ASPARAGINASE FROM *ASPERGILLUS NIGER* EXPRESSED IN *A. NIGER*

New specifications prepared at the 69th JECFA (2008), published in FAO JECFA Monographs 5 (2008). An ADI "not specified" was established at the 69th JECFA (2008).

SYNONYMS

Asparaginase II; L-asparaginase; α-asparaginase

SOURCES

Asparaginase is produced by submerged fed-batch fermentation of a genetically modified strain of *Aspergillus niger* which contains the asparaginase gene derived from *A. niger*. The enzyme is isolated from the fermentation broth by filtration to remove the biomass and concentrated by ultrafiltration. The enzyme concentrate is subjected to germ filtration and is subsequently formulated and standardized to the desired activity using food-grade compounds.

Active principles: Asparaginase

Systematic names and numbers: L-Asparagine amidohydrolase; EC 3.5.1.1; CAS No. 9015-68-3

Reactions catalysed: Hydrolysis of L-asparagine to L-aspartic acid and ammonia

Secondary enzyme activities: No significant levels of secondary enzyme activities.

DESCRIPTION

Yellow to brown clear liquid or off-white granulates

FUNCTIONAL USES

Enzyme preparation.
Used in food processing to reduce the formation of acrylamide from asparagine and reducing sugars during baking or frying.

GENERAL SPECIFICATIONS

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

CHARACTERISTICS

IDENTIFICATION

Asparaginase activity: The sample shows asparaginase activity.
See description under TESTS.

TESTS

Asparaginase activity

Principle
Asparaginase catalyses the conversion of L-asparagine to L-aspartic acid and ammonia. The liberated ammonia subsequently reacts with phenol nitroprusside and alkaline hypochlorite resulting in a blue colour (known as Berthelot reaction). The activity of asparaginase is determined by measuring the absorbance of the reaction mixture at 600 nm.

The asparaginase activity is expressed in ASPU units. One ASPU is defined as the amount of the enzyme required to liberate one micromole of ammonia from L-asparagine per minute under the conditions of the assay (pH=5.0; 37^{o}).

Note: The measuring range of the method is 1.5 – 12 ASPU/ml.

Apparatus
Spectrophotometer (600 nm)
Water bath with thermostatic control (37±0.1^{o})
pH meter
Vortex mixer
Magnetic stirrer
Disposable culture tubes (glass, 10x100 mm)

Reagents and solutions
(Note: use Ultra High Quality water with conductivity of ≤ 0.10 µS/cm)

Phenol nitroprusside solution (Sigma-Aldrich P6994 or equivalent)

Sodium hypochlorite 0.2% in alkali solution (Sigma-Aldrich A1727 or equivalent)

Sodium hydroxide solution 4 M: Weigh 160 g of NaOH pellets. Dissolve in approximately 800 ml of water in a 1 l volumetric flask. Cool down to room temperature, add water to volume and mix until fully dissolved. The solution is stable for 3 months at room temperature.

Citric acid dilution buffer 0.1M, pH 5.00±0.03: Weigh 21.01 g of citric acid monohydrate (analytical reagent grade). Dissolve in approximately 900 ml of water in a 1 l volumetric flask. Adjust the pH to 5.00±0.03 with 4 M NaOH. Add water to volume and mix. The solution is stable for 1 month when stored in a refrigerator.

L-asparagine substrate solution: Weigh 1.50 g of L-asparagine (L-asparagine monohydrate ≥ 99%, Sigma-Aldrich A8381 or equivalent). Dissolve in approximately 80 ml of the citric acid dilution buffer in a 100 ml volumetric flask and stir on a magnetic stirrer until completely dissolved. Add the dilution buffer to volume and mix. The solution should be freshly prepared before the analysis.

TCA stop solution: Weigh 25 g of trichloroacetic acid (Sigma-Aldrich 27242 (Riedel-de Haen) or equivalent). Dissolve in approximately 80 ml of water in a 100 ml volumetric flask. Add water to volume and mix. The solution is stable for 1 year at room temperature.

