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Quality requirements for enzyme preparations—Part 1: Protease preparations

酶制剂质量要求 第1部分：蛋白酶制剂

(English translation)

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Foreword

SAC/TC64/SC5 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.

This document is drafted in accordance with the rules in the GB/T 1.1-2020 *Standardization Work Guidance Part 1 : Structure and Drafting Rules of Standardization Documents.*

This document stipulates the technical requirements related to food quality. Food safety related requirements can be found in the relevant laws and regulations, policies and food safety documents and other documents.

This document is GB/T 23527 *Quality requirements for enzyme preparations p*art 1 . GB/T 23527 issued the following sections :*Part 1: Protease preparations*

This document substitutes replaces GB/T 23527-2009 *Protease preparation*.As compared with GB/T 23527-2009, this document mainly involves the following technical changes:

——Added the terms ' protease preparation ' and ' protease activity unit '(see 3.2, 3.3) ;

——Changed the definition of ' protease activity ' (see 3.4, and 3.2 in 2009 edition) ;

——Changed the ' enzyme activity ' requirement in the physical and chemical requirements (see Table 2, Table 1 in 2009 edition) ;

——Deleted the ' health requirements ' (see 5.3 in 2009 edition) ;

——Changed the test methods of drying weight loss and fineness(see 6.3, 6.4, and 6.3, 6.4 in 2009 edition) ;

——Changed the factory inspection items(see 7.3, 7.3.1 in 2009 edition) ;

——Changed the content of the logo (see 8.1, 8.1 in 2009 edition) ;

——Deleted ' shelf life ' (see chapter 9 in 2009 edition) ;

——Deleted the list of ' categories, codes and producers of protease preparations commonly used in China ' (see Appendix A in 2009 edition) ;

——Added other buffers in the ' protease activity determination Folin method 'y (see A.3.8.4);

——Added other substrates in 'determination of protease activity by UV spectrophotometry ' (see B.3) ;

——Changed reagents in ' Automatic biochemical analysis for the determination of alkaline protease activity ' (see C.3, D.4 in 2009 edition) ;

——Added the test method of 'determination of protease activity by micro-colorimetric method ' (see Appendix D).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights.The issuing body of this document shall not be held responsible for identifying any or all such patent rights.

The document was proposed and prepared by the National Technical Committee 64 on Food Industry of documentization Administration of China (SAC/TC 64).documentdocumentdocumentdocumentdocumentdocument

——The first edition was issued in 2009 as GB/T 23527-2009;

——This is the first revised edition.documentdocumentdocument

Introduction

With the rapid development of the enzyme preparation industry, the variety of enzyme preparations are diversified, the quality of products is improved to a new level, it has made great progress and progress from technology to category. The formulation of GB/T 23527 *Quality requirements for enzyme preparations*, it is the documentization of product quality and detection methods of enzyme preparations. It is the basic work to documentize the order of enzyme preparations and related products industry and promote industrial development.

GB/T 23527 *Quality Requirements for Enzyme Preparations* consists of four parts :

——Part 1 : Protease preparation ;

——part 2 : Lipase preparations ;

——part 3 : α-amylase preparation ;

——part 4 : Immobilized glucose isomerase preparation.

With the product innovation and development of the protease preparation industry, according to the industry product research and collection of product categories, in addition to the protease preparation products from microbial fermentation sources covered by the original document range, there are plant tissue extraction products such as papain, bromelain, fig protease and animal tissue extraction products such as pepsin and trypsin. In addition, according to the needs of product development in the foreign detection method industry, in this revision, the determination conditions of GB/T 23527-2009 enzyme activity determination method were updated and improved, and the micro-colorimetric method was added.

Quality requirements for enzyme preparations— Part 1: Protease Preparations

1. Scope

This document specifies the products classification, requirements, inspection rules, marking, packaging, transportation and storage of protease preparations, and describes the test methods. This document is applicable to the production, inspection and sale of protease preparation prepared through purification after microbial fermentation and or extraction from plant/animal tissues.

2. Normative reference

The following normative document contains provisions which, through reference in this text, constitute the essential provisions of this document. For all the cited documents with date, all the subsequent revision lists (excluding the content of corrigendum) or revised editions are not applicable to this document, however, all the parties who reach an agreement based on this document are encouraged to study whether the latest versions of these documents can be used. For undated references, only the latest editions apply to this document.

GB/T 191 *Packaging - Pictorial marking for handling of goods*
GB/T 601 *Chemical reagent - Preparation of standard titration solution*
GB/T 602 *Chemical reagent - Preparation of standard solution for impurity determination*
GB/T 603 *Chemical reagent - Preparation of preparations and products used in the test method*
GB/T 6682 *Water for analytical laboratory use— specification and test methods*
GB/T 1803 *General test methods for industrial enzyme preparations*

3. Terms and definitions

The following terms and definitions apply to this document.

