Amendment to the Enforcement Ordinance of the Food Sanitation Law and the Standards and Specifications for Foods and Food Additives

The government of Japan will designate Asparaginase from *Aspergillus oryzae* expressed in *Aspergillus oryzae* as an authorized food additive.

Summary

Under Article 10 of the Food Sanitation Law (hereinafter referred to as the "Law"), food additives shall not be used or marketed without authorization by the Minister of Health, Labour and Welfare (hereinafter referred to as "the Minister"). In addition, when specifications or standards are established for food additives based on Article 11 of the Law and stipulated in the Ministry of Health, Labour and Welfare Notification (Ministry of Health and Welfare Notification No. 370, 1959), those additives shall not be used or marketed unless they meet the standards or specifications.

In response to a request from the Minister, the Committee on Food Additives of the Food Sanitation Council that is established under the Pharmaceutical Affairs and Food Sanitation Council has discussed the adequacy of the designation of Asparaginase from *Aspergillus oryzae* expressed in *Aspergillus oryzae* as a food additive. The conclusion of the committee is outlined below.

Outline of conclusion

The Minister, based on Article 10 of the Law, should designate Asparaginase from *Aspergillus oryzae* expressed in *Aspergillus oryzae*, as a food additive unlikely to harm human health, and establish standards for use and compositional specifications, based on Article 11 of the Law (see Attachment).

Attachment

Asparaginase

アスパラギナーゼ

Revision of regulations

Part of the definition and the specifications for Asparginase (*A. oryzae* NZYM-SP-derived) will be added by revision this time. The reagents and test solutions (TS) newly added for testing of *A. oryzae* NZYM-SP-derived asparaginase are given in the last part of this document.

Standards for Use

Not established.

Compositional specifications

Definition Asparaginase is derived from the filamentous fungi (limited to *Aspergillus niger* ASP-72 and *Aspergillus oryzae* NZYM-SP), in which asparaginase productivity is improved by amplifying the asparaginase gene intrinsically occurring in *A. niger* and *A. oryzae.* It is an enzyme that hydrolyzes asparagine into aspartic acid and ammonia. There are two types of Asparaginase: *A. niger* ASP-72-derived and *A. oryzae* NZYM-SP-derived products. It may contain glycerine, dextrin, maltodextrin, salt, or wheat flour.

Asparaginase (*A. niger* ASP-72-derived)

Enzyme Activity Asparaginase has an enzyme activity of not less than 2375 units per gram or milliliter.

Description Asparaginase occurs as a clear, yellow to brown liquid or as pale gray or slightly yellowish white granules.

Identification When tested by the enzyme activity determination, Asparaginase shows activity.

Purity

(1) Lead　Not more than 5.0 μg/g as Pb.

Test Solution　Weigh 0.8 g of Asparaginase into a platinum, quartz, or porcelain crucible or a quartz beaker, and moisten it with a small amount of diluted sulfuric acid (1 in 4). Heat it by increasing the temperature gradually until the sample is carbonized and the white fumes of sulfuric acid are no longer evolved. If necessary, add diluted sulfuric acid (1 in 4) again, and heat until the sample is almost carbonized. For a liquid sample or a sample that is hard to be carbonized, concentrated sulfuric acid may be used, instead of diluted sulfuric acid (1 in 4). After the sample is carbonized, lid the crucible or beaker loosely if necessary, heat in an electric furnace by increasing the temperature gradually, and ignite at 450–600°C to incinerate. If any carbonized residue is present, crush the residue with a glass rod if necessary, moisten with 1 ml of diluted sulfuric acid (1 in 4) and 1 ml of nitric acid, heat until the white fumes of sulfuric acid are no longer evolved, and ignite in the electric furnace to completely incinerate it. To residue, add 10 ml of diluted hydrochloric acid (1 in 4), heat on a water bath, and evaporate to dryness. To the residue, add a small amount of diluted nitric acid (1 in 100), and warm to dissolve it. After cooling, add diluted nitric acid (1 in 100) to make exactly 10 ml.

When incineration is done at 500°C or below, a heat-resistant glass beaker can be used.

Control Solution　To 1 ml of Lead Standard Stock Solution, exactly measured, add water to make exactly 100 ml. To exactly measured 4 ml of this solution, add diluted nitric acid (1 in 100) to make 10 ml.

Procedure　Proceed as directed in Method 1 of the Lead Limit Test.

