SERINE PROTEASE WITH TRYPSIN SPECIFICITY FROM *FUSARIUM OXYSPORUM* EXPRESSED IN *FUSARIUM VENENATUM*

New specifications prepared at the 76th JECFA (2012) and published in FAO JECFA Monographs 13 (2012). An ADI "not specified" was established at the 76th JECFA (2012)

SYNONYMS α-trypsin; β-trypsin; cocoonase; parenzyme; parenzymol; tryptar; trypure; pseudotrypsin; tryptase; tripcellim; sperm receptor hydrolase

SOURCES Produced by submerged fermentation of a genetically modified non-pathogenic and non-toxigenic strain of *Fusarium venenatum* which contains a gene coding for serine protease with trypsin specificity from *Fusarium oxysporum.* The enzyme is secreted into the broth. The cell mass and other solids are secreted into the fermentation broth, separated by vacuum drum filtration or centrifugation. Ultrafiltration and/or evaporation are applied for concentration and further purification. Residual production strain microorganisms are removed by germ filtration. The final product is formulated using food-grade stabilizing and preserving agents and is standardized to the desired activity.

Active principles: Serine protease with trypsin specificity

Systematic names and numbers: EC 3.4.21.4, CAS number: 9002-07-7

Reactions catalysed: Preferential cleavage: Arg, Lys

Secondary enzyme activities: None

DESCRIPTION Brown liquid.

FUNCTIONAL USES Enzyme preparation.
Used in the production of partially or extensively hydrolyzed proteins of vegetable and animal origin. These hydrolyzed proteins may be used for various applications as ingredients in food and beverages, for protein fortification or as ingredients providing functional effects such as emulsification or flavour enhancement.

GENERAL SPECIFICATIONS Must conform to the current edition of the JECFA General Specifications and Considerations for Enzyme Preparations Used in Food Processing.

CHARACTERISTICS

IDENTIFICATION

Serine protease activity with trypsin specificity

The sample shows serine protease activity with trypsin specificity.
See descriptions under TESTS

TESTS

Serine protease activity with trypsin specificity

Principle:
Serine protease with trypsin specificity hydrolyses the substrate Ac-Arg-p-nitro-Anilide (Ac-Arg-*p*NA). The release of *p*-nitroaniline (*p*NA) results in an increase of absorbance at 405 nm and is proportional to the enzyme activity. Enzyme activity is measured in Kilo Microbial Trypsin Units (KMTU). 1 KMTU is the amount of enzyme that releases 1μmol of *p*-nitroaniline from 1mM substrate (Ac-Arg-pNA) per minute at pH 9.0 and temperature 37°.

Reagents and Solutions:

Preparation of 15% Brij 35 solution:
Bring the stock Brij 35 (30% solution) to room temperature. Pour out 1L into a 2L volumetric flask. Make up the volume with demineralized water. Stir vigorously. Store in a bottle with label up to 2 months at 4°.

Preparation of 1M Tris buffer:
Weigh and transfer 121.1g Tris (tris(hydroxymethyl)-aminomethane) into a 1L volumetric flask. Add about 800 ml of deionised water and stir on a magnetic stirrer until dissolved. Make up the volume with deionised water. This solution can be stored at room temperature for up to 1 month.

Preparation of dilution buffer (1.5 mM $CaCl_2$, 0.225 g/l Brij, 100 mM Tris, pH 8.0):

Example
Volume, 10 L: Weigh and transfer 2.20 g $CaCl_2.2H_2O$ into a 10 L volumetric flask. Add 1000 ml of 1 M Tris buffer. Add 15 ml of 15% Brij 35 solution, approximately 7000 ml of deionised water, and 275 ml of 2M hydrochloric acid. Mix until completely dissolved. Ensure that the solution temperature is between 23° to 25°. Adjust pH to 8.00±0.05. Make up the volume with deionised water. Mix thoroughly. This solution can be stored at room temperature up to 8 days. Ensure that the dilution buffer is adjusted to 23-25° prior to use by stirring.

Preparation of 5 mM Ac-Arg-*p*NA solution (Substrate Solution):

It is important to obtain a high quality substrate as well as to prepare, store and measure the substrate solution accurately. This affects the quality of analysis. Frozen substrate must be thawed completely before use, and discarded after.

Weigh and transfer quantitatively 186.0±0.2 mg Ac-Arg-*p*NA into a 100 ml volumetric flask. Make up the volume with dilution buffer. Wrap the flask immediately with aluminum foil to protect from light. Mix for 5 min (max of 10 min). Aliquot the solution into tubes of 10 ml protected from light. Solution can be stored frozen up to 28 days (no yellow colouring appears).
It is recommended to store at least 6 stock samples (at 1g aliquots) per lot for enabling bridging studies.

<u>Preparation of Standard and Sample Solutions:</u>

Preparation of stock standard solution:
Weigh and transfer an amount corresponding to 20.00 KMTU (±0.02 KMTU) of the serine protease with trypsin specificity standard into a 250 ml volumetric flask. Make up the volume with dilution buffer. Mix the solution for about 15 min.

Preparation of sample

Weigh between 0.5 g to 1.78 g of the serine protease with trypsin specificity sample. Ensure that samples are homogeneous. Dilute in a measuring flask with dilution buffer. NOTE: A typical dissolution volume is 250 ml of diluent buffer.

Stock sample solutions can be stored up to 8 h at room temperature. The stock sample solution is further diluted with the dilution buffer, to reach an activity of about 4.7 mKMTU/ml after final dilution. This solution can be stored at room temperature, covered, for up to 6 h.

Procedure

Prepare a standard curve using the stock standard solution as shown below:

Standard #	Dilution Ratio	Stock standard solution, µl	Dilution Buffer, µl	Concentra mKMTU/m
1	50	30	1470	1.6
2	25	60	1440	3.2
3	15	100	1400	5.3
4	12	125	1375	6.7
5	10	150	1350	8.0

Prepared Working standard solutions can be stored, covered, up to 6 h at room temperature.

Place the 5 mM Ac-Arg-*p*NA solution (substrate solution) in a water bath set to 37.0±1.0°. Set the spectrophotometer at 405 nm and the temperature of the cuvette holder at 37.0±0.5°. Pipette 2.5 ml of it into the cuvette. Add 340 ml of the working standard solution, reference standard working solution or sample to the cuvette. Place the cuvette in the spectrophotometer set to 37.0±0.5°. Set and start stopwatch to 12 sec. Read absorbance at 20 sec intervals for 3.5 min at 405 nm.

Calculations

Calculate the average absorbance per minute for each standard via linear regression. Plot the standard curve using the average absorbance per minute calculated against the activity of the standards (mKMTU/ml). Read the absorbance of the sample from the standard curve generated and calculate the enzyme activity as shown below:

$$Activity, KMTU / g = \frac{S \times V \times F}{W \times 1000}$$

where, S, is the reading in mKMTU/ml, from the standard curve, V is the volume of the measuring flask used in ml, F is the dilution factor, W is the weight of the sample in g and 1000 is the conversion factor from mKMTU to KMTU.