3.1.

protease

Enzymes that catalyze the hydrolysis of peptide bonds within protein molecules to convert them into polypeptides or amino acids.

3.2.

protease preparations

Products made from extracted and purified protease as the main catalytic active component through preparation and other processes.

Note: Ingredients that contribute to the storage, stability and use of the product can be added to the preparation process.

3.3.

activity unit of protease

Under certain mild and pH conditions, the amount of protease required to hydrolyze casein to produce 1 μg tyrosine in 1 min was 1 enzyme activity unit.

Note : expressed as ' U '.

3.4.

protease activity

activity of protease preparations

The ability to catalyze the hydrolysis of proteins into polypeptides and amino acids is expressed as the unit of enzyme activity contained in 1 g of solid protease preparation (or 1 mL of liquid protease preparation).

Note : expressed as U/g or U/mL.

4. Product classification

4.1. According to application area

Food industry and other industrial enzyme preparations.

4.2. According to the product form

Solid and liquid enzyme preparations.

4.3. According to applicable pH range

There are three kinds of protease preparations: acid protease preparation, neutral protease preparation and alkaline protease preparation.

5. Requirements

5.1. Sensory requirements

Shall comply with the provisions Table 1.

Table 1 Sensory requirements

Items	Solid form	Liquid form
Color	White to yellowish-brown	Light yellow to yellowish-brown
State	Powder or granule, without agglomeration or deliquescence.	Liquid, allow a few condensate.
Odour	Odourless, microbial fermentation products with special fermentation odour.	

5.2. Physical and chemical requirements

Shall comply with the provisions Table2.

Table 2 Physical and chemical requirements

Item		solid dosage forms	liquid dosage forms
Enzyme activity[a]/ (U/g or U/mL)		Conformity claim	Conformity claim
Loss on drying[b]/%	≤	8.0	—
Fineness (Passes through a test sieve with a mesh size of 0.40 mm.) [b]/%	≥	80	—
[a]Can also be implemented according to the enzyme activity requirements agreed by the supply and demand sides [b]Not applicable to pellet products.			

6. Test methods

6.1. Sensory requirements

Weigh sample 10g or 10mL in clean, dry white porcelain plate, observe, smell, make a judgment, make a record.

6.2. Enzyme activity

According to Appendix A for testing, you can also refer to Appendix B, Appendix C, Appendix D or use other methods for testing. The test results should indicate the method of determination. If necessary, the buffer solution and the substrate should also be marked.

6.3. Loss on drying

According to the test method of drying weight loss in QB/T 1803.

6.4. Fineness

According to the test method of fineness in QB/T 1803, the standard test sieve is selected as ϕ200 × 50-0.40/0.25.

7. Inspection rules

7.1. Batch

A batch of products with the same raw materials, the same formula, the same process and the same production line are continuously produced.Sampling

7.2. Sampling

The sample size for finished goods sampling is provided in Table 3, or determined by the manufacturer and (or) related (relevant) parties. The sampling amount of each minimum outer packaging unit should not be less than 300 g or 300 mL), and when it is insufficient, it is appropriately added according to the proportion. Test after fully mixing.

Table 3 Product sampling table

Batch range (minimum external packaging unit)	Samples (minimum external packaging unit)
≤50	2
51～500	3
＞500	4
Notes: the batch range refers to the minimum number of outer packaging units contained in the batch. The sample size of sampling refers to the minimum number of outer packaging units of the sample.	

7.3. Delivery Inspection

Before the product leaves the factory, the quality supervision and inspection department of the manufacturer shall inspect the product batch by batch according to the provisions of this document, and the product can leave the factory only after passing the inspection and attaching the quality certificate.

7.3.1. Inspection items:

a) Soild inspection items:Sensory requirements、enzyme activity、loss on drying、 fineness.

b) Liquid inspection items: Sensory requirements、enzyme activity...

7.4. Type inspection

Inspection items: all the items required in this document. In general, the type inspection on the same kind of products shall be made at least once a year. In any of the following cases, it is also necessary to make the inspection:

a) Any major change in raw and auxiliary materials

b) Any change in critical process or equipment;

c) Resumed production of the newly trial-produced products or the products under normal production after shutdown for 3 months;

d) Significant difference in the delivery inspection result from the last type inspection results;

e) Sampling inspection required by the national quality supervision and inspection institution according to relevant provisions.

