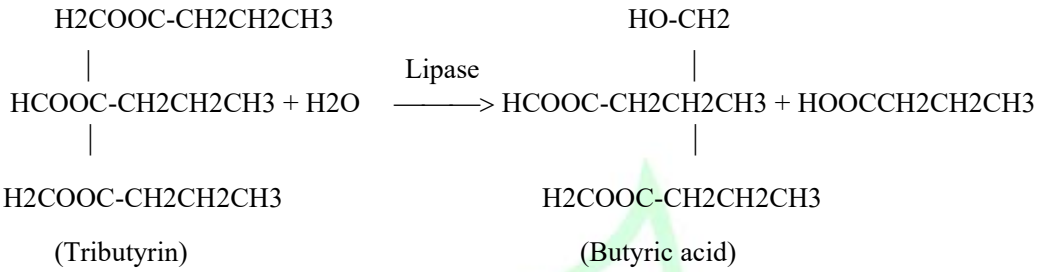
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脂肪酶来源于米曲霉

Principle:

The method is based on the rate at which the enzyme hydrolyzes tributyrin at pH 7.0 to form butyric acid. The butyric acid is titrated with hydroxide, and the consumption of the latter is rec orded as a function of time.



(Tributyrin)　　(Butyric acid)

Reaction conditions

Parameter	Reaction conditions
Temperature	30±1℃
pH-stat titration	pH 7.00
Substrate	0.16M tributyrin(glycerol tributyrate)at start of titration
Reaction time	At least 1.5 minutes Only the linear response from 1.3-2.3 minutes is used to calculate the slope

Definition of unit

1 LU is the amount of enzyme which releases 1 μ mol of titratable butyric acid per minute under the given standard conditions.

Method parameters

Intermediate precision

The intermediate precision (CV% of a single determination (KLU, KLU(LEX), KLU(LPAX), LU-MM, LU-CA) is 3.9%.

Specificity

Any enzyme with esterase activity will add to the measured lipase activity. Any protease in the sample will break down lipase and so reduce the measured lipase activity.

Presence of detergents: The method is strongly influenced by the presence of detergents, with the influence varying from total inhibition to activation depending on the type of detergent and the concentration.

Glassware: It is important that enzyme solutions are handled in glassware as the enzyme adheres to plastic. Enzymes are diluted in glass and the reaction must take place in a glass titration vessel.

The enzyme can be pipetted into the titration vessel using plastic pipette tips provided the delay is not too long.

Range

The analytical range is 1.5–4.0 LU/ml.

Limit of determination

Name	Liquid	Solid
KLU, KLU(LEX), KLU(LPAX)	0.020 KLU/g,which is equivalent to 2.5 g of sample dissolved in 10 ml and further diluted 25 times	0.050 KLU/g,which is equivalent to 1.0 g of sample dissolved in 10 ml and further diluted 25 times
LU-Ca and LU-MM	1 LU-Ca/g,which is equivalent to 2.5g of sample dissolved in 10 ml	2 LU-MM/g,which is equivalent to 1.0g of sample dissolved in 10ml

Equipment

	Equipment
pH-stat titration system	Suggestions: Metrohm titrator Titralab TIM854 titration workstation IMPORTANT:It is critical that the pH electrode is suitable (responds quickly to changes in pH)for pH-stat titration and robust,and that the dispensing of sample and especially titrant(NaOH)is accurate.It is also critical that the titration vessel is made of glass and that efficient stir- ring(rod stirrer with propeller rather than magnets)is integrated.
Emulsifier	Silverson,model L4RT(critical equipment)
Thermostatic water bath able to control temperature to±1℃, thermometer	E.g.,LAUDA MS,Lauda RE304,Haake
Automatic pipettes	-

Reagents

1 M NaOH

Example: Preparation of 1 L

Step	Action
1	Transfer the titrisol solution(1M NaOH)to a 1000-ml graduated flask
2	Fill to the mark with demineralized water
3	Storability:Max.9 days at room temperature