(2) Arsenic　Not more than 4.0 μg/g as As_2O_3 (0.50 g, Method 3, Apparatus B).

Microbial Limits　Proceed as directed under Microbial Limit Tests. The total bacterial count is not more than 50,000/g. *Escerichia coli* and *Salmonella* are negative. For the *Salmonella* test, proceed as directed in the microbial limit tests for Nisin.

Enzyme Activity Determination

(i) Substrate Solution　Weigh 1.50 g of L-asparagine monohydrate, add citric acid–sodium hydroxide buffer (pH 5.0), and dissolve it completely by stirring. Add citric acid–sodium hydroxide buffer (pH 5.0) again to make exactly 100 ml. Prepare fresh before use.

(ii) Sample Solution　Weigh accurately about 2.5 g of Asparaginase, dissolve in 20 ml of citric acid–sodium hydroxide buffer (pH 5.0), and then add citric acid–sodium hydroxide buffer (pH 5.0) to make exactly 25 ml. Dilute this solution with citric acid–sodium hydroxide buffer (pH 5.0) to prepare a solution containing 6 units/ml.

(iii) Control Stock Solution Weigh an amount of *A. niger*-derived asparaginase for enzyme activity determination equivalent to 4000 units, dissolve in 20 ml of citric acid–sodium hydroxide buffer (pH 5.0) , and then add citric acid–sodium hydroxide buffer (pH 5.0) to make exactly 25 ml. Dilute this solution with citric acid–sodium hydroxide buffer (pH 5.0) to prepare a solution containing 6 units/ml.

(iv) Ammonium Sulfate Standard Solutions Weigh accurately about 3.9 g of ammonium sulfate, add 40 ml of citric acid–sodium hydroxide buffer (pH 5.0), and shake for 15 minutes. Again add citric acid–sodium hydroxide buffer (pH 5.0) to make 50 ml. Dilute this solution with citric acid–sodium hydroxide buffer (pH 5.0) 4, 6, 10, 30, and 60 times, respectively, to prepare five standard solutions.

(v) Procedure

Test Solution and Control Solution Place 2.0 ml portions of the substrate solution into two separate test tubes, and warm them at 37°C for 10 minutes. Add 0.100 ml of the sample solution to one test tube and 0.100 ml of the control stock solution to the other, and stir. Warm them at 37°C exactly for 30 minutes, add 0.400 ml portions of trichloroacetic acid solution (1 in 4) to them, stir, and add 2.5 ml of water to each, and again stir. Take 0.100 ml from each test tube, and add 4.0 ml of water and 0.850 ml of basic phenol–nitroprusside TS, and stir. Add 0.850 ml of sodium hypochlorite–sodium hydroxide TS for *A. niger* -derived asparaginase activity determination to each, and allow them to stand at 37°C for 10 minutes. Use them as the test solution and the control solution, respectively.

Reference Solutions Place 2.0 ml portions of the substrate solution into two separate test tubes, and add 0.400 ml portions of trichloroacetic acid solution (1 in 4) to them, and stir. Add 0.100 ml of the sample solution to one test tube and 0.100 ml of the control stock solution to the other, stir, and warm them at 37°C for 30 minutes. Add 2.5 ml of water to each, and stir again. Take 0.100 ml from each test tube, and add 4.00 ml of water and 0.850 ml of phenol-nitroprusside (basic), and stir. Add 0.850 ml of sodium hypochlorite–sodium hydroxide TS for *A. niger*-derived asparaginase activity determination to each, allow them stand at 37°C for 10 minutes. Use them as the reference solutions for the test solution and the control solution, respectively.

Calibration Curve Place 2.0 ml portions of the substrate solution into five separate test tubes, and warm them at 37°C for 10 minutes. To each test tube, add 0.100 ml portion of each of the ammonium sulfate standard solutions with different concentrations, instead of the sample solution, and proceed as directed for the test solution. Measure the absorbance of them against water at 600 nm. Prepare a calibration curve using the absorbance values obtained and the ammonium sulfate concentrations in the ammonium sulfate standard solutions.