7.5. Judgment rules

7.5.1. After the samples are inspected, all the inspected items are qualified, and this batch of products is judged as qualified.

7.5.2. If there are more than two unqualified indexes in the inspection result, it is determined that the batch of products do not meet the requirements of this document. If there are one or two do not meet the requirements, we should re-extract twice the sample size from the same batch of products for re-examination, and the re-examination results shall prevail. If there is still one that does not meet the requirements, it is determined that the product does not meet this document.

7.5.3. When the supply and demand sides have objections to the test results, it can be resolved by consultation between the two sides, or entrust the relevant units to conduct arbitration test, which shall be subject to the arbitration test results.

8. Sign, packaging, transport and storage

8.1. Sign

8.1.1. When using labels for sales packaging, the product type and enzyme activity should be marked.

8.1.2. Packaging, storage and transportation diagrams shall be implemented according to GB/T 191.

8.2. Packaging

Packaging containers should be clean and undamaged.

8.3. Transport

Transport tools should be clean and sanitary. Do not mix or transport with toxic, harmful, corrosive and odorous articles, and avoid damp, pressure and exposure. Handle with care when loading and unloading, and do not directly hook the package.

8.4. Storage

It should be stored in a ventilated, dry and clean warehouse, strictly prevent sun, rain, and fire.Do not put it together with toxic, harmful, corrosive and odorous articles.

Annex A

(Normative)

Determination of protease activity - Folin method

A.1. Principles

Under certain temperature and pH conditions, the protease hydrolyzes the casein substrate to produce the amino acid containing phenolic group. Under the alkaline conditions, Folin reagent is reduced to generate molybdenum blue and tungsten blue. The absorbance of the solution is measured at the wavelength of 680 nm with a spectrophotometer. The enzyme activity is proportional to the absorbance, from which the enzyme activity of the product can be calculated.

A.2. Apparatus and equipment

A.2.1 Electronic balance : Sensitivity is 0.1 mg.

A.2.2 Spectrophotometer.

A.2.3 Constant temperature water bath pot.

A.2.4 pH meter : Accuracy of 0.01.

A.3. Reagents and solutions

The water used in this method should meet the specifications of the third grade water in GB/T 6682 when other requirements are not specified. The reagents used refer to analytically pure when other specifications are not specified. The standard titration solution used in the analysis and the standard solution, preparations and products for impurity determination were prepared according to the provisions of GB/T 601, GB/T 602 and GB/T 603 without specifying other requirements.

A.3.1 Folin reagent

100.0 g of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$), 25.0 g of sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$), 700 mL of water, 50 mL of 85% phosphoric acid and 100 mL of concentrated hydrochloric acid are added into a 2 L mill reflux device. Boiling reflux for 10 h under a low flame, remove the reflux cooler, add 50 g of lithium sulfate (Li_2SO_4), 50 mL of water and several drops of concentrated bromine water (99%) in the fume hood, and then boil them for 15 min to remove the excess bromine (add bromine water if there is still green after cooling, and then boil them to remove the excess bromine), cool them, and dissolve with distilled water to 1,000 mL. Mix well and filter. The prepared reagent shall be golden yellow and stored in brown bottle.

A.3.2 Folin solution for use

Take 50 mL Folin reagent (A.3.1) and 100 mL water, mixe well.

Note :It can also be formulated with a commercial Folin solution.

A.3.3 Sodium carbonate solution (0.4 mol/L)

Weigh 42.4 g of anhydrous sodium carbonate (Na_2CO_3), dissolve with water to 1 L

A.3.4 Trichloroacetic acid (0.4 mol/L)

Weigh 65.4 g of trichloroacetic acid, dissolve with water to 1 L.

A.3.5 Sodium hydroxide solution (20 g/L)

Weigh 20.0 g of sodium hydroxide tablets, add 900 mL of water and stir to dissolve. Allow the solution to reach room temperature and then dissolve with water to 1 L, and stir well.

A.3.6 Hydrochloric acid solution (1.0 mol/L)

Move 8.3 mL of concentrated hydrochloric acid into a volumetric flask containing 80 mL of water and shaken well.

A.3.7 Hydrochloric acid solution (0.1 mol/L)

Move 0.83 mL of concentrated hydrochloric acid into a volumetric flask containing 80 mL of water and shake well.

A.3.8 Buffer Solution

A.3.8.1 Phosphate buffer (pH 7.5, Suitable for neutral protease preparations)

Weigh 6.02 g of disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$) and 0.5 g of sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$), respectively, dissolve with water to 1000 mL. The pH was adjusted to 7.50 ± 0.05 by hydrochloric acid solution (A.3.6) or sodium hydroxide solution (A.3.5).

