Determination of protease activity, AU(A) – analysis by Konelab

Scope

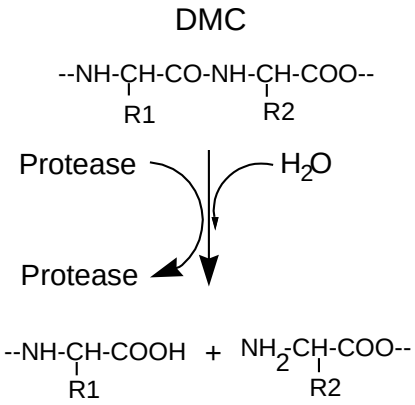
Novozymes Enzyme QC laboratories involved in analysis of samples from Novozymes production.

Application

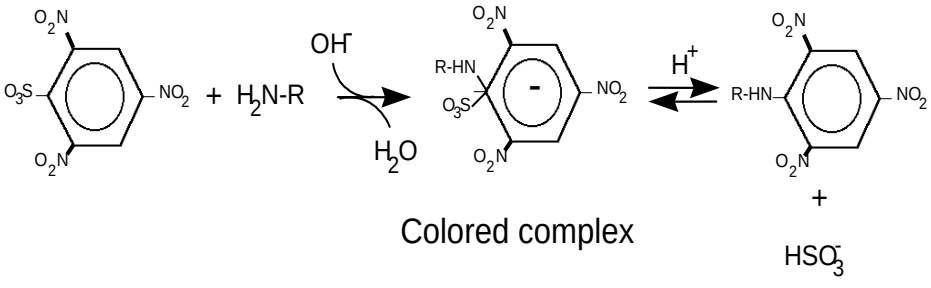
The method is used to determine the protease activity in samples.

Principle

Dimethyl casein (DMC) is hydrolyzed by the proteolytic enzyme to small peptides as shown below:



The primary amino groups formed in this process react with trinitrobenzene sulfonic acid (TNBS), forming a colored complex as shown below:



This color development is monitored by the Konelab, so the change in absorption per time unit can be calculated. This increase in absorption is proportional to the reaction rate, and thus to the enzyme activity.

Reaction conditions	
Temperature	50°C
pH	8.3
Substrate concentration	0.25%
Enzyme concentration	0.006–0.017 mAU(A)/ml
Reaction time	8 minutes
Measuring time	2 minutes (seven measurements with an interval of 18 seconds)
Wavelength	405 nm

Definition of unit

The activity is determined relative to a protease A standard. The result is given in the same units as the standard, which is designated:
AU(A) – (**A**nson **U**nit (**A** standard).

Method parameters

Specificity

Serine endopeptidases with activity at pH 8.3 will be measured. Primary amines will interfere.

Range

The range is 0.072–0.216 mAU(A)/ml in the final dilution.

Limit of determination

The limit of determination is 0.001 AU(A)/g (for a minimum preparation of 1 g liquid sample in 10 ml).

The limit of determination is 0.002 AU(A)/g (for a minimum preparation of 1 g solid sample in 25 ml).

Accuracy and intermediate precision:

The accuracy is 99%.
The intermediate precision is 4%.

Equipment

Equipment	
Konelab 30 Analyzer	Thermo Fisher Scientific *NOTE:* Critical equipment
Diluter	e.g., Hamilton
Analytical balance	e.g., Sartorius, Mettler
Balance	e.g., Sartorius
pH meter	e.g., Radiometer, Metrohm
Thermometer (0–100°C)	-
Magnetic stirrer plates	-

Chemicals

Name	Chemical formula	Brand	Working environment guideline
Brij 35 (30% w/v)	-	e.g., Sigma B4184	Harmful
N,N-dimethyl casein (DMC)	-		-
Sodium tetraborate decahydrate	$Na_2B_4O_7$, 10 H_2O	e.g., Merck art. no. 6308	Avoid contact with eyes and skin
Sodium dihydrogen phosphate monohydrate	NaH_2PO_4, H_2O	e.g., Merck art. no. 6346	-
Sodium sulfite	Na_2SO_3	e.g., Merck art. no. 6657	-
2,4,6-trinitrobenzene sulfonic acid (TNBS)	$C_6H_3N_3O_9S$	e.g., Fluka BioChemica art no. 92822 1 M solution	Toxic, explosive in dry form, corrosive

NOTE: Always check out the Material Safety Data Sheet (MSDS) for the chemicals marked with an environment guideline.

