation of gastrointestinal food digestion

André Brodkorb[1*], Lotti Egger[2], Marie Alminger[3], Paula Alvito[4], Ricardo Assunção[4], Simon Ballance[5], Torsten Bohn[6], Claire Bourlieu-Lacanal[7], Rachel Boutrou[8], Frédéric Carrière[9], Alfonso Clemente[10], Milena Corredig[11], Didier Dupont[8], Claire Dufour[12], Cathrina Edwards[13], Matt Golding[14], Sibel Karakaya[15], Bente Kirkhus[5], Steven Le Feunteun[8], Uri Lesmes[16], Adam Macierzanka[17], Alan R. Mackie[18], Carla Martins[4], Sébastien Marze[19], David Julian McClements[20], Olivia Ménard[8], Mans Minekus[21], Reto Portmann[2], Cláudia N. Santos[22,23], Isabelle Souchon[24], R. Paul Singh[25], Gerd E. Vegarud[26], Martin S. J. Wickham[27], Werner Weitschies[28] and Isidra Recio[29]

[1]Teagasc Food Research Centre, Moorepark, Fermoy, Ireland. [2]Agroscope, Bern, Switzerland. [3]Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden. [4]National Institute of Health Doutor Ricardo Jorge, Lisbon and CESAM, University of Aveiro, Aveiro, Portugal. [5]Nofima AS, Ås, Norway. [6]Luxembourg Institute of Health, Strassen, Luxembourg. [7]INRA/Montpellier SupAgro, Montpellier, France. [8]STLO, INRA, Agrocampus Oeust, Rennes, France. [9]Aix-Marseille, CNRS, UMR7281 Bioénergétique et Ingénierie des Protéines, Marseille, France. [10]Estación Experimental del Zaidin, Consejo Superior de Investigaciones Científicas (CSIC), Granada, Spain. [11]Department of Food Science, Aarhus University, Tjele, Denmark. [12]SQPOV, INRA, Avignon, France. [13]Quadram Institute Bioscience, Norwich, UK. [14]Riddet Institute, Massey University, Palmerston North, New Zealand. [15]Department of Food Engineering, Faculty of Engineering, Ege University, Izmir, Turkey. [16]Israel Institute of Technology, Technion City, Haifa, Israel. [17]Faculty of Chemistry, Gdansk University of Technology, Gdansk, Poland. [18]School of Food Science & Nutrition, University of Leeds, Leeds, UK. [19]BIA, INRA, Nantes, France. [20]Chenoweth Lab, Department of Food Science, University of Massachusetts, Amherst, MA, USA. [21]Triskelion, Zeist, the Netherlands. [22]Instituto de Biologia Experimental e Tecnológica, Oeiras, Portugal. [23]Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal. [24]GMPA, AgroParisTech, INRA, Université Paris-Saclay, Thiverval-Grignon, France. [25]Departments of Biological and Agricultural Engineering, and of Food Science and Technology, University of California, Davis, Davis, CA, USA. [26]Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Aas, Norway. [27]Reacta Biotech Limited, The Langley Building, Manchester, UK. [28]Ernst Moritz Arndt University of Greifswald, Greifswald, Germany. [29]Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM), Madrid, Spain. *e-mail: andre.brodkorb@teagasc.ie



Supplementary Figure 1

Oral bolus hydration in vivo.

Bolus hydration (g of saliva/g of foods) in vivo just before swallowing, for various foods based on published data[116–123]. Supplementary Methods (enzyme activity assays) were adapted from Minekus et al.[27] under a Creative Commons Attribution 3.0 license (https://creativecommons.org/licenses/by/3.0/legalcode).