CAUTION: Corrosive

0.025 M degassed CO2-free NaOH (titrant)

Example: Preparation of 1 L

Step	Action
1	Transfer 25.00 ml of 1M NaOH to a 1000-ml graduated flask
2	Fill to the mark with demineralized water
3	Degas the final solution. Do not decant the solution after degassing,and make sure that it is installed tight- ly in the titration equipment
4	Storability:Max.9 days after opening in a tightly sealed bottle joined to the bu- rette at room temperature

0.005M NaOH(for rinsing titration vessel and tubing)

Example:Preparation of 5L

Step	Action
1	Transfer 25 ml of 1M NaOH to a 5000-ml container
2	Fill to the mark with demineralized water
3	Storability:Max.6 months at room temperature

Gum arabic emulsifier (0.6% w/v)

Example: Preparation of 3 L

Step	Action
1	Add 180 ml of demineralized water to a 400-ml beaker
2	Place the beaker on a stirrer with a 4-cm magnetic stirring bar
3	Start the stirrer at high speed and gently pour 18.0±0.1 g of gum arabic in quanti-tatively,rinsing the weighing beaker with demineralized water.Make sure that it does not clot and leave it stirring
4	Weigh out 53.7 g of NaCl±0.1g and transfer quantitatively to a 3-L graduated flask
5	Weigh out 1.20±0.05 g of $KH_2PO_4$and transfer quantitatively to the same flask.
6	Add demineralized water to approx.350 ml
7	Leave to stir until completely dissolved

8	Measure out 1620 ml of 87%glycerol(for 85%,measure 1650 ml+60 ml)at room temperature and transfer to the same flask.Rinse with demineralized water as thoroughly as possible
9	Remove the gum arabic from the stirrer and let it aspirate.Make sure that there are no clots
10	Add the gum arabic solution to the 3-L graduated flask
11	Stir until completely dissolved
12	Adjust the pH to 4.5±0.05 using HCl or NaOH
13	Fillup to 3 L with demineralized water
14	Storability:Max.9 days after preparation stored at room temperature

Tributyrin reagent (0.17 M tributyrin, emulsion)

Example: Preparation of 1200 ml (max. allowed volume for one preparation – otherwise homog enization is inefficient)

Step	Action
1	Weigh out 62.5±0.1 g of glycerol tributyrate(as density is 1032 g/ml and purity 99%)directly into the emulsifier container
2	Measure out 200 ml of gum arabic emulsifier at room temperature and transfer to the same container.Rinse the weighing container with water,adding a total of 940 ml of demineralized water
3	Homogenize the mixture for 3 min using the Silverson emulsifier L4RT on the set- ting 7000 rpm,starting timing when the emulsifier reading has reached 7000 rpm Emulsion stirring speed and time are critical factors
4	Leave to stir for 20 min using an ordinary magnetic stirrer
5	Adjust the pH to 4.75±0.05 using HCl or NaOH(if an automated titrator,e.g., Metrohm,is used,this is not necessary)
6	Storability:Max.1 day at room temperature(prepare fresh each day)

0.1 M glycine buffer

Example: Preparation of 5 L

Step	Action
1	Weigh out 37.54g of glycine buffer and transfer to a 5000-ml graduated flask
2	Weigh out 18.5 g of NaOH and transfer to the same volumetric flask
3	Fill with demineralized water to 4500 ml
4	Stir until completely dissolved
5	Adjust the pH to 10.8±0.05
6	Fill to the mark with demineralized water
7	Storability:Max.31 days at room temperature

0.01M glycine buffer

Example:Preparation of 1 L

Step	Action

1	Measure 100 ml of 0.1 M glycine buffer into a graduated flask
2	Transfer to a 1000-ml graduated flask
3	Fill with demineralized water to 950 ml
4	Stir for at least 5 minutes
5	Adjust the pH to 10.8±0.05
6	Fillto the mark with demineralized water
7	Storability:Prepare fresh each day;store at room temperature

