SYKAM
CHROMATOGRAPHY

STANDARD OPERATION PROCEDURE

Protein sample preparation by acidic hydrolysis with oxidation

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1. Version History

Modification correlated to last version should be indicated in document.

Date	Status / Version	Author	Modifications
18.03.2016	0.1	Jasper Hölzer	Created

2. Scope of Application

Sample preparation of proteins and peptides by acidic hydrolysation with previous oxidation (without tryptophan)

3. Description

This procedure describes the preparation of protein and peptide samples for the qualitative and quantitative determination of amino acids including methionine and cystine by oxidation and subsequent acidic hydroxylation.

For the analysis of the proteinogenic amino acids including methionine and cystine it is required to oxidize the sample before dissociating the polymer to single amino acids. During the oxidation disulphide bridges of cystine within the protein will be cracked and the oxygen sensitive amino acids methionine and cysteine will be transduced in their oxidized derivatives methionine-sulfone and cysteinic acid. Thereby a differentiation between cysteine and cystine concentration in the protein is not possible anymore.

When a hydrolysation is performed without forced oxidation it is nearly impossible to avoid oxidation processes of methionine and cystine which would cause miscalculations of the respective concentration. To achieve approximate results for these amino acids without a forced oxidation as described in this SOP it is important to reduce the oxygen concentration in the headspace of the hydrolysation vessel as good as possible. However, the quantitative determination of these amino acids is limited if using this method. Thus, if the exact quantification of methionine and cystine is not necessary, the simple acidic hydrolysation procedure can be used (see SOP: Protein sample preparation by acidic hydrolysis, Sykam QM-4159).

The oxidized proteins and peptides are digested completely by hydrolysing the peptide bond between two amino acids catalysed by hydrochloric acid.

For reliable quantitative results it is important that the sample preparation procedure is strictly complied regarding reaction times and temperatures.

Related to the acidic media used for hydrolysis, amino acids such as asparagine and glutamine will be transduced into the acid derivatives aspartic acid and glutamic acid.

Tryptophan is not stable to persist the acidic hydrolysis and will be decomposed completely during hydrolysation. If the concentration of tryptophan has to be determined it will be required to perform a basic hydrolysation (see SOP: Protein sample preparation by basic hydrolysis, Sykam QM-4161). On the other hand the basic hydrolysation will degrade all other amino acids.

If the duration between sample preparation and measurement is longer than a few hours the prepared sample should be store at 2-8 °C or colder.

4. Required Resources

4.1 Equipment

- hydrolysis vessels* or Pyrex glass bottles with screw cap, volume: ≥ 50 ml
- Pyrex glass bottles with screw cap, volume: 50 to 100 ml
- beakers*, volume: 250 ml
- volumetric glass flasks*, volume: 200 ml
- three volumetric glass flasks, volume: 1000 ml
- two plastic bottle for chemicals, volume: 1000 ml
- dash bottle, volume: 1000 ml
- adjustable micro pipets (e.g. Eppendorf pipets), maximum volumes 1000 µl, 200 µl and if internal standard should be used 10 µl
- oven, block thermostat, microwave oven or similar heating device for hydrolysis vessels
- pH meter incl. pH probe and temperature sensor
- magnetic stirrer
- PTFE coated magnetic stir bar
- PTFE coated stir bar catcher
- vortex stirrer for micro centrifuge tubes and vials
- centrifuge** for micro centrifuge tubes, volume: 1.5 ml, rotation speed: ≥14.000 rpm
- refrigerator at 2-8 °C or similar cooling device

4.2 Chemicals

- deionized water or higher grade (conductivity ≤ 0.5 µS)
- hydrochloric acid, (e.g. 32 w/v%), analysis grade or higher
- phenol, analysis grade or higher
- hydrogen peroxide solution 30 w/v%
- sodium pyrosulfite (Na2S2O5), analysis grade or higher
- sodium hydroxide, analysis grade or higher
- amino acids used as internal standard (ISTD) (e.g. D,L-Norleucine, CAS: 616-06-8)

