Supplementary Material

Protocol for enzyme assays

Enzyme assays are based on commonly used protocols, cited here, and those recommended by commercial enzyme providers. Below is a detailed description of the enzyme assays referred to in the manuscript.

To get adequate measurement of enzyme activity, the assays have to be conducted in large excess of substrate. So it is generally advised to test several concentrations of enzyme and check that the recorded activity increases linearly with the concentration of enzyme in the reaction mixture.

Care should be taken when diluting enzyme powders containing protease activity (*e.g.* pure protease powder or pancreatin) to disperse the powder following a well-defined procedure. We recommend 10 minutes dissolution in an aqueous solution with a pH adjusted to limit the proteolytic activity (pH 6.5 for pepsin for instance). After dissolution the enzyme preparation must be stored on ice again to limit proteolytic degradation.

For enzyme activity assays based on spectrophotometric measurements, the absorbance should be inferior to 1 (DO < 1) according to the Beer Lambert's law recommendation. Before adding the enzyme, check the absorbance of the mix of buffer and substrate. If absorbance is above 1, dilute the substrate.

Please refer to Material Safety Data Sheets (MSDS) for hazards and appropriate handling precautions.

Purified Water refers to water obtained with a deionizing system, presenting a resistivity superior or equal to 18 MΩ cm at 25ºC.

α-AMYLASE enzymatic Assay (EC 3.2.1.1)

References: According to Bernfeld [1]

Method: Spectrophotometric Stop Reaction

Principle:

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

Unit definition: One unit liberates 1.0 mg of maltose from starch in 3 minutes at pH 6.9 at 20°C

Conditions:

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

Protocol

Preparation of reagents

Substrate: soluble potato starch

Prepare 100 mL of a 20 mM sodium phosphate buffer containing 6.7 mM NaCl. Adjust it to pH 6.9 at 20°C with 1 M NaOH. Prepare a 1.0% w/v soluble starch solution in the sodium phosphate buffer pH 6.9 by dissolving 0.25 g soluble potato starch (for instance ref S2630 Sigma-Aldrich) in an initial volume of 20 mL of buffer. Cover the beaker to avoid water evaporation and bring to boil while stirring and maintain just below boiling temperature for 15 minutes. After cooling to room temperature, complete the starch solution to the appropriate volume (25 mL) by adding 5 mL of purified water.

Preparation of colour reagent solution:

Prepare a 5.3 M sodium potassium tartrate solution in 2 M NaOH. Dissolve 0.8 g NaOH in 10 mL purified water. Heat the solution to reach a temperature ranging between 50 to 70°C. Dissolve 12.0 g of sodium potassium tartrate tetrahydrate (MW: 282.22 g/mol), in 8.0 mL of warm 2 M NaOH solution. Maintain at this temperature while constant stirring to dissolve but do not boil.

Prepare a 96 mM 3,5-dinitrosalicylic acid (MW: 228.12 g/mol) solution by dissolving 438 mg of acid in 20 mL of purified water. Heat the solution to reach a temperature ranging between 50 to 70°C. Maintain at this temperature while constant stirring to dissolve but do not boil.

Heat 12 mL of purified water to 60°C, add slowly 8 mL of the 5.3 M Sodium Potassium Tartrate Solution. Then add 20 mL of the 96 mM 3,5-dinitrosalicylic acid solution. Stir until complete dissolution. The solution can be stored up to 6 months in amber flask at room temperature.

Prepare 10 mL of 0.2 % w/v maltose standard (for instance ref M5885 Sigma-Aldrich).

Enzyme: Just before use, prepare a solution containing approximately 1 unit/mL of α- amylase in purified water.

Assay:

Set the spectrophotometer at 540 nm and 20°C.

Set a bench top shaking incubator fitted with sample holder at 20°C. Heat a water bath at 100°C, fit it with a sample holder to stop the reaction. Prepare a container filled with ice to cool the samples.

Pipette into 15 mL cap covered containers 1 mL of substrate (solution of potato starch), mix by swirling and incubate at 20°C for 3-4 minutes to achieve temperature equilibration.

Test: Then add 500 µl to 1 mL of enzyme solution, mix by swirling and incubate at 20°C for exactly 3 minutes. After 3 minutes, stop the solution by adding 1 mL of colour reagent solution, cap the reaction vessel, place in the boiling water bath, complete the volume of enzyme added to 1 mL (for instance if 700 µL has been added, add 300 µL of enzyme solution). Boil for exactly 15 minutes and cool on ice for a few minutes. Add 9 mL of purified water. Mix by inversion. Pipette 3 mL of the reaction mixture in a cuvette of spectrophotometer and record the absorbance at 540 nm, noted A_{540} Test.