References

116. Watanabe, S. & Dawes, C. The effects of different foods and concentrations of citric acid on the flow rate of whole saliva in man. *Arch. Oral Biol*. **33**, 1–5 (1988).
117. Saint-Eve, A., Panouille, M., Capitaine, C., Deleris, I. & Souchon, I. Dynamic aspects of texture perception during cheese consumption and relationship with bolus properties. *Food Hydrocoll.* **46**, 144–152 (2015).
118. Motoi, L., Morgenstern, M. P., Hedderley, D. I., Wilson, A. J. & Balita, S. Bolus moisture content of solid foods during mastication. *J. Texture Stud.* **44**, 468–479 (2013).
119. Moongngarm, A., Bronlund, J., Grigg, N. & Sriwai, N. Chewing behavior and bolus properties as affected by different rice types. *Int. J. Med. Biol. Sci.* **6**, 51–56 (2012).
120. Loret, C. et al. Physical and related sensory properties of a swallowable bolus. Physiol. Behav. **104**, 855–864 (2011).
121. Jourdren, S. et al. Breakdown pathways during oral processing of different breads: impact of crumb and crust structures. *Food Funct.* **7**, 1446–1457 (2016).
122. Drago, S. R. et al. Relationships between saliva and food bolus properties from model dairy products. *Food Hydrocoll.* **25**, 659–667 (2011).
123. Doyennette, M. et al. Main individual and product characteristics influencing in-mouth flavour release during eating masticated food products with different textures: mechanistic modelling and experimental validation. *J. Theor. Biol.* **340**, 209–221 (2014).

Supplementary Methods - Enzyme assays

Enzyme and bile assays are adapted from Minekus et al.[1], namely: α-amylase (EC 3.2.1.1), pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), pancreatic lipase (EC 3.1.13) and bile salts (according to supplier's protocol). The assay for gastric lipase has been adapted from Carrière et al.[2] and merged with that for pancreatic lipase.

α-Amylase Activity Assay (EC 3.2.1.1)

References: according to Bernfeld[3]

Method: Spectrophotometric Stop Reaction

Principle:

Starch + H_2O $\xrightarrow{\alpha\text{-Amylase}}$ Reducing Groups (Maltose)

Unit definition: One unit releases 1.0 mg of maltose from (potato) starch in 3 minutes at pH 6.9 and 20°C.

Conditions: T = 20°C, pH = 6.9, A_{540nm}, light path = 1 cm

Procedure

Preparation of reagents

Substrate: soluble potato starch (1.0% w/v)

Preparation of substrate solution:

Prepare 100 mL of a 20 mM sodium phosphate buffer containing 6.7 mM NaCl. Adjust the pH to 6.9 at 20°C with 1 M NaOH. Dissolve 0.25 g soluble potato starch (ref S2630 Sigma-Aldrich) in 20 mL of the sodium phosphate buffer, pH 6.9. Heat the covered beaker while stirring and maintain the solution just below boiling temperature for 15 minutes. Cool to room temperature and complete the starch solution to the appropriate volume (25 mL) by addition of H_2O.

Standard Curve: Prepare 10 mL of 0.2 % w/v maltose standard (M5885 Sigma-Aldrich).

Enzyme: Shortly before the assay, prepare an enzyme solution of an estimated activity of 1 unit/mL of α-amylase in purified H_2O

Assay solution: Colour reagent solution 3,5-dinitrosalicylic acid (DNS)

Prepare a 5.3 M sodium potassium tartrate solution in 2 M NaOH by dissolving 0.8 g NaOH in 10 mL H_2O and heating the solution at a temperature ranging between 50 to 70°C. Add 12.0 g of sodium potassium tartrate tetrahydrate (in 8.0 mL of warm 2 M NaOH solution, maintain the temperature constant while stirring to dissolve the tartrate but do not boil it.

Prepare a 96 mM DNS solution by dissolving 438 mg of DNS in 20 mL of H_2O. Heat the solution at a temperature between 50 to 70°C. Maintain at this temperature while constant stirring to dissolve DNS but do not boil it.

Heat 12 mL of purified water to 60°C and add slowly 8 mL of the 5.3 M the sodium potassium tartrate solution. Add 20 mL of the 96 mM 3,5-dinitrosalicylic acid solution and stir until complete dissolution. The solution can be stored in an amber flask at room temperature for one month.

Assay:

Set the spectrophotometer at 540 nm and 20°C. Set a bench top shaking incubator fitted with a sample holder at 20°C, a heating bath or block at 100°C to stop the reaction, and an ice-bath to cool the sample.

