



National Standard of the Peoples Republic of China

GB/T 33682-

Purity and enzymatic activity
determination of Nitrile hydratase

腈水合酶纯度和活性的测定

(English Translation)

(报批稿)

Issue date

Implementation date

Issued by the State Administration for Market Regulation
and the National Standardization Administration

Foreword

This standard is drafted in accordance with the rules given in the GB/T 1.1-2009 *Directives for standardization-Part 1: Structure and drafting of standards*.

This standard was prepared by SAC/TC 387 (National Technical Committee 387 on Biochemistry Products and Testing Technology of Standardization Administration of China) .

Shenzhen Second People's Hospital is in charge of this English translation. In case of any doubt about the contents of English translation, the Chinese original shall be considered authoritative.

Purity and enzymatic activity determination of Nitrile hydratase

1 Scope

Describes the principle of nitrile hydratase purity and activity determination, enzyme activity unit definition, reagents or consumables, instrumentation, sample preparation, test procedure, and result calculation. It is suitable for the determination of the purity and activity of nitrile hydratase which catalyzes the conversion of acrylonitrile to acrylamide.

2 Normative references

The contents of the following documents constitute indispensable provisions of this document through normative references in the text. Among them, for cited documents with date, only the version corresponding to that date applies to this document; for cited documents without date, the latest version (including all revision sheets) applies to this document.

GB/T 6682 Specification and test methods for water used in analytical laboratories.

3 Terms and Definitions

The following terms and definitions apply to this document.

3.1

Nitrile hydratase (NHase)

Metalloenzymes with the ability to catalyze the nitrile to amide compounds.

Note 1: Most NHase consists of two subunits, α and β , and generally exists in the form of $\alpha_2\beta_2$ tetramer.

Note 2: NHase active centers contain different metal ions and NHase are generally classified as iron-type nitrile hydratase (Fe-NHase) and cobalt-type nitrile hydratase (Co-NHase).

3.2

NHase purity

The measure of nitrile hydratase purity is specific activity, which refers to the amount of nitrile hydratase activity contained in each milligram of enzyme protein, i.e.:

Nitrile Hydratase Purity = Amount of Nitrile Hydratase Vigor Units (U) / Amount of Protein (mg)

3.3

NHase activity

The activity of nitrile hydratase to catalyze the acrylonitrile to acrylamide is expressed primarily in terms of viability units.

3.4

NHase activity units

A unit that indicates how much nitrile hydratase is present.

4 Abbreviations

The following abbreviations apply to this document.

Nitrile hydratase (NHase)

Sodium dodecyl sulfonate polyacrylamide gel electrophoresis (SDS-PAGE)

bovine serum albumin (BSA)

Liquid Chromatography (LC)

Acrylamide-bisacrylamide(Acr-Bis)

Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)

Sodium dodecyl sulfonate (SDS)

Ammonium persulphate (APS)

tetramethylethylenediamine (TEMED)

phosphate buffered saline (PBS)

5 Theory

5.1 Principle of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method for the determination of nitrile hydratase purity

Based on the differences in charge number and molecular weight of different proteins, a certain concentration of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) can separate proteins with different molecular weights in a certain range of time, and the purity of the nitrile hydratase was calculated after staining, decolorization, imaging and data processing steps.

5.2 Principle of Nitrile Hydratase Activity Assay

Under appropriate conditions, nitrile hydratase catalyzes the isocratic formation of acrylamide from acrylonitrile. The generated acrylamide was quantified by liquid chromatography with external standard method, and the enzyme activity of nitrile hydratase was calculated according to the definition of enzyme activity unit.

6 Reagents or consumables

Unless otherwise specified, the reagents used in this method are analytical reagent, and the test water is the first grade water specified in GB/T 6682.

6.1 30% (w/v) methylenebisacrylamide solution (Acr-Bis) (Appendix A, section A.1)

6.2 1.0 mol/L tris(hydroxymethyl) aminomethane hydrochloride solution (Tris-HCl), pH 6.8 (Appendix A, Section A.2)

- 6.3 1.5 mol/L tris(hydroxymethyl) aminomethane hydrochloride solution (Tris-HCl), pH 8.8 (Appendix A, section A.3)
- 6.4 10% (w/v) sodium dodecyl sulfate (SDS) solution (Appendix A, Section A.4)
- 6.5 10% (w/v) ammonium persulfate (APS) solution (Appendix A, section A.5)
- 6.6 10x Tris-glycine electrode buffer (see Appendix A, section A.6)
- 6.7 5× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) spiking buffer (Appendix A, Section A.7)
- 6.8 Staining solution (Appendix A, section A.8)
- 6.9 Decolorizing solution (Appendix A, paragraph A.9)
- 6.10 1.0 mg/mL bovine serum albumin standard solution (Appendix A, section A.10)
- 6.11 0.1 mol/L potassium phosphate buffer (pH = 7.4) (Appendix A, section A.11)
- 6.12 10 mmol/L potassium phosphate buffer (pH = 7.4) (Appendix A, section A.12)
- 6.13 100 mmol/L acrylonitrile (Appendix A, section A.13)
- 6.14 Chromatographically pure acetonitrile
- 6.15 100 mmol/L acrylamide (Appendix A, section A.14)
- 6.16 Organic microporous filter membranes: pore size 0.45 μm
- 6.17 Mobile phase of liquid chromatography (see Appendix A, paragraph A.14)