4.3 Consumables

- disposable syringe, volume: ≥ 5 ml
- disposable syringe filter, pore size: ≤ 0.45 µm
- tips for micro pipets, in required sizes
- sample vials, glass, screw cap with perforated Silicon/PTFE septum (Sykam Cat. No.: S000212)
- Sykam Sample Dilution Buffer for hydrolysate samples (SDB/Na) (Sykam Cat. No.: S000020)
- Amino acid standard mix for oxidized hydrolysis
  - Sykam Standard Type H-Ox: 20 amino acids (concentration in µmol/ml)
    Standard for oxidized hydrolysates (Sykam Cat. No.: S000030)

* one per sample which are prepared simultaneously

** if alternative sample post preparation procedure is used

5. Procedure

5.1 Pre-preparation:

1. Oxidation Solution 1

   Prepare a solution of 23 M formic acid with an addition of 0.55 w/v% phenol. Therefore fill 1068 g formic acid (100 v/v%, 1.2 kg/l, MW_{HCOOH} 46.03 g/mol) and 5.7 g phenol in a 1 litre volumetric flask and fill up to 1 litre with deionized water.

2. Oxidation Solution 2

   Note: this solution must be prepared freshly for usage!
   Prepare a mixture of 90 v/v% of Oxidation Solution 1 with 10 v/v % of hydrogen peroxide solution with a concentration of 30 w/v% in a Pyrex bottle of 50 to 100 ml. For each sample preparation 5 ml of this solution are required (e.g. for two samples mix 9 ml of Oxidation Solution 1 with 1 ml of hydrogen peroxide solution).
   This mixture must rest for 30 minutes at room temperature before usage!

3. Acidic Hydrolysis Solution

   Prepare a solution of 6 M hydrochloric acid with an addition of 0.1 w/v% Phenol. Therefore fill 590 ml hydrochloric acid (32 w/v%, 1.16 kg/l, MW_{HCl} 36.46 g/mol) and 1 g Phenol in a 1 litre volumetric flask and fill up to 1 litre with deionized water.

4. Basic Neutralization Solution 1

   Prepare a solution of 7.5 M sodium hydroxide in water. Therefore weigh in 300 g sodium hydroxide (MW_{NaOH} 40.00 g/mol) in a 1 litre volumetric flask and add about 800 ml deionized water. As soon as the solution reaches room temperature again fill up to 1 litre with deionized water. Transfer this solution in a 1 litre plastic bottle.

Note: the plastic bottle must be resistible against highly basic solutions!

5. Basic Neutralization Solution 2

   Prepare a solution of 1 M sodium hydroxide in water. Therefore weigh in 40 g sodium hydroxide (MW_{NaOH} 40.00 g/mol) in a 1 litre volumetric flask and add about 500 ml deionized water. As soon as the solution reaches room temperature fill up to 1 litre with deionized water. Transfer this solution in a 1 litre plastic bottle.

Note: the plastic bottle must be resistible against highly basic solutions!

6. Internal Standard Solution

   For preparation of internal standard solutions see SOP: Preparation procedure for Amino Acid Standard Additions QM-4101.

All these solutions except internal standard solutions can be stored for about 2 years. For advised storage time of internal standard solution see related SOP.

5.2 Sample pre-preparation:

- Use representative subset of the sample and homogenize it by grinding till the particle size is approximately ≤ 0.5 mm.
- If the sample material has a high moisture concentration,
  - weigh in a few gram or less of sample,
  - dry the sample at a temperature of maximum 50 °C or freeze dry till weight remains constant,
  - determine the weight loss during drying for later calculations if required.
- If the sample material has a high fat content (> 5 %)
  - weigh in a few grams or less of dry sample,
  - reduce the fat concentration by extraction using petroleum ether (e.g. liquid-liquid extraction (LLE) or Soxhlet extraction),
  - remove solvent completely after extraction by evaporation at room temperature,
  - determine the weight loss during defatting for later calculations if required.
- Determine the Kjeldahl-nitrogen content, if possible.
- Otherwise determine the overall amino acid content in a pre-test measurement and estimate the Kjeldahl-nitrogen content from this value. The conversion factor for amino acids to Kjeldahl-nitrogen is 0.16 (this factor may vary depending on the kind of sample material). Also for pre-test measurements follow this SOP.