Test: Pipette 1 mL of substrate solution (potato starch) into cap covered tubes (15 mL), mix and incubate at 20°C for 5 min to achieve temperature. Add 0.5 – 1 mL of enzyme solution (according to the scheme below), mix and incubate at 20°C for exactly 3 minutes. Immediately thereafter, stop the reaction by addition of 1 mL of DNS solution. Complete the enzyme volume added to 1 mL, cap the tube, place it at 100°C (heating bath or block) and boil it for exactly 15 minutes. Cool the tube for a few minutes on ice and add 9 mL of H_2O. Mix the reaction and pipette 3 mL in a cuvette and record the absorbance at 540 nm.

Blank: For blank tests, follow the same procedure but no enzyme is added before the 3 minutes incubation time.

Pipetting scheme for three different enzyme concentrations:

Volumes in mL	1st enzyme concentration	2nd enzyme concentration	3rd enzyme concentration	Blank
Substrate (potato starch)	1.00	1.00	1.00	1.00
Enzyme solution	0.50	0.70	1.00	-
DNS	1.00	1.00	1.00	1.00
2nd addition of enzyme	0.50	0.30	-	1.00
H_2O	9.00	9.00	9.00	9.00

Standard Curve with maltose:

Dilute the maltose solution (0.2% w/v) according to the scheme in H_2O

Volumes in (mL)	D1	D2	D3	D4	D5	D6	D7	Std. Blank
Maltose solution	0.05	0.20	0.40	0.60	0.80	1.00	2.00	-
H_2O	1.95	1.80	1.60	1.40	1.20	1.00	-	2.00

1mL DNS reagent solution is added to each maltose standard, thereafter the tubes are boiled for 15 minutes, cooled on ice to room temperature and 9mL of H_2O are added.

Calculations

Standard Curve:

$$\Delta A_{540}\text{Standard} = \Delta A_{540}\text{Standard} - \Delta A_{540}\text{Std. Blank}$$

Plot the ΔA_{540}nm of the Standards versus the quantity of maltose [mg] and establish a linear regression:

$$\Delta A_{540}\text{Standard} = \text{a} \times [\text{maltose}] - \text{b}$$

Enzyme activity:

$$\Delta A_{540}\text{Sample} = \Delta A_{540}0\text{Test} - \Delta A_{540}\text{Test Blank}$$

$$\frac{\text{Units}}{\text{mg powder}} = \frac{[\,A_{540}\ \text{Test} - A_{540}\ \text{Test Blank}] - \text{b}}{(\text{a} \times\ \text{X}\,)}$$

a: slope of the linear regression for standards ΔA_{540}nm *vs* the quantity of maltose (mg).

b: intercept of the linear regression for standards ΔA_{540}nm *vs* the quantity of maltose (mg).

X: quantity of amylase powder (mg) added before stopping the reaction.

Pepsin Activity Assay (EC 3.4.23.1)

References: adapted from Anson *et al.* [4,5]

Method: Spectrophotometric Stop Reaction

Principle:

Haemoglobin + $H_2O \xrightarrow{\text{pepsin}}$ TCA soluble tyrosine containing peptides

Unit definition: One unit will produce a ΔA_{280} of 0.001 per minute at pH 2.0 and 37°C, measured as TCA-soluble products. These units are often referred to “Sigma” or “Anson” pepsin units.

Conditions: T = 37°C, pH = 2.0, A_{280nm}, light path = 1 cm

Procedure:

Preparation of reagents

Substrate: Prepare a haemoglobin solution by dispersing 0.5 g haemoglobin (bovine blood haemoglobin, ref H2500 Sigma-Aldrich) in 20 mL purified water, adjust to pH 2 with 300 mM HCl and complete the volume to 25 ml to obtain a solution at 2% w/v haemoglobin at pH 2.

Enzyme: Prepare a stock solution of 1 mg/mL pepsin (porcine pepsin, ref. P6887 Sigma-Aldrich) in 10 mM Tris buffer, 150 mM NaCl at pH 6.5. The stock solution has to be stored on ice or refrigerated at 4°C. Just before the assay, a range of 5 to 10 concentrations of pepsin in 10 mM HCl has to be prepared. For instance, dilute the pepsin stock solution to prepare the following enzyme assay solutions: 5, 10, 15, 20, 25, 30 µg/mL.