Standard

The standard is available upon request

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Stock solution 1:
Weigh out an amount of enzyme standard corresponding to 50012 LU units into a glass weighing boat</td></tr>
<tr><td>2</td><td>Dissolve the standard in 0.1M glycine buffer in a 50-ml measuring flask</td></tr>
<tr><td>3</td><td>Stir the solution for at least 15 minutes.
Storability:24 hours at room temperature</td></tr>
<tr><td>4</td><td>Stock solution 2:
Transfer 2.0 ml of stock solution 1 to a 100-ml measuring flask using an automatic pipette with plastic tip.Fill with demineralized water.
Stir for approx.5 minutes</td></tr>
<tr><td>5</td><td>Working solutions:
The standard solutions are prepared by diluting stock solution 2 with demineral- ized water according to this table:
<table>
<tr><th>Std no.</th><th>LU/ml</th><th>ml stock solution 2</th><th>Demineralized water up to(ml)</th></tr>
<tr><td>1</td><td>0.2000</td><td>0.250</td><td>25</td></tr>
<tr><td>2</td><td>0.5001</td><td>0.625</td><td>25</td></tr>
<tr><td>3</td><td>1.000</td><td>1.25</td><td>25</td></tr>
<tr><td>4</td><td>2.001</td><td>2.50</td><td>25</td></tr>
<tr><td>5</td><td>3.001</td><td>3.75</td><td>25</td></tr>
<tr><td>6</td><td>4.001</td><td>5.00</td><td>25</td></tr>
</table>
Stir for approx.5 minutes
NOTE:Automatic pipettes with plastic tips are used for all dilutions.
Stability:24 hours at room temperature</td></tr>
</table>

QC sample

The QC sample is available upon request.

Prepare a QC sample with known enzyme content in the same way as for the samples below.

Samples

Dissolve max. 2.5 g (liquid) or 1.0 g (granulated) sample into min. 10 ml of buffer regardless of the unit (KLU(LEX), KLU(LPAX), LU-Ca, LU-MM, or KLU).

Different lipases require special sample preparation to give the correct results, either to activate the enzyme, to protect it from inactivation, or to release the enzyme from the sample matrix.

Step	Action
1	Weigh out approx.1 g of sample precisely and dissolve in a 50-ml measuring flask. Liquid samples are weighed directly into the flask.Dry samples are weighed directly into the flask or into a glass weighing boat and then transferred to the flask
2	Dilute the sample in a measuring flask with the buffer specified below for each lipase. IMPORTANT:Samples requested as KLU(LEX)are dissolved in a max.volume given that the second dilution must be at least 25times as for other samples
3	Stir for at least 15 minutes. Storability:KLU,KLU(LEX),and KLU(LPAX):24 hours at room temperature.LU-MM and LU-CA:should be analyzed as soon as possible
4	The samples are further diluted using the buffer specified below for each lipase using an automatic pipette with plastic tip.KLU,KLU(LEX),and KLU(LPAX)samples should as a minimum be diluted 1:25 for the second dilution.There is no minimum second dilution for LU-CA and LU-MM. Stir before use. NOTE:If possible,the activity of the final dilution should be approx.2.5 LU/ml for all analyses. Storability:KLU,KLU(LEX),and KLU(LPAX):24 hours at room temperature.LU-MM and LU-CA:Should be analyzed as soon as possible

Procedure

Preparation of the system

The titration vessel and tubing are rinsed through in the following order:

Ethanol (96% or 99%)

An appropriate soap solution (e.g., 1% RBS)

Hot water

Demineralized water

Fresh substrate

The pH electrode is calibrated. The sensitivity of the pH electrode is checked with buffer solution pH 4 and 7 once a day just before the first run and must be between 95% and 102%.

If this is not the case, the pH electrode is rinsed in accordance with the manufacturer’s instruc tions and recalibrated.

Analysis

The pH electrode is preserved in saturated KCl or Metrohm no. 6.2323.000 overnight to prevent drift when starting analyses.

N2 is blown over the reaction solution to eliminate CO2 uptake from the air. It is important to ensure that the temperature of the substrate is 30　1　C before and during the analysis. It is best kept this way by heating the substrate by passing it through a coil placed in a thermostatic water bath during analysis.

The titration vessel and sample tubing are rinsed with 0.005 M NaOH in between each sample.