7 Instrumentation

- 7.1 Electronic balance: 0.01 g and 0.0001 g sensitivity.
- 7.2 Centrifugal vacuum concentrators
- 7.3 Metal baths
- 7.4 Vortex oscillator
- 7.5 Ultraviolet-visible spectrophotometer
- 7.6 Electrophoresis apparatus
- 7.7 Gel scanning device
- 7.8 Decolorizing shaker
- 7.9 Pipette
- 7.10 Liquid chromatograph with UV-visible detector (variable wavelength detector) or diode array detector (diode array detector)

7. 11 Chromatographic columns, which shall meet the following requirements, with C18 as the stationary phase.

8 Sample Preparation

The sample to be tested was accurately weighed and prepared as a 1 mg/mL sample solution by dissolving in 10 mmol/L PBS solution. If the sample to be tested was a solution, it was concentrated into lyophilized powder by centrifugal vacuum concentrator.

9 Analytical steps

9.1 Determination of protein content

Determination according to SN/T 3926.

9.2 Determination of the Nitril Hydratase Purity

9.2.1 Electrophoresis sample preparation

Accurately pipette 20 μL of 1.0 mg/mL of the sample and pipette 5 μL of 5 \times electrophoresis (SDS-PAGE) spiking buffer into a centrifuge tube and mix them, then heat them in a metal bath at 100 $^{\circ}\text{C}$ for 10 min, and then cool them down and set them aside.

9.2.2 Preparation of electrophoretic separation gel and concentrated gel

Assemble the gel-making device and prepare 5% concentrated gel and 12% separation gel solution according to Table 1, respectively.

Table 1 Concentrated gum, separating gum formula

	5% gel concentrate (μL)	12% separating gel (μL)
30% Acr-Bis	750	4000
1.5 mol/L Tris-HCl (pH8.8)	0	2500
1.0 mol/L Tris-HCl (pH6.8)	780	0
10% APS	60	100
10% SDS	60	100
water	4440	3400
TEMED	6	5

9.2.3 Electrophoretic Sampling

The volume of the sample was 10 μL with 3 replicates for each sample.

9.2.4 Electrophoresis

Pipet 10 μL of nitrile hydratase sample to be tested, add 40 μL of 5 \times sampling buffer, and then put it into boiling water for 5-10 min, and then spot the sample into the wells of the gel, and start the electrophoresis once all the samples are spotted. When the samples were in the concentrated gel, the voltage was adjusted to 80 V. After the

samples entered the separation gel, the voltage was adjusted to 120 V. The electrophoresis was terminated when the indicator bromophenol blue moved to the lowest end of the protein gel. Then the gel was stained by Kaumas Brilliant Blue R-250 for 2-4 h. After the staining, the gel was removed from the staining solution and put into the decolorizing solution for decolorization, during which the decolorization was carried out by using a decolorizing shaker to oscillate the decolorization until the background blue color was completely cleaned up, the gel appeared transparent, and the bands were clearly visible to terminate the decolorization.

9.3 Determination of Nitril Hydratase Activity

9.3.1 Liquid Chromatography Reference Conditions

Detection wavelength is 215 nm.

The column temperature was 40 °C.

The mobile phase was water acetonitrile volume ratio of 2:1, isocratic elution.

Injection volume: 2 µL.

Flow rate: 0.2 mL/min.

9.3.2 Enzyme-catalyzed conversion of acrylonitrile to acrylamide

Accurately pipette 10 µL of nitrile hydratase at a concentration of 0.5 mg/mL into a 1.5 mL centrifuge tube and place it on a metal bath at 25 °C. And then adding 490 µL of 100 mmol/L acrylonitrile into the same centrifuge tube, vortex the tube thoroughly and react for 10 min at 25 °C, then adding 0.1 mol/L phosphoric acid solution to terminate the reaction. The supernatant was collected and passed through 0.45 µm organic microporous filter membrane. At the same time, acrylamide solutions with concentrations of 0.5, 1, 2, 5 and 10 mmol/L were prepared according to Table 2 and loaded into the LC sample chamber as the standard samples, respectively.