A.3.8.2 Sodium lactate buffer (pH 3.0, Suitable for acidic protease preparations)

Take 4.71 g of lactic acid (80% ～ 90%) and 0.89 g of sodium lactate (70%), add water to 900 mL, and then stir them until it is uniform. Adjust the pH to 3.0 ± 0.05 with lactic acid or sodium lactate to a constant volume of 1000 mL.

A.3.8.3 Boric acid buffer solution (pH 10.5, Suitable for alkaline protease preparation)

Weigh 9.54 g of sodium borate and 1.60 g of sodium hydroxide, add 900 mL of water, and then stir them until it is uniform. Adjust pH = 10.5 ± 0.05 with 1 mol/L hydrochloric acid solution (A.3.6) or 0.5 mol/L sodium hydroxide solution (A.3.5) to a constant volume of 1000 mL.

A.3.8.4 Other buffer solutions

Manufacturers and users may also explore the use of the following buffer solutions (A.1) or other suitable buffer systems.

Table A.1 Other buffer solution reference preparation method

Buffer system	Refer to preparation method
Sodium acetate buffer (pH=4.7)	Weigh 4.10 g of anhydrous sodium acetate and add it into 900 mL of water; after full dissolution, add 3.00 g of glacial acetic acid. Adjust pH = 4.7 ± 0.05 with acetic acid solution (A.3.6) or sodium hydroxide solution (A.3.5) to a constant volume of 1000 mL.
phosphate buffered saline (pH=6.0)	Weigh 17.9 g of disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$) and add it into 800 mL of water; after full dissolution, adjust pH to 6.0 ± 0.05 with hydrochloric acid solution to a constant volume of 1000 mL.
Tris buffer (pH=8.5)	Weigh 6.06 g of 2-amino-2- (hydroxymethyl) -1,3-propanediol (Tris base) in 800 mL of water; after sufficient dissolution, adjust pH to 8.5 ± 0.05 with hydrochloric acid solution (A.3.6), and then dissolve with water to 1000 mL.

A.3.9 Substrate solution (10.0 g/L)

A total of 1.00 g casein substrate for protease preparation (accurate to 0.001 g) was weighed in a beaker, and about 80 mL of the corresponding buffer solution was added. The mixture was boiled in a boiling water bath for 30 min, and stirred continuously until the casein was completely dissolved. Cooled to room temperature, transferred to a 100 mL volumetric flask,

adjusted to the corresponding pH with hydrochloric acid solution (A.3.6) or sodium hydroxide solution (A.3.5), and diluted with the corresponding pH buffer solutionThe solution is stored at 4 ° C, and the shelf life is 3 days. Re-confirm and adjust pH to specified value before use.

Casein from different sources or batches has an effect on the test results. If different caseins are used as substrates, the results should be compared with the above protease-specific casein substrates before use.

A.3.10 L-tyrosine standard stock solution (100 μg/mL)

0.100 g of L-tyrosine (accurate to 0.001 g) was dried to a constant weight at 105 ° C and put into a beaker. 60 mL of hydrochloric acid solution (A.3.6) was added. After being fully dissolved, it was transferred to a 100 mL volumetric flask. The hydrochloric acid solution was diluted and shaken well. Then draw 10.00 mL to 100 mL volumetric flask. The solution was diluted with 0.1 mol / L hydrochloric acid solution (A.3.7) and shaken well.

A.4 Analytical procedure

A.4.1 Preparation of standard curve

A.4.1.1 L-tyrosine series of standard working solutions are prepared according to table A.2

Table A.2 L-tyrosine series standard working solution

Tube No.	Concentration of L-tyrosine standard solution mg/mL	The volume of L-tyrosine standard stock solution (A.3.9) mL	Volume of water added mL
0	0	0	10
1	10	1	9
2	20	2	8
3	30	3	7
4	40	4	6
5	50	5	5

A4.1.2 The standard solution of 1.00 mL table A.2 was taken into the glass test tube, and 5.00 mL sodium carbonate solution (A.3.3) and 1.00 mL Folin reagent solution (A.3.2) were added. The mixture was shaken and mixed, and the reaction was carried out in a water bath at 40 ° C ± 0.2 ° C for 20 min, and the solution was taken out and cooled to room temperature. The absorbance values of a series of standard working solutions at 680 nm were determined by 10 mm colorimetric cup with 0 tube as blank. The standard curve was drawn with the absorbance value as the ordinate and the concentration of L-tyrosine as the abscissa.

A4.1.3 Using the regression equation, the amount of tyrosine (μg) when the absorbance is 1 is calculated, which is the absorbance constant K value. The K value should be in the range of 95 ~ 100. If it does not meet, the reagent needs to be re-configured for testing.