5.3 Sample hydrolysation:

- Weigh in 0.1 to 1.0 g of the sample material, it should be equivalent to approximately 10 mg Kjeldahl-nitrogen.
  - For pre-test sample preparation weigh in 0.1 to 0.3 g depending on the expected protein content.
- Transfer the sample into a reaction vessel or Pyrex flask.
- Add 5 ml of Oxidation Solution 2 to the sample and swivel gently.
  - Note: This reaction can be very intensive. The mixture can squirt out of the reaction vessel!
  - Close reaction vessel, but not completely. The emitted gas needs to escape to avoid overpressure in the vessel.
  - When sample and Oxidation Solution are mixed properly, the reaction must proceed for 1 hour at room temperature.

  Note: In reference literature this reaction is carried out for 16 hours in ice bath.
  - The reaction mixture should be swivelled from time to time.
- Stop the oxidation reaction by adding 0.9 g sodium pyrosulfite ($Na_2S_2O_5$)

  Note: This reaction can be very intensive. The mixture can squirt out of the reaction vessel!

  Note: To avoid intoxications the neutralization reaction must be performed in a fume hood due to the emitted sulfur dioxide!
  - Swivel the sample mixture gently till reaction is finished (no bubbles)
- After neutralization add 25 ml of Hydrolysis Solution (preparation see above subsection: Pre-preparation)
- At this point of sample preparation an internal standard can be added if required (e.g. add 1000 µl of internal standard solution (e.g. norleucine) with a concentration of 4 µmol/ml to the sample). Notice the change in dilution factor.

- Mix the sample carefully with the hydrolysis solution by swivelling.
  - Take care that no sample residues stick to the inner wall of the flask.
- Heat the sample in the closed vessel for 24 h at a temperature of 110 ± 2 °C. See different time and energy settings for microwave hydrolysis in related manual.
  - If not using pressure resistant vessels release overpressure after 30 minutes of heating.

  !!!BE CAREFUL FLASK MAY EXPLODE!!!
- After thermal treatment take the flask out of the oven and cool it down to room temperature by dint of cold water or ice cooled water. If not cooled automatically by microwave system.

5.4 Sample post preparation:

Transfer the hydrolysate solution quantitatively into a 250 ml beaker with as less as possible SDB/Na (Sample Dilution Buffer). For convenience the SDB/Na can be filled into a dash bottle.

- Use a PTFE stirring bar and a magnetic stirrer to mix the hydrolysis solution during pH adjustment.
- Use a temperature probe (e.g. from pH meter) to observe the temperature of the hydrolysis solution during pH adjustment.
- Add slowly about 18 ml of Neutralization Solution 1 (preparation see above subsection: Pre-preparation).
  - Thereby the temperature should not exceed 40 °C.
- Use a calibrated pH probe to adjust the pH value of the hydrolysis solution more precisely.
  - Note that the pH value is highly influenced by the temperature and the temperature-correction for the pH meter may cover only a narrow operating range.
- Continue pH adjustment by adding carefully Neutralization Solution 1 or 2 to the hydrolysis solution in order to achieve a pH value of 2.1 to 2.3.
- Transfer the pH-adjusted solution with SDB/Na quantitatively into a 200 ml volumetric flask.
  - Thereby rinse the stirring bar and the stir bar catcher as well as the temperature and pH probe with SDB/Na. For convenience the SDB/Na can be filled into a dash bottle.
- Fill the graduated flask up to 200 ml with SDB/Na at the respective calibration temperature of the flask.
- Alternative pH adjustment (simplified but not that precise, retention times may vary)
  - Transfer the hydrolysate solution quantitatively into a 200 ml volumetric flask with SDB/Na (Sample Dilution Buffer), for convenience the SDB can be filled into a dash bottle.
  - Add slowly about 18.5 ml of Neutralisation Solution 1 (preparation see above subsection: Pre-preparation).
  - Thereby the temperature should not exceed 40 °C.
  - Fill the graduated flask up to 200 ml with SDB/Na at the respective calibration temperature of the flask.