Table 2 Table of standard solution preparation for liquid chromatography

Acrylamide standard solution	concentration	10 mmol/L PBS	
stock solution	100 mmol/L	--	
S1	10 mmol/L	stock solution 100 µL	900 µL
S2	5 mmol/L	stock solution 50 µL	950 µL
S3	2 mmol/L	S1 200 µL	800 µL
S4	1 mmol/L	S1 100 µL	900 µL
S5	0.5 mmol/L	S1 50 µL	950 µL

10 Analysis of results

10.1 Purity Calculation

After electrophoretic decolorization, the gel was placed in the protein imprinting imaging system for imaging. Taking pictures to record the experimental bands and use the electrophoretic image analysis software p calculate the proportion of nitrile hydratase in the samples (%) after the selection of an appropriate exposure degree .Average of the three replicates of the samples times the protein content in the samples is the actual

average relative purity of the samples (expressed as a percentage) according to the formula (1):

$$P = R \times C \times 100\%$$

(1)

where:

P: relative purity of nitrile hydratase;

R: the proportion of nitrile hydratase in the sample;

C: protein content in the sample of nitrile hydratase.

The relative purity of the sample to be tested is the product of the average of the three replicates of the sample and the protein content of the sample to be tested, and the result is expressed as a percentage (%), and the results of the calculation are retained two decimal places.

10.2 Acrylamide Calculation

The standard working curve was plotted according to the concentration of acrylamide standard solution and the corresponding peak area, and the amount of acrylamide in the reaction system was calculated by the standard curve equation (2):

$$x = (y - b)/a \quad (2)$$

where:

x: concentration of acrylamide generated (mmol/L);

y: integrated area of acrylamide ;

a: slope of the standard working curve;

b: intercept of the standard working curve;

Where the standard chromatogram of acrylamide is shown in Appendix B.1.

10.3 Activity Calculations

Defined as the amount of enzyme required to catalyze the production of 1 μmol of amide per minute at 25 °C. Equation (3) was calculated as follows:

$$EA \text{ (U/mg)} = x \times v_1 / (t \times c \times v_2) \quad (3)$$

where:

EA: enzyme activity (U/mg)

x: concentration of acrylamide generated (μmol/mL)

v₁: volume of reaction system for catalytic generation of acrylamide (mL)

t: reaction time (min)

c: volume of nitrile hydratase to be tested added to the reaction (mL)

v₂: concentration of nitrile hydratase to be tested added to the reaction (mg/mL)

10.4 repeatability

The absolute difference between two independent determinations obtained under conditions of repeatability shall not exceed 10% of the arithmetic mean.

Appendix A

(Normative)

Reagent Preparation

A.1 30% (w/v) methylenebisacrylamide solution (Acr-Bis)

Weigh 29.00 g of acrylamide and 1.00 g of N,N'-methoxybisacrylamide, add 60 mL of water, stir well to dissolve, and then volume to 100 mL. Filter with 0.45 μ m microporous filter membrane to remove the bacteria and impurities, then store in a brown bottle at 4 °C to keep away from light. If there is any precipitation, it should be filtered and the solution should be reconstituted after two months.

A.2 1.0 mol/L Tris-hydroxymethylaminomethane hydrochloride solution (Tris-HCl), pH 6.8

Weigh 12.12 g of tris(hydroxymethyl)aminomethane (Tris) and dissolve it in 80 mL of water, stir well to dissolve, adjust the pH to 6.8 with HCl and condense it to 100 mL, keep it at room temperature for spare use.

A.3 1.5 mol/L tris-hydroxymethylaminomethane hydrochloride solution (Tris-HCl), pH 8.8

Weigh 18.17 g of tris-hydroxymethylaminomethane dissolved in 80 mL of water, stir well to dissolve, adjust the pH to 8.8 with HCl and condense to 100 mL, store at room temperature for spare use.

A.4 10% (w/v) sodium dodecyl sulfate (SDS) solution

Weigh 2.00 g of sodium dodecyl sulfate and dissolve it in about 16 mL of water, ultrasonically dissolve it and then volume it to 20 mL, store it at room temperature and keep it aside.

A.5 10% (w/v) ammonium persulfate (APS) solution

Weigh 0.10 g of ammonium persulfate in a 1.5 mL centrifuge tube, add 1 mL of water to dissolve, ready to use.

A.6 10-fold Tris-glycine electrode buffer

Weigh 30.00 g Tris, 144.00 g Glycine, 10.00 g SDS dissolved in 800 mL of water and volume to 1000 mL, store at room temperature, dilute 10 times when used.