Notes: L-tyrosine diluent should be measured immediately after dilution.

A.4.2 Testing of samples

A.4.2.1 Preparation of enzyme solutions to be tested

Weigh 1.0 g of protease sample (accurate 0.0001 g). Add 80 mL of corresponding buffer solution, stir to dissolve for not less than 30 min, then transfer to a 100 mL volumetric flask, dilute to volume with corresponding buffer solution and mix well. Transfer an appropriate amount of

enzyme solution, add the corresponding buffer solution to dilute it to a certain concentration. The recommended concentration range is 10 U/mL-15 U/mL for enzyme activity.

A.4.2.2. Determination

A4.2.2.1 Place the casein solution in a water bath with constant temperature of 40℃ ± 0.2℃ for preheating for 5 min;

A4.2.2.2 Operate according to the Table A.3 procedures.

A4.2.2.3 Neutral proteases such as Bacillus subtilis No.1.398 and Actinomycetes No.166 source, in addition to the standard curve and sample determination of the reaction temperature and color temperature of 30 ℃ ± 0.2 ℃, other operations are the same as above.

Table A.3 Procedures

Reagent A (blank)	Reagent B (for protease sample, it is necessary to make three parallel samples)
Add 1.00 mL of enzyme solution, 40℃ ± 0.2℃, 2 min	
Add 2.00 mL of trichloroacetic acid (shake well)	Add 1.00 mL of casein solution (shake well)
40℃±0.2℃, 10 min	
Add 1.00 mL of casein solution (shake well)	Add 2.00 mL of trichloroacetic acid (shake well)
Take it to rest for 10 minutes and filter (slow qualitative filter paper)	
Take 1.00 mL filtrate, add 5.0 mL sodium carbonate solution, perform color development at 40℃ ± 0.2℃ for 20 min	
Measure the absorbance of tube B with a 10 mm cell at 680 nm using tube A as blank.	

A.4.3 Calculation

The enzyme activity of the sample was calculated according to (A.1).

$$X_1 = \frac{c_1 \times V_1 \times 4 \times n_1}{m_1 \times 10} \quad \text{(A.1)}$$

Where: X_1—— the enzyme activity of sample, enzyme activity unit per gram (U/g) ;

c_1—— the concentration of L-tyrosine produced by protease hydrolysis was obtained from the document curve in micrograms per milliliter(μg/mL).document

V_1—— the volume of volumetric flask used to dissolve the sample,in the unit of mL.;

4 —— the total volume of reaction reagent (mL) ;

n1—— the dilution multiple of the sample

m1——mass of sample in grams,in the unit of g.

10——reaction time, in the unit of minutes (min) .The results are expressed to integers.

A.5 Precision

The absolute difference between two independent determinations obtained under repeatability conditions should not exceed 5% of the mean value.

Annex B

(Normative)

Determination of protease activity - UV spectrophotometry

B.1 Principles

Under certain temperature and pH conditions, protease hydrolyzes casein or hemoglobin substrate to generate tyrosine, then add trichloroacetic acid to terminate the enzyme reaction, precipitate the unhydrolyzed substrate, and determine the tyrosine in filtrate by using ultraviolet spectrophotometry at 275 nm. The enzyme activity is calculated according to the proportional relationship between absorbance and enzyme activity.

The conversion coefficients between the UV results and Folin results are different for different protease preparations. If necessary, relevant parties can determine the conversion factor of two methods according to the statistics of test results.

B.2 Apparatus and Equipment

The same with A.2

B.3 Reahents and solutions

The same with A.3

According to different properties of protease, in addition to the casein substrate used in A~~B~~.3, it is also feasible to select the hemoglobin substrate specified in B.1 or other suitable substrates.

Table B.1 Hemoglobin substrate reference preparation method

Substrate	Refer to preparation method
Hemoglobin	Weigh 4.0 g hemoglobin (with the accuracy of 0.001 g), add 100 mL water and stir for 10 min. Adjust the pH to 1.70 ± 0.05 with 0.1 mol/L hydrochloric acid solution. Stir for 10 min to dissolve completely, adjust pH to 4.70 ± 0.05 with 0.5 mol/L sodium acetate solution, transfer to a 200 mL volumetric flask, and dilute to the scale with water.

B.4 Analytical procedure

B.4.1 Calculation of K value

Different concentrations of L-tyrosine standard solution were prepared according to Table A.1 of Folin method, and then the absorbance (A) was determined directly by ultraviole t spectrophotometer, and the K value was calculated. K value should be in the range of 13 0 ~ 135, if not, need to re-configure the reagent, test

B.4.2 Preparation of enzyme solutions to be tested

The same with A.4.2.1. The final concentration of sample diluent shall be within the range of 10 U/mL~ 20 U/mL.