  Note: Using this simplified method for pH adjustment may result in variations of the final pH value. Thus may cause variations of retention times in the acidic part of the separation.
- If the sample is not measured within a few hours, store the volumetric flask at a temperature of 2 to 8 °C until usage.
- Cleaning of sample solution from particles can be performed by one of the following steps:

  - Take an aliquot of the hydrolysed sample solution with a syringe and percolate the solution through a 0.45 µm pore size syringe filter.
  - Discard the first percolated millilitre of the solution!
  - Alternative: Take an aliquot of the hydrolysed sample solution and transfer it to a centrifuge tube and centrifuge solution at 14.000 rpm for 10 minutes.
- Transfer a defined aliquot of the filtrated/cleaned sample solution to a sample vial dilute it with SDB/Na.
  - The dilution factor should be chosen to obtain sufficiently strong signal intensities in the range of the calibration (e.g. 500 µl sample diluted with 500 µl SDB/Na).
- If using an internal standard (e.g. norleucine) at this point of sample preparation dilute the sample in the same way but with the following changes.
  - Transfer the same aliquot of filtrated hydrolysed sample solution into a vial and add 10 µl of internal standard solution (e.g. norleucine) with a concentration of 10 µmol/ml. Dilute the sample with a volume of SDB/Na which is reduced by the volume of the internal standard (e.g. 500 µl sample add 10 µl internal standard solution diluted with 490 µl SDB/Na).
- Mix the solution for homogeneity (e.g. vortex mixer)
- These solutions are now ready for injection.

6. Additional Information

- In case of massive retention time variations in the beginning of a sample chromatogram compared to a standard measurement check the adjustment of the pH value after dilution with SDB/Na. The pH value should be in the range of about 2.10 to 2.30.
- When a hydrolysation is performed without forced oxidation it is nearly impossible to avoid oxidation processes of these amino acids which would cause miscalculations of the respective concentration. To achieve approximate results for these amino acids without a forced oxidation as described in this SOP it is important to reduce the oxygen concentration in the headspace of the hydrolysation vessel as good as possible. Due to this the quantitative determination of these amino acids is limited. In case that these amino acids are not of higher interest the simple acidic hydrolysation procedure can be used (see SOP: Protein sample preparation by acidic hydrolysis, Sykam QM-4159).
- The amino acids asparagine and glutamine will transduce in to their acid derivatives aspartic acid and glutamic acid.
- Tryptophan is not stable to persist the acidic hydrolysis and will decompose completely during hydrolysation. If the concentration of tryptophan has to be determined, it will be required to perform additionally a basic hydrolysation (see SOP: Protein sample preparation by basic hydrolysis, Sykam QM-4161). On the other hand this basic hydrolysation will degrade most of the other amino acids.
- If the time period between sample preparation and measurement is longer than a few hours, the prepared sample should be store at 2-8 °C or colder. The dilution factor for the described sample preparation depends on the following preparation steps:
  - mass change factor after drying
  - mass change after defatting
  - sample amount used for hydrolysis
  - dilution by oxidation solution
  - dilution by hydrolysis solution
  - if applied dilution by internal standard addition before hydrolysis
  - dilution after pH adjustment
  - factor of dilution for injection (including ISTD if applied)

In Case of ambiguities don’t hesitate to contact labsupport@sykam.com

7. Literature

[1] Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed (Text with EEA relevance)

[2] SOP: Protein sample preparation by acidic hydrolysis, Sykam QM-4159

[3] SOP: Protein sample preparation by basic hydrolysis, Sykam QM-4161

[4] SOP: Preparation procedure for Amino Acid Standard Additions QM-4101