Reagents/substrates

Preparation of reagents and substrates must be documented according to valid procedures.

Brij 35 solution 15% w/v

EXAMPLE: Preparation of 2,000 ml Brij 35 15%:

Step	Action
1	Transfer 1,000 ml Brij 35 30% w/v to a 2,000-ml measuring flask. Brij 35 30% can be heated to 35–45°C
2	Rinse the Brij flask with demineralized water and transfer the water to the 2,000-ml flask
3	Fill up to 2,000 ml with demineralized water and mix vigorously
4	Brij 35 solution can be stored for 2 months in a refrigerator

TNBS, 2,4,6-trinitrobenzene sulfonic acid solution 0.1% w/v

EXAMPLE: Preparation of 100 ml TNBS 0.1%:

Step	Action
1	Pipette 0.345 ml 1 M TNBS into a 100-ml measuring flask
2	Fill up to 100 ml with demineralized water and mix
3	Measure pH. pH is approx. 2.5 ± 0.3 in a TNBS solution 0.1%
4	TNBS solution must be prepared every day. *NOTE:* The TNBS solution is stored in a refrigerator, shielded against light (brown bottle or aluminum foil)

IMPORTANT: In dry form, TNBS is classified as an explosive, and it is very important to prevent TNBS from drying out. Rinse empty TNBS bottles with water before disposal. Keep waste containers with cuvettes closed in order to prevent the TNBS in these from drying out. Do not store these waste containers for too long.

DMC, N,N-dimethyl casein solution 0.32%

EXAMPLE: Preparation of 1,000 ml DMC solution 0.32%:

Step	Action
1	Dissolve 3.20 g N,N-dimethyl casein (DMC) in approx. 200 ml boiling demineralized water in a beaker. Keep the beaker with the DMC on the heat while stirring for 20 minutes. Keep the temperature close to boiling point
2	Dissolve 25.92 g $Na_2B_4O_7$, 10 H_2O, and 13.30 g NaH_2PO_4, 1 H_2O in 400 ml demineralized water in a 1,000-ml measuring flask
3	After cooling, transfer the DMC solution to the 1,000-ml measuring flask
4	Fill up to 1,000 ml with demineralized water
5	Mix well and filter the solution twice through a Whatman filter no. 54 or a filter with similar pore size
6	Add 1.2 ml Brij 35 solution 15% and mix. Brij 35 is added after filtration to avoid formation of foam during filtration
7	Check the pH of the solution; pH must be 8.00 ± 0.05. Adjust if necessary with HCl or NaOH
8	Fill the substrate into smaller vessels and store for 24 hours in a refrigerator. Then freeze the substrate
9	The DMC can be stored for 1 year in a freezer and for 1 week in a refrigerator after thawing. *IMPORTANT:* When removing DMC solution 0.32% from a freezer, stir the solution after thawing until it is clear

Sodium sulfite stock solution 20% w/v

EXAMPLE: Preparation of 10 L sodium sulfite stock solution 20% w/v:

Step	Action
1	Dissolve 1,000 g Na_2SO_3 by vigorous agitation in a beaker that has first been filled with approx. 3.5–4 L demineralized water. Prepare this solution twice. *IMPORTANT:* Sprinkle Na_2SO_3 carefully into the water in the beaker while stirring
2	Stir until the sodium sulfite in the beakers has dissolved
3	Transfer the sodium sulfite solution in the two beakers to a 10-L measuring flask
4	Fill up to 10 L with demineralized water and mix
5	The stock solution can be stored at room temperature for 1 month in a closed vessel

Sodium sulfite solution 2%

EXAMPLE: Preparation of 10 L sodium sulfite solution 2%:

Step	Action
1	Measure out 1,000 ml sodium sulfite stock solution 20% and transfer it into a 10-L measuring flask containing approx. 8 L demineralized water
2	Add 15 ml Brij 35 15%
3	Fill up to 10 L with demineralized water and mix
4	Sodium sulfite solution 2% can be stored for 2 days at room temperature

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 aerosol/dust generation.