Standard solution: Weigh to ± 0.1 mg approximately 3.9 g of ammonium sulfate (analytical reagent grade) with an officially certified content. Dissolve in approximately 40 ml of the citric acid dilution buffer in a 50 ml volumetric flask by stirring on a magnetic

stirrer for about 15 min. Add the dilution buffer to volume and mix. Make five dilutions with the dilution buffer and calculate the concentration of each dilution based on the certified content of ammonium sulfate. The table below provides an example.

Label	Dilution factor	Concentration, mg/ml
S1	60	1.3
S2	30	2.6
S3	10	7.8
S4	6	13.0
S5	4	19.5

Control sample solution: Weigh to ± 0.1 mg an amount of an asparaginase preparation with known activity (for example, 18930 ASPU/g; batch KFP0445A/DIV/4; expiration date January 2020; available from DSM Food Specialties) approximately equivalent to 4000 ASPU. Dissolve in approximately 20 ml of the citric acid dilution buffer in a 25 ml volumetric flask. Add the dilution buffer to volume, and mix. Dilute the solution with the dilution buffer to a final activity of approximately 6 ASPU/ml.

Test sample solution: Weigh to ± 0.1 mg approximately 2.5 g of an asparaginase preparation. Dissolve in approximately 20 ml of the citric acid dilution buffer in a 25 ml volumetric flask. Add the dilution buffer to volume and mix. Dilute the solution with the dilution buffer to a final activity of approximately 6 ASPU/ml.

Procedure
Standard curve:

1. Label five test tubes according to the concentrations of the standard solutions (S1 to S5). Pipette 2.0 ml of the substrate solution to each tube. Incubate in the water bath for 10 minutes. To each tube, add 100 µl of the appropriate standard solution and mix. Incubate the tubes in the water bath exactly for 30 min. Add 0.4 ml of the TCA stop solution to stop the reaction. Add 2.5 ml of water and mix. This is the reaction mixture.

2. Prepare five test tubes (labeled S1 to S5). Add to each tube 800 µl of water and 20 µl of the appropriate reaction mixture. To develop colour, add 170 µl of the phenol nitroprusside solution, mix and add 170 µl of the alkaline sodium hypochlorite solution. Mix and incubate in the water bath for 10 min. Transfer the content of each tube to the spectrophotometer cuvette and measure the absorbance at 600 nm after zeroing the instrument against air.

3. Use linear regression to prepare the standard curve. Plot the absorbance against the concentration of ammonium sulfate in

the standard solutions (mg/ml). Use the slope of the standard curve (ml/mg) to calculate the activity of the control and test samples.

(NOTE: The standard curve should be prepared immediately prior to sample analysis.)

Control and test samples:

1. For all control and test samples, follow the procedure described in steps 1 and 2 above for the standard solutions.

2. Use a blank for each control and test sample. To prepare the blank, pipette into a test tube 2.0 ml of the substrate solution and 0.4 ml of the TCA stop reagent. Mix and add 100 µl of either the control or test sample solution. Mix and incubate in the water bath for 30 min. Add 2.5 ml of water and continue as described in step 2 of the procedure for the standard solutions.

Calculations

Calculate the activity of each control and test sample in activity units per gram of the enzyme preparation (ASPU/g) using the following formula:

$$\text{ASPU/g} = \frac{A \times V \times Df \times 2 \times 10^6}{a \times M \times W \times 30 \times 10^3}$$

Where:

A is the absorbance of the sample minus the absorbance of the blank

V is the initial volume of the sample solution (25 ml)

Df is the dilution factor

2 accounts for 2 moles of ammonia per 1 mole of ammonium sulfate

10^6 is the conversion factor from moles to µmoles

a is the slope of the standard curve (ml/mg)

M is the molar mass of ammonium sulfate (132.14 g/mol)

W is the sample weight (g)

30 is the reaction time (min)

10^3 is the conversion factor from milligrams to grams