Assay:

Set the spectrophotometer at 280 nm and 20°C. Set a bench top shaking incubator fitted with a sample holder at 37°C.

Test: Pipette 500 µL of haemoglobin solution into 2 mL Eppendorf tubes and incubate in a shaking incubator at 37°C for 3-4 minutes to reach the assay temperature.

Add 100 µL of pepsin assay solutions for each concentration and incubate them for 10 minutes exactly. To stop the reaction, 1 mL of 5% w/v TCA (Trichloroacetic Acid) is added in each tube. In order to get a clear soluble phase available for absorbance measurement, centrifuge the Eppendorf tubes at 6,000 × *g* for 30 minutes to precipitate remaining haemoglobin; remove the pellet.

Place the soluble phase into quartz cuvettes and read the absorbance at 280 nm (A_{280} Test).

Blank: For blank tests, the same procedure is followed but the pepsin is added after the addition of TCA, which stops the reaction. The blank absorbance is noted A_{280} Blank.

Because, the absorbance is a function of the pepsin concentration, a linear curve has to be obtained. If no linear part is found, it can be due to a large amount of enzyme, and therefore it is necessary to use more dilute enzyme assay solutions.

Calculations:

$$\text{Units/mg} = \frac{[\text{A280 Test} - \text{A280 Blank}] \times 1{,}000}{(\Delta t \times \text{X} \times 0.001)}$$

Δt: duration of the reaction, i.e. 10 minutes

X = amount of pepsin powder (µg) in 1mL in the assay solution (i.e., 5, 10, 15, 20, 25, 30 µg)

1,000 = dilution factor to convert µg to mg

0.001 = ΔA_{280} per unit of pepsin

Check that the activity obtained is the same for each tested concentration of pepsin, to make sure that you are in the linear part of the pepsin concentration curve.

Gastric and pancreatic lipase activity assay (EC 3.1.1.3)

References: Gargouri et al.[6]; Moreau et al.[7]; Carrière et al. [2,8], Erlanson and Borgström [9]

Method: pH titration

Principle:

Tributyrin + H_2O $\xrightarrow{\text{lipase}}$ butyric acid + glycerol

The gastric and pancreatic lipase activity assay are conducted by pH titration and tributyrin as substrate. The free fatty acids released by the lipases are titrated at a constant pH by sodium hydroxide (0.02 - 0.1 N) during at least 5 min. The concentration of NaOH is adjusted to allow the titrator to keep the pH as constant as possible during the titration.

Unit definition: One unit releases 1 µmol of butyric acid per minute at 37°C at the pH of the assay: 6.0 for Human Gastric Lipase, 5.5 for Rabbit Gastric Lipase and 8 for Pancreatic Lipase. These units are often referred to International Units. Both, purified Human and Rabbit Gastric Lipases show a specific activity of approx. 1,200 U/mg protein on tributyrin[7,10] and human Pancreatic Lipase has a specific activity of ca. 8,000 U/mg of protein on tributyrin [2]

Procedure:

Preparation of reagents:

Assay solution: Prepare 200 mL of the following aqueous solutions which vary for gastric or pancreatic lipase:

	Gastric Lipase		Pancreatic Lipase	
	Concentration [mg/L]	Corresponding weight [mg] for 200 mL	Concentration [mg/L]	Corresponding weight [mg] for 200 mL
NaCl	9,000 (150 mM)	1,800	9,000 (150 mM)	1,800
Sodium tauro-deoxycholate	1,000 (2 mM)	200	2,000 (4 mM)	400
BSA	100 (1 µM)	20	-	-
$CaCl_2$	-	-	200	40
Tris-(hydroxymethyl)-aminomethane	-	-	36	7.20
pH	adjust with HCl (0.1M) at pH 5.5 (RGE) or pH 6 (HGL)		adjust with HCl (0.1 M) at pH 8	

Titration Solution: Prepare a solution of 0.1 N sodium hydroxide (NaOH) by dissolving 2 g NaOH in 500 mL of purified water. It is recommended to perform a back titration using 0.1 N HCl to confirm the precise molarity of the NaOH titration solution. Alternatively, commercial NaOH stock solutions can be used.