Avoid inhalation of dust aerosols and contact with skin and eyes.
Handling of chemicals and disposals of waste must be performed according to the MSDS for the product.

Standard

Protease A standard is available upon request.

Stock solution

Step	Action
1	Weigh out 1.08 AU-A of the protease A standard
2	Dissolve the standard in sodium sulfite solution 2% in a 500-ml measuring flask
3	Stir on a magnetic stirrer for 15 minutes
4	*NOTE:* The stability is set to 7 hours

Enzyme standards

Step	Action
1	The standard solutions are then prepared by diluting the stock solution with sodium sulfite solution 2% on the diluter directly into the sample cups. The standard curve is a 7-point curve with a factor 3 between lowest and highest standard points according to this table.

No.	Dilution factor	Enzyme stock solution µl	Diluent sodium sulfite 2% µl	Activity mAU(A)/ml
1	30	40	1,160	0.072
2	24	50	1,150	0.090
3	20	60	1,140	0.108
4	16	70	1,130	0.135
5	15	80	1,120	0.144
6	12	100	1,100	0.180
7	10	120	1,080	0.216

NOTE: Standard final solutions in sample cups are stable for 30 min after dilution

QC sample

The preparation of the QC sample must be documented according to valid procedures.

QC sample

QC sample for AU(A) is available upon request.

Step	Action
1	Weigh out 2.11 AU(A) of the QC sample
2	Dissolve the QC sample in sodium sulfite solution 2% in a 250-ml measuring flask
3	Stir on the magnetic stirrer for 15 minutes
4	Dilute the stock solution 60 times with sodium sulfite solution 2% on the diluter. *IMPORTANT:* The final solution in the sample cup is stable for 30 minutes after dilution

Samples

The lowest dilution of liquid samples: 10 times.
The lowest dilution of solid samples: 25 times.

Weighing and dilution of protease samples are described in the following:

Step	Action
1	Weigh out 0.5–1.5 g of sample
2	Dilute the sample in a measuring flask with sodium sulfite solution 2%
3	Stir for approx. 15 minutes on a magnetic stirrer
4	The samples are further diluted with sodium sulfite solution 2% on the diluter. The dilution on the diluter is made directly into the sample cup. *NOTE:* If possible, the activity in the final dilution should be approx. 0.144 mAU(A)/ml)
5	Each weighing is analyzed once on the Konelab. *IMPORTANT:* Sample final solutions in sample cups are stable for 30 minutes after dilution

Blank

Sodium sulfite solution 2% is used as a blank sample.

Procedure

Step	Action
1	Prepare all samples.
2	Place the reagents in the Konelab:
3	Place blank, standards, QC sample, and samples in the Konelab in the stated order. *NOTE:* 19 samples can be analyzed in one run
4	Start and run the Konelab

Reagent	Konelab name	Reagent container volume	Syringe speed	Stability on board the Konelab
DMC 0.32%	DMC	60	Normal	1 day
TNBS solution 0.1%	TNBS	20	Normal	4 hr

Configurations

The Konelab reagent and test definition for AU(A) is shown in appendix 1.

Calculation

Step	Action
1	The activity of the enzyme samples is determined relative to the standard curve
2	On the basis of the results in A/min for the six enzyme standards, a standard curve is drawn with the activities of the standards in mAU(A)/ml as the x-values and the A/min of the standards as the y-values. A logit/log4 algorithm is used
3	The enzyme activity of the diluted samples is read from the standard curve. Calculation of activity of a sample in AU(A)/g is performed as stated in the formula: $\text{Activity AU(A)/g} = \frac{S \cdot V \cdot F}{W \cdot 1000}$ S = Reading from the standard curve in mAU(A)/ml V = Volume of the measuring flask in ml F = Dilution factor for second dilution W = Weight of sample in g 1000 = Conversion factor from mAU(A) to AU(A) *EXAMPLE:* 0.5416 g sample is dissolved in a 250-ml measuring flask and further diluted 30 times using a diluter. On the Konelab an A/min of 0.2224 is measured. From the standard curve an activity of 0.1216 mAU(A)/ml is calculated . $Activity = \frac{0.1216 \cdot 250 \cdot 30}{0.5416 \cdot 1000} = 1.68\ AU(A)/g$