Blank: For blank assays, the protocol is similar but no enzyme is added before the 3 minute incubation. It is only after the addition of the colour reagent and after putting the reaction vessel in the boiling bath, that 1 mL of enzyme solution is added.

After blank procedure, the absorbance, A_{540} Blank is similarly recorded.

To test 3 concentrations of enzymes, the following tests can be run successively:

	1st Concentration of enzyme	2nd Concentration of enzyme	3rd Concentration of enzyme	Test Blank
Substrate: Potato starch solution [mL]	1.00	1.00	1.00	1.00
Enzyme solution [mL]	0.50	0.70	1.00	(-)
Colour reaction [mL]	1.00	1.00	1.00	1.00
2nd addition of enzyme [mL]	0.50	0.30	(-)	1.00
Purified water [mL]	9.00	9.00	9.00	9.00

Standard Curve to quantify the liberated maltose

A standard curve is established by diluting the maltose solution (0.2 % w/v) as followed in purified water

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

1 mL of colour reagent solution is added for each maltose concentration.

After this addition the maltose solutions are boiled in the water bath for 15 minutes, cooled on ice to room temperature and 9 mL of purified water is added.

Mix by inversion and record the A_{540} for the Standards and Standard Blank.

Calculations:

Standard Curve

ΔA_{540}Standard = A_{540} Std. - A_{540} Std. Blank

Plot the ΔA_{540nm} of the Standards *vs.* quantity of maltose [mg]. Establish the corresponding linear regression:

$$\Delta A540\ Standard = a \times [maltose] + b$$

Enzyme activity determination

ΔA_{540} Sample = A_{540} Test - A_{540} Test Blank

$$\frac{Units}{mg\ powder} = \frac{[\ (A540\ Test\ -\ A540\ Test\ Blank) - b]}{(a\ \times\ X\ )}$$

a: slope of the linear regression established for ΔA_{540nm} of the Standards *vs.* quantity of Maltose [mg]

b: intercept of the linear regression established for ΔA_{540nm} of the Standards *vs.* 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.* [2, 3]

Method: Spectrophotometric Stop Reaction

Principle:

Haemoglobin + H_2O $\xrightarrow{pepsin}$ 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

Protocol

Preparation of reagents

Substrate: a 2 % w/v haemoglobin (bovine blood haemoglobin, ref H2500 Sigma-Aldrich) solution is prepared by dispersing 0.5 g haemoglobin in 20 mL purified water and then diluting this solution in 5 mL of 300 mM HCl in order to get a solution at 2 % w/v haemoglobin at pH 2.

Enzyme: A stock solution of Pepsin (porcine pepsin, ref P6887 Sigma-Aldrich) is dissolved in 150 mM NaCl adjusted to pH 6.5 using 100 mM NaOH. 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, we suggest for instance to dilute the pepsin at: 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 and incubate in shaking incubator at 37°C for 3-4 minutes to achieve temperature equilibration.

Add 100 µl of pepsin solutions for each concentration which is tested and incubate for 10 minutes exactly. To stop the reaction, 1 mL of TCA (5% w/v trichloroacetic acid) is added in each tube. In order to get a clear soluble phase available for absorbance measurement, a centrifugation step is useful. Place eppendorf in a centrifuge at 6,000 g during 30 minutes to precipitate haemoglobin.

Remove the soluble phase and place it in 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.

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

Calculations:

$$Units/mg = \frac{[\,A280\ Test - A280\ Blank] \times 1000}{(\Delta t \times\ X)}$$

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

X: concentration of pepsin powder in the final reaction mixture (quartz cuvette) [mg/mL]

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 evolution of pepsin concentration.

Trypsin Activity Assay (EC 3.4.21.4)

References: adapted from Hummel [4] and also advised by the Worthington laboratory

Method: Continuous Spectrophotometric Rate Determination

Principle:

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

With TAME: p-Toluene-Sulfonyl-L-arginine methyl ester

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

Conversion of unit: 1 TAME Unit = 19.2 USP/NF Units = 57.5 BAEE Units

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

Protocol

Preparation of reagents

Substrate: TAME (p-Toluene-Sulfonyl-L-arginine methyl ester) (ref T4626 Sigma-Aldrich) at 10 mM is prepared and dissolved in purified water.

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

Prepare a 46 mM TRIS/HCl buffer, containing 11.5 mM $CaCl_2$ adjust its pH at 8.1 at 25°C.

Assay:

Set the spectrophotometer at 247 nm and 25°C.