B.4.3 Assay

The operation is the same as that in A.4.2.2 for taking liquid, reaction and static precipitation, until filtration. Use an ultraviolet spectrophotometer to determine the absorbance of filtrate at the wavelength of 275 nm.

Notes: If the results are not parallel, it is feasible to return the sample solution added with trichloroacetic acid to water bath for incubation for 30 min, and then determine the absorbance.

B.5 Calculation

The enzyme activity of the final dilution of the sample is read from the document curve as U/mL. The enzyme activity of the sample is calculated according to formula (B.1):

$$X_2 = \frac{A_2 \times V_2 \times n_2 \times 4}{1 \times m_2 \times 10} \quad \text{(B.1)}$$

Where:

X_2—— the enzyme activity of sample, in the unit of U/g;

A_2—— the activity of the final dilution of the sample obtained from the document curve, in the unit of U/mL;

V_2—— the volume of volumetric flask used to dissolve the sample, in the unit of mL;

~~N~~n_2—— Dilution factor;

4——the total volume of the reagent , in the unit of mL.

l——the volume of the enzyme solution absorbed, in the unit of mL

m_2——mass of sample, in the unit of grams (g);

10——reaction time, in the unit of minutes (min) .

Results are rounded to integers.

B.6 Precision

The absolute difference between two independent determinations obtained under repeatability conditions should not exceed 5% of the mean value.

Annex C

(Normative)

Determination of alkaline protease activity - automatic biochemical assay

C.1 Principles

Proteolytic enzymes can hydrolyze N-succinyl-propionyl-propionyl-prolyl-phenylalanine p-nitroanilide substrate. The yellow p-nitroaniline released during this process has absorption at 405 nm, and the enzyme activity is calculated by measuring the change in absorbance per unit time.

C.2 Apparatus and Equipment

C.2.1 Automatic biochemistry analyzer: it is required to have sampling and stirring system, temperature control system (accuracy 0.3 ℃) and detection system(the detection system requires continuous detection of changes in absorbance at 405 nm) .

C.2.2 Analytical balance: sensitivity is 0.1 mg.

C.2.3 pH meter: accuracy of 0.01.

C.3 Reagents

The water used in this method should conform to the specification of water in GB/T 6682 when no other requirements are specified. The reagents used in this method refer to the analytical pure when no other specifications are specified.

C.3.1 Sodium citrate buffer (50 mmol/L, pH=6.0)

Accurately weigh 10.50 g of citric acid monohydrate and 0.74 g of calcium chloride dihydrate in a 1 L volumetric flask, add 450 mL of water to dissolve, add 5.33 g of sodium hydroxide, and continue to add water to about 980 mL. After complete dissolution, 0.76 g of 30% polyoxyethylene lauryl ether (Brij L23) is added.pH is adjusted to 6.0 ± 0.05 with 4 mol/L sodium hydroxide solution, dilute with water to the scale.

C.3.2 CHES buffer (100 mmol/L, pH=9.0)

Accurately weigh 2.07 g of CHES (N-cyclohexyl-2-aminoethanesulfonic acid) into a 100 mL volumetric flask, and dissolve with 70 mL of water. Add 73.5 mg of calcium chloride dihydrate and 76.2 mg of 30% Brij L23 (polyoxyethylene lauryl ether). Finally, 4mol / L sodium hydroxide solution was used to adjust the pH to 6.0 ± 0.05, and the water was diluted to the groove.

C.3.3 Substrate stock (50 g/L)

Accurately weigh 50 mg of N-succinyl-propionyl-amino-prolyl-phenylalanine p-nitroanilide substrate (with the accuracy of 0.1 mg) into a 10 mL beaker, add about 1 mL of DSMO, and stir until it is completely dissolved. Transfer it into a centrifuge tube and protect from light.

C.3.4 Substrate working solution (0.3 g/L)

Transfer 120 μL of the substrate stock solution (C.3.3) into a 20-mL volumetric flask, dilute to the scale with CHES buffer (C.3.2) and mix.

C.4 Analytical procedure

C.4.1 Preparation of standard curve

Standard stock solution : A certain amount of known active protease standard (accurate to 0.1 mg) was weighed in a 500 mL volumetric flask, and the sodium citrate buffer solution (C.3.1) was dissolved and diluted to obtain a standard stock solution. The amount of the standard should be weighed to make the protease activity in the standard stock solution 4.7 U / L.