Determination Measure the absorbance (A_T and A_C) of the test solution and the control solution against water at 600 nm. Also measure the absorbance (A_{BT} and A_{BC}) of the reference solutions for the test solution and the control solution against water at 600 nm. Measure the slope, a (ml/mg), of the calibration curve. Calculate the enzyme activity of *A. niger*-derived asparaginase for enzyme activity determination used for the preparation of the control solution by the formula given below. When the obtained activity is in the range of 91 to 109% of the labeled value, determine the enzyme activity of the sample also by the formula. One unit of enzyme activity is equivalent to the amount of the enzyme required to liberate 1 μmol of ammonia per minute from L-asparagine when the enzyme activity is determined as directed in the Procedure.

$$\text{Enzyme activity (unit/g)} = \frac{A \times D_f \times 25 \times 2 \times 10^3}{a \times W \times 132.14 \times 30}$$

A = the value obtained by deducting the absorbance (A_{BT} or A_{BC}) of the corresponding reference solution from the absorbance (A_T or A_C) of the test solution or the control solution, whichever is appropriate,

D_f = the dilution factor of the sample solution or the control stock solution,

W = the weight (g) of the sample or *A. niger*-derived asparaginase for enzyme activity determination.

Asparginase (*A. oryzae* NZYM-SP-derived)

Enzyme Activity Asparaginase has an enzyme activity of not less than 3,500 units per gram or milliliter.

Description Asparaginase occurs as a light brown liquid or as white to grayish white granules.

Identification When tested by the enzyme activity determination, Asparaginase shows activity.

Purity

(1) Lead Not more than 5.0 μg/g as Pb.

Weigh 0.8 g of Asparaginase, and proceed as directed in Purity (1) for Asparaginase (*A.* niger ASP-72-derived).

(2) Arsenic Not more than 4.0 μg/g as As_2O_3 (0.50 g, Method 3, Apparatus B).

Microbial Limits Proceed as directed under Microbial Limit Tests. The total bacterial count is not more than 50,000/g. *Escerichia coli* and *Salmonella* are negative. For the

Salmonella test, proceed as directed in the microbial limit test for Nisin.

Enzyme Activity Determination

(i) Substrate Solution Weigh 0.25 g of L-asparagine monohydrate, add 15 ml of MOPS buffer (0.1 mol/L, pH7.0), and dissolve it completely by stirring. Cover the container to block out light. Refer the resulting solution to as Solution A. To Solution A, add 0.011 g of β-nicotinamide adenine dinucleotide disodium salt hydrate (reduced form), 0.063 g of disodium 2-ketoglutarate, and an appropriate amount of L-glutamic acid dehydrogenase (bovine liver-derived) equivalent to not less than 1680 units, and stir well to dissolve them. Add MOPS buffer (0.1 mol/L, pH7.0) to make exactly 25 ml. Prepare fresh before use.

(ii) Sample Solution Weigh accurately about 1.0 g of Asparaginase, and dissolve it in acetate buffer (0.1 mol/L, pH5.0, containing polyoxyethylene(23) lauryl ether) to make exactly 100 ml. Dilute this solution with acetate buffer (0.1 mol/L, pH5.0, containing polyoxyethylene(23) lauryl ether) to prepare a solution containing 0.6 units/ml.

(iii) Standard Stock Solutions Weigh an amount of *A. oryzae*-derived aparaginase for enzyme activity determination equivalent to 775 units, dissolve in acetate buffer (0.1 mol/L, pH5.0, containing polyoxyethylene(23) lauryl ether) to make exactly 100 ml. Dilute this solution with acetate buffer (0.1 mol/L, pH5.0, containing polyoxyethylene(23) lauryl ether) 8, 10, 15, 20, and 30 time to prepare five solutions containing 0.9688, 0.7750, 0.5167, 0.3875, and 0.2583 units/ml, respectively.

(iv) Procedure

Test Solution Transfer 4.6 ml of the substrate solution into a test tube, and warm at 37.0 ± 0.5°C for 8 minutes. Add 0.400 ml the sample solution, shake, and warm at 37.0 ± 0.5°C for 90 seconds.

Standard Solutions Transfer 4.6 ml portions of the substrate solution into five separate test tubes, and warm at 37.0 ± 0.5°C for 8 minutes. To each test tube, add 0.400 ml of each of the standard stock solutions with different concentrations, instead of the sample solution, and proceed as directed for the test solution.

Calibration Curve Measure the absorbance of the standard solutions against water at 340 nm and prepare the calibration curve from the absorbance values obtained and the enzyme activity in 1 ml (unit/ml) of each standard stock solution.