Pipette into quartz cuvettes a mix of 2.6 mL of 46 mM Tris/HCl buffer (pH 8.1) and 0.3 mL of the substrate (10 mM TAME), mix by inversion and incubate in spectrophotometer at 25°C for 3-4 minutes to achieve temperature equilibration.

Test: Then add 100 µl of trypsin solutions for each concentration which is tested and record the absorbance increase at 247 nm, noted ΔA_{247}, during 10 min in continuum until levelling off. Determine the slope ΔA_{247} Test from the initial linear portion of the curve. If no linear part is found repeat the test using less enzyme.

Blank: For blank assays, the protocol is similar but no enzyme is added and the absorbance is similarly recorded, however it stabilises quicker (within 5 min). ΔA_{256} Blank is obtained, it should be close to zero.

Calculations:

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

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

ΔA_{247}: slope of the initial linear portion of the curve, [unit absorbance/minute] for the Test (with enzyme) and Blank

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 same activity is obtained for the two tested concentrations.

Chymotrypsin activity assay (EC 3.4.21.1)

References: adapted from Hummel[4] and Wirnt[5] **Method:** Continuous Spectrophotometric Rate Determination

Principle:

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

With BTEE: N-Benzoyl-L-Tyrosine Ethyl Ester

Unit Definition: One unit of chymotrypsin hydrolyses 1.0 µmole of BTEE per minute at pH 7.8 at 25ºC.

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

Protocol:

Preparation of reagents:

Enzyme: Prepare 1 mM HCl to dissolve the enzyme. Chymotrypsin (bovine chymotrypsin, ref C7762 Sigma-Aldrich) is dissolved at least at 2 concentrations ranging between 10-30 µg/mL in 1 mM HCl.

Prepare a 80 mM Tris/HCl buffer, containing 100 mM calcium chloride. Adjust pH at pH=7.8 at 25°C.

Substrate: The substrate BTEE (N-Benzoyl-L-Tyrosine Ethyl Ester) (ref B6125 Sigma-Aldrich) is dissolved at a concentration of 1.18 mM in methanol/purified water as follows: Weigh 18.5 mg of BTEE, dilute it in 31.7 mL of absolute methanol and complete to 50 mL with deionized water in a 50 mL Class A volumetric flask. Invert the flask several times to ensure complete mixing.

Assay:

Set the spectrophotometer at 256 nm and 25°C.

Pipette into quartz cuvettes a mix of 1.5 mL of Tris/HCl buffer and 1.4 mL of substrate (BTEE), mix by inversion and incubate in spectrophotometer at 25°C for 3-4 minutes to achieve temperature equilibration.

Test: Then add 100 µl of chymotrypsin and record the absorbance increase at 256 nm during 10 min in continuum until levelling off. Determine the slope ΔA_{256} Test from the initial linear portion of the curve. If no linear part is found repeat the test using less enzyme.

Blank: For blank assays, the protocol is similar but no enzyme is added and the absorbance is similarly recorded but get stable very quickly (within 5 min). ΔA_{256} Blank is obtained, it should be close to zero.

Calculations:

Obtain the slopes ΔA_{256} [unit absorbance/minute] for both the blank and test reactions using the maximum linear rate and over at least 5 minutes:

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

Δ A_{256}: slope of the initial linear portion of the curve, [unit absorbance/minute] for the Test (with enzyme) and Blank.

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

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

Check that the same activity is obtained for the two tested concentrations.

Pancreatic Lipase activity assay (EC 3.1.13)

References: Carrière and co-workers[6-8] and Erlanson & Borgström [9] **Method:** pH-stat titration

Principle:

Tributyrin + H_2O pancreatic lipase and cofactor: colipase> butyric acid + *sn*-2 monobutyrin

The pancreatic lipase activity assays can be conducted using the pH-stat technique using tributyrin as substrate. The free fatty acids released by the lipase are titrated at a constant pH by sodium hydroxide (NaOH, 0.1N) during the course of the hydrolysis.

Unit definition: The pancreatic lipase activity must be expressed in international units (U) with 1 U = 1 µmol butyric acid released per minute at 37°C and at pH 8.0; specific activity of pure Human Pancreatic Lipase is 8,000 U/mg protein on tributyrin[7].

Conditions: The titration is performed at 37°C and at pH 8.0, a pH value at which gastric lipase is not active and carboxyl ester lipase is poorly active.