Approval

Standard curve:

Parameter	Requirement
Curve appearance	The standard curve must be a smooth hollow curve. The rates for the standards must be at the level that is normal for the protease A standard curve using the relevant DMC and TNBS batches
r^2	$\geq$ 0.995. If r^2 is < 0.995, one standard may be removed

Approval of QC sample:

Parameter	Requirement
QC sample	The result of the QC sample must not deviate from the control limits, which are set to: Declared value ± 8%.

Approval of samples:

Parameter	Requirement
Sample	CV of six weighings (= three weighings on two different standard curves) $\leq$ 7.2.

Statement of analysis results

The analysis result is stated with three significant digits.

Configurations

AU(A) test definition:

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Test definition              Konelab Arena 6.5                    Page:    1

AU-A                         Novozymes   S/N: S14 19 151
                             Enzym Kemisk Laboratorium
Date :   2006-09-13
Time :   15.52
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Full name          EB-SM-0218
Online Name        AU-A              Test In Use         YES
Test type          Photometric                           LOW    HIGH
                                     Test limit          0.00000 *      Abs/min
Result unit        Abs/min           Initial absorbance  0.000   *      A
Number of Decim.   5                 Dilution limit      *       *      Abs/min
                                     Secondary dil 1+    0.0     0.0
                                     Critical limit      *       *      Abs/min
                                     Reflex test limit   *       *      Abs/min
                                     Reflex test

Acceptance               Automatic   Reference class     LOW     HIGH   In Use
Dilution 1+              0.0

Sample type              Sample type 5 Correction factor  1.00
                                     Correction bias     0.00    Abs/min
                                     Temperature         50.0    °C

Calibration type         None
Factor                   1.00        Bias                      0.00

Bias correction in use   NO

Cd reduction             NO

Manual QC in Use         NO          Routine QC in Use          NO


Blank                    None
                                     Fixed cuvette

Reagent                  DMC         Volume (ul)               180
Disp. with               Extra       Add. Volume (ul)          50
Wash reagent             DMC
Reagent wash             Before dispense

Incubation                           Time (sec)                480

Reagent                  TNBS        Volume (ul)               36
Disp. with               Extra       Add. Volume (ul)          50
Wash reagent             TNBS
Reagent wash             Before dispense

Incubation                           Time (sec)                60

Sample                               Volume (ul)               18
Disp. with               Extra       Add. Volume (ul)          50
Dilution with            Water       Wash reagent              None

Incubation                           Time (sec)                360

Measurement              Kinetic
Wavelength (nm)          405 nm      Side wavel. (nm)          None
Curve type               Nonlinear
Nonlinearity limits
Conc. (Abs/min)          0.00000     Percent (%)               25
Time (sec)               120         Points and Intervals      7 / 18.0 (sec)
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Reagent definitions

DMC:

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Reagent definition          Konelab Arena 6.5                    Page:    1

                            Novozymes   S/N: S14 19 151
                            Enzym Kemisk Laboratorium


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Reagent                     DMC           Lot        Expiry date (dd.mm.yy)
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Stable on board (days)      1
Alarm limit (ml)            2.0

Information


Vial volume                 60 ml
Barcode id

Syringe speed               Normal
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TNBS:

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Reagent definition          Konelab Arena 6.5                    Page:    1

                            Novozymes   S/N: S14 19 151
                            Enzym Kemisk Laboratorium


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Reagent                     TNBS          Lot        Expiry date (dd.mm.yy)
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Stable on board (days)      1
Alarm limit (ml)            1.0

Information


Vial volume                 20 ml
Barcode id

Syringe speed               Normal
```

Validity

Valid from May 2010.

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