Step	Action
1	15 ml of tributyrin reagent(emulsion)is poured into the titration vessel.The pH of the sample solution must be lower than 7.0 as the titration starts.Before adding the sample,the substrate must be titrated to just below 7.0
2	1.0 ml of sample solution is added to the titration vessel.The pH is maintained at pH 7.0 during titration.The amount of titrant added per min to maintain a con- stant pH is measured
3	The mean slope of the linear range of the titration curve is printed from the titra- tor (if different equipment is used,the data may be transferred in another way). The reaction must give a linear output for at least 1.5 minutes for the results to be used(only the linear response from 1.3-2.3 min is used to calculate the slope)
4	A blind sample(demineralized water)is analyzed,the standard curve is analyzed (one tube for each standard),and the level control is analyzed at the beginning of each run just after the standard.The samples(one tube per sample)are then ana- lyzed. It is important that the samples are produced in runs and are not analyzed using the same standard curve during the whole day.In other words,the size of the run has to be known from the beginning.Max.run size is 92 samples,excluding the blind sample,the standard curve samples,and the QC sample.If the samples are analyzed later on the same day,the standard solutions must be reanalyzed
5	After the last sample has been analyzed,the system is rinsed: 1.As in between each sample. 2.Substrate container and tubings are emptied and rinsed with ethanol. 3.If the system is not in use for more than approx.1 week,all sensitive parts containing NaOH or HCl are rinsed with demineralized water to avoid pre- cipitations of salts

Calculation

Step	Action
1	The activity of the enzyme samples is determined relative to the standard curve
2	On the basis of the result in ml of titrant added per min for the six standard dilu- tions,a standard curve is drawn with the activities of the standards in LU/ml as the x- values and the associated mean slope(ml/min)of the standards as the y-values.The standard curve fit should be linear

3	The enzyme activity of the diluted samples is read from the standard curve.The ac- tivity of a sample in KLU/g is calculated using the formula:
	$KLU/g = \frac{S \cdot V \cdot F}{W \cdot 1000}$ S = Reading from the standard curve in LU/ml V = Volume of the measuring flask in ml F = Dilution factor for first and second dilutions W = Weight of sample in g or ml 1000 = Conversion factor LU to KLU
	If the results are given in LU/g or LU/ml,the calculation factor is omitted from the formula

Approval of analytical run

Standard curve:

Parameter	**Requirement**
Limits for upper and lower y-axis measurements	Std 1:<0.02 ml/minutes Std 6:0.14-0.18 ml/minutes
Quality of fit(lower r^2limit)	0.9945
Curve appearance	The standard curve fit should be linear

Approval of titration curve for standards,QC sample,and samples:

Parameter	Requirement
Quality of fit(lower r^2limit)	≥0.9995 for values in the range of standards 3-6. For values in the range of standards 1-2,r^2≥0.990 is acceptable If the correlation coefficient is less than stated above,the sys- tem must be investigated for defects

QC sample:

The measured activity of the QC sample must be the declared value +/- 2 standard deviations.

Samples:

The analytical result (= average of two weighings on two different standard curves) must have CV ≤ 7.0%.

Statement of analysis results

The results are stated with three significant digits.

Results < limit of determination for KLU, KLU(LEX), and KLU(LPAX) are given as < 0.02 KLU/g for liquid samples and 0.05 KLU/g for solid samples.

Results < limit of determination for LU-CA and LU-MM are given as < 1 LU/g for liquid samples and < 2 LU/g for solid samples.

Configurations

The parameters of titration:

Parameter	**Value**
Titrant conc.	1.0000 ml/minute
Result unit	ml/minute
Set point	7.000 pH
Result based on	Slope
Calculation factor	1.0000
Blank value	0.0000
Calc.	Start:1.3 End:2.3 minutes
Temp.	30℃
Speed of stirring propeller	10

Handling of enzymes and chemicals

Enzymes and enzyme solutions should be handled in a fume hood or in closed containers.

Avoid inappropriate handling of enzymes and enzyme solutions, which may result in aero sol/dust generation.

Avoid inhalation of dust aerosols and contact with skin and eyes.

Handling of chemicals and disposal of waste must be performed according to valid procedures.