Enzyme: Prepare a 1 mg/mL solution by dissolving 5 mg of lipase (e.g. rabbit gastric extract powder, RGE25-100MG Lipolytech, France) in 5 mL of purified water. Store on ice. Perform the assay with at least 2 different amounts of the enzyme solution, i.e. 50 and 100 µL, at 1 mg/mL.

Substrate: Use tributyrin of purity grade (≥99%; ref T8626 Sigma-Aldrich)

Assay:

Set a thermo-regulated pH-stat device to 37°Cfitted with a jacketed and capped reaction vessel (20-70 mL) and mechanical stirrer, preferentially with a 3-pale propeller.

Pour 14.5 mL of the assay solution and 0.5 mL of tributyrin into the titration vessel. Make sure the volume of the assay is enough to ensure adequate pH-measurement, i.e., the pH electrode is correctly immersed. By switching on the mechanical stirring of the apparatus, tributyrin will get dispersed to form a fine oil-in-water emulsion after 3-5 min at 37°C.

Switch on the automated delivery of titrant solution (0.1 N NaOH) to monitor the pH and adjust it at the selected pH end-point of titration, i.e., pH 5.5 for rabbit gastric lipase, pH 6.0 for human gastric lipase or pH 8.0 for pancreatic lipase. Add 50 or 100 µL of the enzyme solution. Monitor the rate of titrant solution (NaOH) which is required to maintain the pH constant at 37°C due to the release of free fatty acids. These conditions allow measuring linear kinetics of free fatty release for at least 5 minutes.

If pancreatic lipase does not contain colipase, add colipase at a molar excess (ratio of 2:1 colipase:lipase) before adding the enzyme.

Calculations:

$$\frac{\text{Units}}{\text{mg powder}} = \frac{\text{R(NaOH)} \times 1000}{\text{v} \times \text{[E]}} \times \text{F}$$

R(NaOH): Rate of NaOH delivery in µmol NaOH per minute, *i.e.*, µmol free fatty acid titrated per minute

v: volume [µL] of enzyme solution added in the pH-stat vessel

[E]: concentration of the enzyme solution [mg powder/mL]

F: correction factor to take into account the partial ionization (and titration) of fatty acids at the pH of the assay. Only for the titration of butyric acid at pH 5.5, a correction factor F of 1.12 has to be applied.

Check that the activity obtained is the same for each tested concentration of lipase, to make sure that you are in the linear part of the enzyme concentration curve.

Trypsin Activity Assay (EC 3.4.21.4)

References: adapted from Hummel[11] and following recommendations from the Worthington laboratory

Method: Kinetic spectrophotometric rate determination

Principle:

TAME + H_2O $\xrightarrow{\text{trypsin}}$ p-Toluene-Sulfonyl-L - Arginine + Methanol

Unit definition: One unit hydrolyses 1 µmol of p-toluene-sulfonyl-L-arginine methyl ester (TAME) per minute at 25°C and pH 8.1

Unit conversion: 1 TAME Unit = 19.2 USP/NF Units = 57.5 BAEE Units

Conditions: T = 25°C, pH = 8.1, A_{247}nm, Light path = 1 cm

Preparation of reagents

Substrate: TAME (ref. T4626 Sigma-Aldrich) at 10 mM is prepared and dissolved in purified water.

Enzyme: Prepare at least 2 concentrations of trypsin (porcine trypsin, ref. T0303 Sigma-Aldrich) ranging between 10-20 µg/mL in 1 mM HCl.

Assay solution: 46 mM Tris/HCl buffer, containing 11.5 mM $CaCl_2$ at pH at 8.1 and 25°C.

Assay:

Set the spectrophotometer at 247 nm and 25°C.

Test: Pipette 2.6 mL of assay solution and 0.3 mL of the substrate (10 mM TAME) into quartz cuvettes, mix by inversion and incubate in spectrophotometer at 25°C for 3-4 minutes to achieve the temperature.