A.7 5 \times sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) spiking buffer

Take 1.25 mL of 1 mol/L Tris-HCl (pH 6.8), 0.50 g of SDS, 2.5 mL of glycerol, 25.0 mg of bromophenol blue, and dissolve in water to 5 mL, then dispense 500 μ L into each tube, and store in the refrigerator at 4 °C. Add 0.0780 g of dithiothreitol (DTT) to each tube before use.

A.8 Staining solution

Staining solution A: Caulmers Brilliant Blue G-250 0.10 g, ethanol 50 mL, 85% (m/v) phosphoric acid 25 mL, water to 1000 mL, keep in reserve.

Staining solution B: Kaomas Brilliant Blue R-250 0.50 g, glacial acetic acid 25 mL, ethanol 250 mL, water to 500 mL, stored at room temperature.

A.9 Decolorizing solution

Take 100 mL of glacial acetic acid, 400 mL of methanol, and quantify with water to 1 L. Store at room temperature.

A.10 1.0 mg/mL bovine serum albumin standard solution

Weigh 10.0 g of bovine serum albumin with $\geq 98\%$ purity, add water to 20 mL, prepare to a concentration of 1 mg/mL, and store at $-20\text{ }^{\circ}\text{C}$.

A.11 0.1 mol/L potassium phosphate buffer (pH=7.4)

Weigh 17.42 g of K_2HPO_4 , add water to 100 mL, get 0.1 mol/L. Weigh 13.61 g of KH_2PO_4 , add distilled water to 100 mL, transfer 80.2 mL of K_2HPO_4 and 19.8 mL of KH_2PO_4 to make 0.1 mol/L potassium phosphate buffer, store at $4\text{ }^{\circ}\text{C}$.

A.12 10 mmol/L potassium phosphate buffer

Take 100 mL of 0.1 mol/L potassium phosphate buffer and add water to make 1 L. Store at $4\text{ }^{\circ}\text{C}$.

A.13 200 mmol/L 3-cyanopyridine (nicotinonitrile)

Weigh 2.08 g of nicotinonitrile solid, dissolve in 10 mmol/L potassium phosphate buffer (pH=7.4) and volume to 100 mL.

A.14 100 mmol/L acrylamide

Weigh 0.7108 g of acrylamide dissolved in 100 mL of 10 mM PBS solution.

A.15 Liquid Chromatography Mobile Phase

Chromatography grade acetonitrile and ultrapure water were prepared in a 1:2 (V/V) mixture.

Appendix B

(Informative)

Chromatogram of the standard for acrylamide

See Figure B.1 for the chromatogram of the standard for acrylamide.

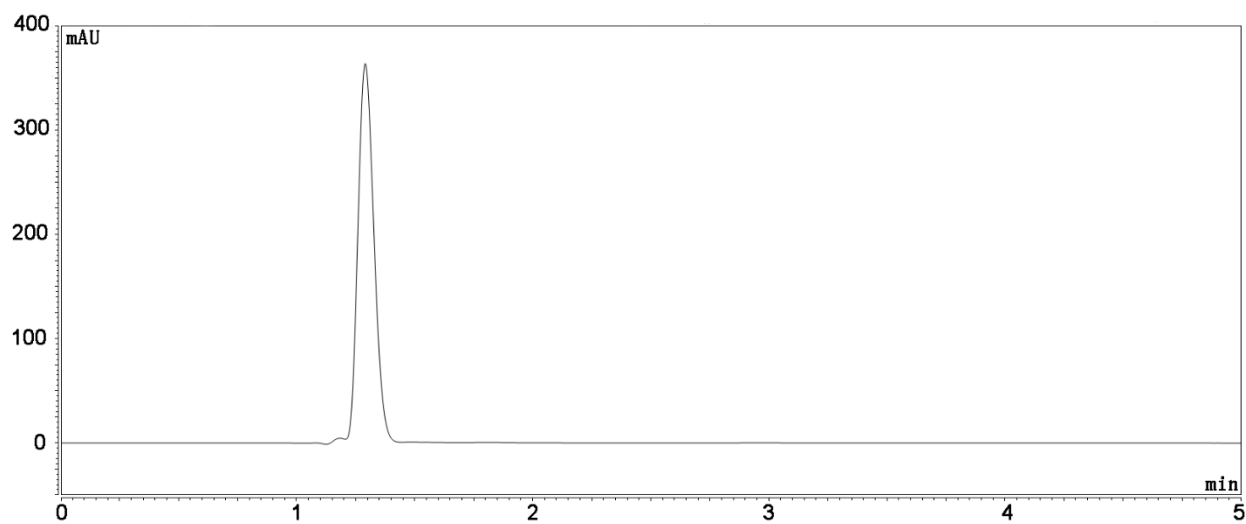


Figure B.1 Liquid chromatogram of 2 mM acrylamide standard