Determination Measure the absorbance, A, of the test solution against water at 340 nm. Determine the enzyme activity, U (unit/ml), of the sample solution from absorbance A and the calibration curve. Then calculate the enzyme activity of the sample by the following formula. One unit of enzyme activity is equivalent to the

amount of the enzyme required to liberate 1 μmol of ammonia per minute from L-asparagine when the enzyme activity is determined as directed in the procedure.

$$\text{Enzyme activity (unit/g)} = \frac{\text{U} \times \text{D} \times 100}{\text{Weight (g) of the sample}}$$

U = the enzyme activity of the sample solution,

D = the dilution factor of the sample solution.

Reagents and Test Solutions (TS)

Acetate Buffer (0.1 mol/L, pH5.0, containing polyoxyethylene(23) lauryl ether)
To 500 ml of acetate buffer (1 mol/L, pH 5.0), add 3500 ml of water and 7.5 ml of polyoxyethylene(23) lauryl ether TS. Adjust the pH to 5.0 with sodium hydroxide solution of an appropriate concentration, and dilute with water exactly to 5000 ml.

Acetate buffer (1 mol/L, pH 5.0) Dissolve 88.8 g of sodium acetate trihydrate in 1800 ml of water. Adjust the pH to 5.0 with acetic acid and dilute with water to exactly 2000 ml.

Asparaginase (*A. niger*-derived) for Enzyme Activity Determination
Obtained from the filamentous fungi, *A. niger* ASP-72, in which asparaginase productivity is improved by amplifying the asparaginase gene intrinsically occurring in *Aspergillus niger*. Occurs as a clear, yellow to brown liquid or as pale gray or slightly yellowish white granules. Has an enzyme activity whose number of units is known. One unit of Asparaginase for Enzyme Activity Determination corresponds to the amount of the enzyme required to liberate 1 μmol of ammonia in one minute at pH 5.0 and at 37°C when L-asparagine as the substrate is used.

Asparaginase (*A. oryzae*-derived) for Enzyme Activity Determination Obtained from the filamentous fungi, *A. oryzae* NZYM-SP, in which asparaginase productivity is improved by amplifying the asparaginase gene intrinsically occurring in *Aspergillus oryzae*. Occurs as a light brown liquid or as white to grayish white granules. It has an enzyme activity whose number of units is known. One unit of this substance is equivalent to the amount of the enzyme required to liberate 1 μmol of ammonia in one minute at pH 5.0 and at 37°C when L-asparagine as the substrate is used.

Disodium 2-ketoglutarate $C_5H_4Na_2O_5$ A white powder. Soluble in water.

L-Glutamic Acid Dehydrogenase (bovine liver-derived) Obtained from bovine liver. It has an enzyme activity whose number of units is known. One unit of enzyme activity

is equivalent to the amount of the enzyme required to liberate 1 μmol of L-glutamic acid per minute at pH 7.3 and at 25°C when 2-ketoglutaric acid as the substrate is used.

3-(*N*-Morpholino)propanesulfonic Acid $C_7H_{15}NO_4S$ A white crystalline powder. Freely soluble in water and practically insoluble in ethanol (99.5).

Melting point 275–280°C.

MOPS Buffer (0.1 mol/L, pH7.0) Dissolve 21 g of 3-(*N*-morpholino)propanesulfonic acid in 900 ml of water. Adjust the pH to 7.0 with sodium hydroxide solution of an appropriate concentration, and dilute with water to exactly 1000 ml.

β-Nicotinamide Adenine Dinucleotide Disodium Salt Hydrate (reduced form) $C_{21}H_{27}N_7Na_2O_{14}P_2$ A white to pale yellow powder. Soluble in water.

Polyoxyethylene(23) Lauryl Ether $(C_2H_4O)_nC_{12}H_{26}O$ Use lauromacrogol specified in the Japanese Pharmacopoeia.

Polyoxyethylene(23) Lauryl Ether TS Add water to 15 g of polyoxyethylene(23) lauryl ether to make 100 ml.

Sodium Hypochlorite–Sodium Hydroxide TS for Asparaginase (*A. niger*-derived) Activity Determination To 2.5 ml of sodium hypochlorite TS, add water to make 10 ml. Standardize the resulting solution using 3 ml of it, as directed in Assay for "Sodium Hypochlorite" in the Monographs, and adjust it to make a solution of 0.32 to 0.38 mol/L sodium hypochlorite. Adjust its pH to 12.5 with sodium hydroxide solution with an appropriate concentration. To 3 ml of this solution, add 85 ml of water, and adjust its pH to 12.5 with sodium hypochlorite solution with an appropriate concentration. Add water to make 100 ml. Store in a cool, dark place.