Add 100 µl of each concentration of trypsin solutions and record in continuum the absorbance increase at 247 nm (ΔA_{247}) during 10 min, until levelling off. Determine the slope ΔA_{247} from the initial linear portion of the curve. If no linear part is found, repeat the test with a lower or higher amount of enzyme.

Blank: For blank assays, follow the same protocol by replacing the enzyme with buffer (equilibration is usually reached faster, 5 min). The blank slope, ΔA_{247}, should be close to zero.

Calculations:

The slopes ΔA_{247} [unit absorbance/minute] are established for both the blank and the test reactions by using the maximum linear rate over at least 5 minutes:

$$\text{Units/mg} = \frac{[\,\Delta\text{A247 Test} - \Delta\text{A247 Blank}] \times 1000 \times 3}{(540 \times \text{X})}$$

ΔA_{247}: slope of the initial linear portion of the curve, [unit absorbance/minute] for the Test (with enzyme) and ΔA_{247} Blank without enzyme

540: molar extinction coefficient (L/(mol × cm) of TAME at 247 nm.

3: Volume (in millilitres) of reaction mix

X: quantity of trypsin in the final reaction mixture (quartz cuvette) [mg]

Check that the activity obtained is the same for each tested concentration of trypsin, to make sure that you are in the linear part of the enzyme concentration curve.

Chymotrypsin activity assay (EC 3.4.21.1)

References: adapted from Hummel[11] and Rick[12]

Method: Kinetic spectrophotometric rate determination

Principle:

BTEE + H_2O $\xrightarrow{\text{chymotrypsin}}$ N - Benzoyl - L - Tyrosine + Ethanol

Unit Definition: One unit of chymotrypsin hydrolyses 1.0 µmol of N-Benzoyl-L-Tyrosine Ethyl Ester (BTEE) per minute at pH 7.8 and 25ºC.

Conditions: T = 25°C, pH = 7.8, A_{256}nm, Light path = 1 cm

Preparation of reagents:

Substrate: Dissolve the substrate, BTEE (ref. B6125 Sigma-Aldrich), at a concentration of 1.18 mM in methanol/purified water. Weigh 18.5 mg of BTEE, dissolve it in 31.7 mL of absolute methanol and complete to 50 mL with deionized water in a 50 mL volumetric flask.

Enzyme: The enzyme is dissolved in 1 mM HCl. Prepare at least 2 concentrations of chymotrypsin (porcine chymotrypsin, ref. C7762 Sigma-Aldrich) ranging between 10-30 µg/mL in 1 mM HCl.

Assay solution: 80 mM Tris/HCl buffer, containing 100 mM $CaCl_2$ at pH at 7.8 and 25°C.

Assay:

Set the spectrophotometer at 256 nm and 25°C.

Test: Mix 1.5 mL of the assay solution and 0.3 mL of the substrate (1.18 mM BTEE) into quartz cuvette, mix by inversion and incubate in spectrophotometer at 25°C for 3-4 minutes to achieve temperature equilibration. Add 100 µl of each concentration of the chymotrypsin solutions and record the absorbance increase ΔA at 256 nm (ΔA_{256}) during 10 min in continuum, until levelling off. Determine the slope ΔA_{256} from the initial linear portion of the curve. If no linear part is found repeat the test with a lower or higher amount of enzyme.

Blank: For blank assays, follow the same protocol by replacing the enzyme with buffer only (equilibration is usually reached faster, 5 min). The blank slope ΔA_{256} Blank should be close to zero.

Calculations:

The slopes Δ_{A256} [unit absorbance/minute] are established for both the blank and the test reactions by using the maximum linear rate over at least 5 minutes:

$$\text{Units/mg} = \frac{[\,\Delta\text{A256 Test} - \text{A256 Blank}] \times 1000 \times 3}{(964 \times \text{X})}$$

ΔA_{256}: slope of the initial linear portion of the curve, [unit absorbance/minute] for the Test (with enzyme) and ΔA_{256} Blank without enzyme

964: molar extinction coefficient L/(mol × cm) of BTEE at 256 nm.