The range of the quasi-curve should be 0.12U / L ~ 0.47U / L. Within this range, the user of the method can select 6 different concentrations to prepare the standard curve working solution. According to the different characteristics of the product, the user of the method can choose other standard curve range. The correlation coefficient R^2 of the standard curve should be greater than 0.9945.

Note:Standard stock solution and standard working solution should be prepared now.

C.4.2 Sample Solution preparation

Weigh a certain amount of protease sample, dissolve and dilute it with sodium citrate buffer (C.3.1). The dilution ratio should make the enzyme activity of final diluent is around 0.29 U/L.

Minimum dilution factor of sample is 25.

C.4.3 Steps and parameters

C.4.3.1 Steps

C4.3.1.1 Add 160 μL of substrate to the cuvette;

C4.3.1.2 Keep the substrate warm for 480 s and the temperature shall reach 37 ℃;

C4.3.1.3 Add 20 μL each of the blank, document, document control and sample to the cuvette;

C4.3.1.4 The first measurement is started after 63 s of reaction and then measure once per 18 s intervals.Total of 9 absorbances are measured for each sample.

C.4.3.2 Parameters

C.4.3.2.1 Equilibrium substrate

Temperature: 37℃;

Heat preservation time: 480 s;

Amount of the substrate added: 160 μL;

C.4.3.2.2 Enzyme reaction cycle

Temperature: 37℃;

Time: 63 s;

Sample: 20 μL.

C.4.3.2.3 Determination cycle

Determination mode: kinetic method;

Wavelength: 405 nm;

Curve type: non-linear;

Time: 150 s;

Readings: 9 times;

Interval: 18 s.

C.5 Calculation

C.5.1 Calculation of standard curve

The standard curve was drawn with the change rate of absorbance (OD / min) as the ordinate and the enzyme activity (U / L) of the standard working solution of protease as the abscissa. The standard curve should be a smooth and concave curve..

C.5.2 Calculation of enzyme activity of sample

documentThe enzyme activity of the sample is calculated according to formula (C.1):

$$X_3 = \frac{A_3 \times V_3 \times n_3}{m_3 \times 1000} \cdots\cdots\cdots\cdots\cdots\cdots\cdots \quad (C.1)$$

Where:

X_3—— the enzyme activity of sample, in the unit of U/g;

A_3—— the activity of the final dilution of the sample obtained from the document curve, in the unit of U/L;

V_3—— the volume of volumetric flask used to dissolve the sample, in the unit of mL;

n_3—— dilution multiple;

m_3——Mass of sample, in the unit of grams (g);

1000 —— Conversion coefficient per milliliter of enzyme activity unit and per liter of enzyme activity unit.

C.5.3 Expression of results

The measurement results of the samples are expressed as the arithmetic mean with three significant figures.documentdocumentdocument

C.6 Precision

The absolute difference between two independent determinations obtained under repeatability conditions should not exceed 5% of the mean value.

Annex D

(Normative)

Determination of protease activity - Microcolorimetric method

D.1 Principles

Under certain temperature (Such as 70 ℃) and pH conditions, protease hydrolyzes the substrate (N-succinyl-propionyl-propionyl-prolyl-phenylalanine p-nitroaniline) to produce yellow p-nitroaniline. The absorbance of the solution is measured with a spectrophotometer at a wavelength of 414 nm. The enzyme activity is proportional to the absorbance, from which the enzyme activity of the product can be calculated.

D.2 Apparatus and Equipment

D.2.1 Analytical balance: Sensitivity is 0.1 mg.
D.2.2 UV-VIS spectrophotometer is equipped with a 200 uL microcuvette.
D.2.3 Thermostatic water bath.
D.2.4 pH meter: accuracy of 0.01.

D.3 Reagents and solutions

D.3.1 Sodium hydroxide solution (10 mol/L)

Weigh 40.0 g of sodium hydroxide, add 90 mL of water and stir to dissolve. Allow the solu tion to reach room temperature and then dilute with water to 100 mL, and stir well.

D.3.2 HEPES buffer (0.1 mol/L, pH=7.5)

Accurately weigh 5.98 g of 4-hydroxyethylpiperazineethiosulfonic acid in 200 mL of water, stir to dissolve, adjust the pH of the solution to 7.5 ± 0.05 with 10 mol/L sodium hydroxide solution, and dissolve with water to 250 mL.

Note: Manufacturers and users may also explore the use of other suitable buffer systems.