Protocol

Preparation of reagents:

Assay solution: Prepare 200 mL of the following aqueous solution by dissolving Tris-(hydroxymethyl)-aminomethane, electrolytes (NaCl, $CaCl_2$) and biliary salts (Sodium taurodeoxycholate) in purified water according to the following proportions:

	Concentration [mg/L]	Corresponding weight [mg] for 200 mL
Tris-(hydroxymethyl)-aminomethane	36	7.20
NaCl	9000	1800
$CaCl_2$	200	40
Sodium taurodeoxycholate	2080	420

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

Enzyme: Dissolve 5 mg of enzyme powder in 5 mL of purified water. Store on ice. At least 2 amounts of enzyme solution (1 mg/mL) have to be tested.

Assay:

Switch on a thermo-regulated pHstat device, such as Tritino or Dosino from Metrohm, fitted with a jacketed and capped reaction vessel and mechanical stirrer.

Blank: Pour into the titration vessel 15 mL of the aqueous phase and add 0,5 mL of tributyrin. Make sure the volume of the assay is high enough to ensure adequate pH-measurement (microelectrode should be correctly immersed). Switch on magnetic stirring (tributyrin will get dispersed) and let equilibrate at 37°C.

Monitor the rate of titrant solution (NaOH) delivery which is required to maintain a pH of 8 at 37°C.

Test: Pour into the titration vessel 14.5 mL of the assay solution and add 0.5 mL of tributyrin. Ensure to use the correct flask to ensure adequate pH-measurement (microelectrode should be correctly immersed). Switch on mechanical stirrer (tributyrin will be dispersed) and let equilibrate at 37°C.

Add 10 to 100 µL of a diluted solution of pancreatic lipase containing colipase. If the lipase powder does not contain colipase make sure colipase is added in functional excess (colipase/lipase molar ratio 2:1) before adding the enzyme to allow measuring maximum activity. Monitor the rate of titrant solution (NaOH) delivery, which is required to compensate for the liberation of free fatty acids and maintain a pH of 8 at 37°C. These conditions allow measuring linear kinetics of free fatty release for at least 5 minutes.

Calculations:

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

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

v: volume [µL] of enzyme solution added in the pHstat vessel

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

Check that the same activity (U/mg) is obtained for the both tested enzyme amounts.

Pancreatin activity

To measure the trypsin and chymotrypsin activity of the pancreatin (porcine pancreatin (8 x USP specifications, ref P7545 Sigma-Aldrich), the protocols are the same as described here above. Pancreatin is dissolved in 1 mM HCl (pH 3). The dissolution of pancreatin is difficult. After dissolution, keep on mixing during 10 minutes using a magnetic stirrer and then store the pancreatin solution on ice or at refrigerated temperature 4°C. Dilute the enzyme to a concentration ranging between 0.1 to 1 mg/mL and measure at least 3 different dilutions of the pancreatin in this range. Just before adding the enzyme to the reaction vessel, vortex pancreatin a few seconds. For measuring the lipase activity in pancreatin, it is recommended to dissolve in 150 mM NaCl and adjust pH at 6.8 (pancreatic lipase is degraded at low pH), and follow the above procedure[10].

Bile salts assays

The concentration of bile salts in the bile (fresh or commercial) is tested using a commercial kit (bile acid kit, ref 1 2212 99 90 313, DiaSys Diagnostic System GmbH, Germany or similar). Follow the supplier's protocol. Prepare and dilute the bile at different concentrations. Check that the concentration of bile salts measured is less than 150 µmol/L in your bile solution, otherwise dilute the bile to be in this range.

References

1. P. Bernfeld, in *Methods Enzymol.*, Academic Press, 1955, vol. Volume 1, pp. 149-158.
2. M. L. Anson, *The Journal of General Physiology*, 1938, **22**, 79-89.
3. M. L. Anson and A. E. Mirsky, *The Journal of General Physiology*, 1932, **16**, 59-63.
4. B. C. Hummel, *Canadian Journal of Biochemistry and Physiology*, 1959, **37**, 1393-1399.
5. R. Wirnt, in *Methods of Enzymatic Analysis*, Academic Press, New York, 1974, pp. 1006-1012.
6. J.-C. B. Bakala N'Goma, S. Amara, K. Dridi, V. Jannin and F. Carrière, *Therapeutic Delivery*, 2012, **3**, 105-124.
7. F. Carrière, J. Barrowman, R. Verger and R. Laugier, *Gastroenterology*, 1993, **105**, 876-888.
8. E. Ville, F. Carrière, C. Renou, R. Laugier and eacute, *Digestion*, 2002, **65**, 73-81.
9. C. Erlanson and B. Borgström, *Scand. J. Gastroenterol.*, 1970, **5**, 293.
10. P. Capolino, C. Guérin, J. Paume, J. Giallo, J.-M. Ballester, J.-F. Cavalier and F. Carrière, *Food Digestion*, 2011, **2**, 43-51.