3: Volume (in millilitres) of reaction mix

X: quantity (mg) of chymotrypsin in the final reaction mixture (quartz cuvette)

Check that the activity obtained is the same for each tested concentration of chymotrypsin, to make sure that you are in the linear part of the enzyme concentration curve.

Pancreatin

The amount of pancreatin is normalized to the trypsin activity. However, to digest fat containing food, the lipase activity should be recorded as well. Therefore, to measure the enzyme activities of the pancreatin (porcine pancreatin 8 x USP specifications, ref P7545 Sigma-Aldrich), the protocols are the same as described above. For trypsin (or chymotrypsin) Pancreatin is dissolved in 1 mM HCl (pH 3). Pancreatin is difficult to dissolve, mix during 10 minutes using a magnetic stirrer and then keep the solution on ice or at refrigerated temperature 4°C to prevent loss of activity. Dilute the pancreatin to a concentration ranging between 0.1 to 1 mg/mL and measure at least 3 different dilutions. Vortex pancreatin before

pipetting it to the enzyme reaction vessel. To measure the lipase activity in pancreatin, dissolve it in 150 mM NaCl at pH 6.8 (pancreatic lipase is degraded at low pH), and follow the above procedure to record lipase activity.

Bile salts in bile

The concentration of bile salts in the bile (fresh or commercial) can be measured with a commercial kit (bile acid kit, 1 2212 99 90 313, DiaSys Diagnostic System GmbH, Germany, MAK309-1KT, Merck or similar) according the supplier's protocol. Measure the bile at different concentrations bearing in mind the linearity range of the kit.

REFERENCES

1 Minekus, M. *et al.* A standardised static in vitro digestion method suitable for food - an international consensus. *Food & Function* **5**, 1113-1124, doi:10.1039/C3FO60702J (2014).

2 Carrière, F., Barrowman, J. A., Verger, R. & Laugier, R. Secretion and contribution to lipolysis of gastric and pancreatic lipases during a test meal in humans. *Gastroenterology* **105**, 876-888 (1993).

3 Bernfeld, P. in *Methods Enzymol.* Vol. Volume 1 149-158 (Academic Press, 1955).

4 Anson, M. L. & Mirsky, A. E. The estimation of pepsin with hemoglobin. *J. Gen. Physiol.* **16**, 59-63, doi:10.1085/jgp.16.1.59 (1932).

5 Anson, M. L. The Estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *J. Gen. Physiol.* **22**, 79-89, doi:10.1085/jgp.22.1.79 (1938).

6 Gargouri, Y. *et al.* Importance of human gastric lipase for intestinal lipolysis: an in vitro study. *Biochim. Biophys. Acta* **879**, 419-423, doi:10.1016/0005-2760(86)90234-1 (1986).

7 Moreau, H., Gargouri, Y., Lecat, D., Junien, J.-L. & Verger, R. Purification, characterization and kinetic properties of the rabbit gastric lipase. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism* **960**, 286-293, doi:10.1016/0005-2760(88)90036-7 (1988).

8 Carriere, F. *et al.* Purification and biochemical characterization of dog gastric lipase. *The FEBS Journal* **202**, 75-83, doi:10.1111/j.1432-1033.1991.tb16346.x (1991).

9 Erlanson, C. & Borgström, B. Tributyrine as a substrate for determination of lipase activity of pancreatic juice and small intestinal content. *Scand. J. Gastroenterol.* **5**, 293 (1970).

10 Gargouri, Y. *et al.* Kinetic assay of human gastric lipase on short- and long-chain triacylglycerol emulsions. *Gastroenterology* **91**, 919-925, doi:10.5555/uri:pii:0016508586906955 (1986).

11 Hummel, B. C. W. A modified spectrophotometric determination of chymotrypsin, trypsin, and thrombin. *Can. J. Biochem. Physiol.* **37**, 1393-1399, doi:10.1139/o59-157 (1959).

12 Rick, W. in *Methods of Enzymatic Analysis (Second Edition)* (ed Hans Ulrich Bergmeyer) 1006-1012 (Academic Press, 1974).