D.3.3 Substrate stock solution (2.0 mmol/L)

Weigh 10.0 mg of N-succinyl-propionamide-propionamide-prolinamide-phenylalanine-p-nitroaniline in a centrifuge tube, add 8 mL of HEPES buffer (D.3.2) , heat in a water bath at 60℃ for 30 min ~ 90 min. After complete dissolution of the substrate, transfer it into an ice water bath for 5 minutes. At this time, the substrate solution should be a translucent and uniform liquid. If the solution turns yellow, it indicates that the substrate has been decomposed and needs to be prepared again. This solution is stored in an ice-water bath. It can be stored at -20℃ for 30 days and cannot be repeatedly frozen and thawed.

D.3.4 Protease document stock solution (40 u/mL)

Weigh an appropriate amount of protease document with known enzyme activity, accurate to 0.1mg, dissolve it with HEPES buffer (D.3.2) and dilute to 10 mL, so that the concentration of protease document solution is 40 u/mL.

D.4 Analytical procedure

D.4.1 Substrate use solution

A certain amount of HEPES buffer solution (D.3.2) was added to the substrate reserve solution (D.3.3), and then 20 μL of the diluted substrate solution was added to 980 μL of HEPES

buffer solution until the mixture was mixed. The absorbance was measured at 313 nm wavelength between 0.38 and 0.40.

D.4.2 Preparation of standard curve

D.4.2.1 Protease series standard solutions: prepared according to Table D.1.

Table D.1 Protease series standard solution

Tube No.	Concentration of protease standard solution U/mL	The volume of standard stock solution (D.3.4) μL	Volume of buffer solution (D.3.2) μL
0	0	0	1000
1	1.4	35	965
2	1.8	45	955
3	2.4	60	940
4	3.6	90	910
5	4.4	110	890
6	5.8	145	855

D.4.2.2 Preheating : Put the standard solution of protease series into 70 ℃ ± 0.2 ℃ constant temperature water bath, preheat for 90 min, and inactivate other proteases in the solution.

D.4.2.3 Operate according to the Table D.2 procedure:

Table D.2 Operating procedure

Procedure	Tube A (blank)	Test tube B (standard solution, need to do 3 parallel samples)
1	45 μL Protease Series Standard Solution	45 μL Protease Series Standard Solution
2	120 μL substrate solution	120 μL , Accurate reaction 20min
3	/	70℃±0.2℃, react accurately for 20 min;
4	Quickly transfer to ice water bath and hold for 2 min.	
5	The absorbance of test tube B was measured at 414 nm in 100 μL to 10 mm micro-cuvette with test tube A as blank; The standard curve was drawn with absorbance as ordinate and enzyme activity concentration as abscissa.	

D.4.3 Determination of samples

D.4.3.1 Preparation of enzyme solutions to be tested

Weigh 1.0 g of protease sample, accurate to 0.0001 g. Add 80 mL of corresponding buffer solution, stir to dissolve for not less than 30 min, then transfer to a 100 mL volumetric flask, dilute to volume with corresponding buffer solution and mix well. Transfer an appropriate amount of enzyme solution, add the corresponding buffer solution to dilute it to a certain concentration. The recommended concentration range is 1.5 U/mL- 5 U/mL for enzyme activity.

D.4.3.2 Determination

For the heat-resistant protease sample, prior to the determination step, firstly place the sample solution in a constant temperature water bath at 70℃ ± 0.2℃, preheat for 90 min,

so that other proteases in solution are inactivated, Operate according to theTable D.3 procedure.

Table D.3 Ppearting procedure

Procedure	Tube A (blank)	Tube B (sample solution, it is necessary to make three parallel samples)
1	45 uL sample solution	45 uL sample solution
2	120 uL substrate solutions	120 uL substrate solutions
3	/	70℃±0.2℃, react accurately for 20 min;
4	Quickly transfer to the ice-water bath and keep for 2 min;	
5	Pipette 100 μL into a 10 mm microcuvette, respectively, and measure the absorbance of tube B at 414 nm with tube A as blank.	

D.4.4 Calculation

The enzyme activity of the sample was calculated according to Equation (D.1) :

$$X_4 = \frac{A_3 \times V_3 \times n_4}{m_4} \cdots\cdots\cdots\cdots\cdots\cdots\cdots \quad (D.1)$$

Where:

X_4—— the enzyme activity of sample, in the unit of U/g;

A_3—— the vitality of the final diluent of the sample obtained from the standard curve., in the unit of U/mL;

V_3—— the volume of volumetric flask used to dissolve the sample, in the unit of mL;

n_4—— dilution ratio of sample;

m_4—— Mass of sample, in the unit of grams (g);

The results are expressed to integers.

D.5 Precision

The absolute difference between two independent determinations obtained under repeatability conditions should not exceed 5% of the mean value.