NISIN

Prepared at the 77th JECFA (2013), and published in FAO JECFA Monographs 14 (2013), superseding specifications for Nisin prepared at the 71st JECFA (2009). An ADI of 0–2 mg/kg bw was established at the 77th JECFA.

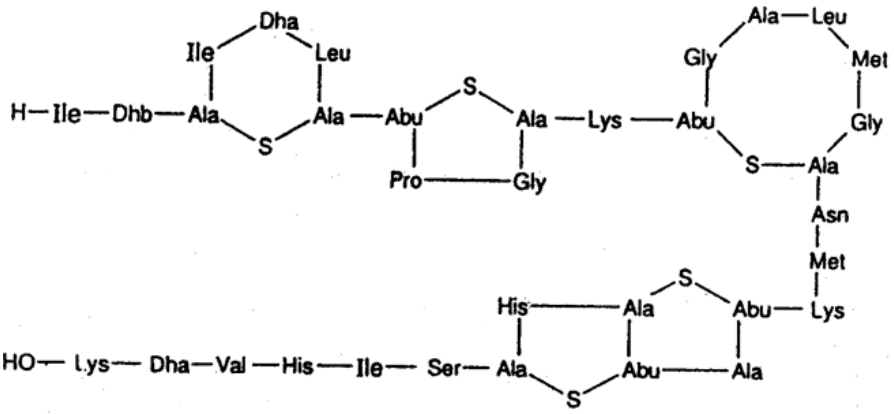
SYNONYMS INS No. 234

DEFINITION Nisin is a mixture of closely related antimicrobial polypeptides produced by strains of *Lactococcus lactis* subsp. *lactis* under appropriate fermentation conditions. The major polypeptide from the fermentation is Nisin A.
Nisin is produced in a sterilized medium of non-fat milk solids or non-milk-based fermentation source, such as yeast extract and carbohydrate solids. The fermentation process is controlled for time and pH, until optimum nisin production has been achieved. The nisin is then concentrated, recovered and purified from the fermentation medium by various methods, such as sterile injection, membrane filtration, acidification, salting out, ultrafiltration or spray-drying. The purified nisin is then standardized with sodium chloride to achieve desired activity levels of nisin preparation.
Nisin is stable at ambient temperatures and when heated under acidic conditions (up to pH 3). Nisin is commercially available as nisin preparation, which contains 2.5% w/w nisin, >50% sodium chloride; the remaining components of the preparation are milk solids and products of fermentation that include proteins and carbohydrates.
The activity of nisin is measured in International Units (IU). 1 IU of nisin is equivalent to 0.025 µg.

C.A.S. Number 1414-45-5

Chemical formula $C_{143}H_{230}O_{37}N_{42}S_7$ (Nisin A)

Structural formula



Abu=alpha-aminobutyric acid, Dha=dehydroalanine, Dhb=dehydrobutyrine (Nisin A)

Formula weight 3354.12 (Nisin A)

Assay Not less than 900 IU of nisin per milligram (or 22.5 microgram/milligram)

DESCRIPTION White to light brown micronized powder

FUNCTIONAL USES Antimicrobial preservative

CHARACTERISTICS

IDENTIFICATION

Solubility (Vol. 4)	Soluble in water and insoluble in non-polar solvents
Differentiation from other antimicrobial substances	Passes tests See description under TESTS
Nisin activity	The sample shows nisin activity See description under METHOD OF ASSAY

PURITY

Loss on drying (Vol. 4)	Not more than 3.0% (105°, 2 h)
Sodium Chloride	Not less than 50% See description under TESTS
Lead (Vol. 4)	Not more than 1 mg/kg Determine using an AAS (Electrothermal Atomization technique) appropriate to the specified level. The selection of sample size and method of sample preparation may be based on principles of methods described in Volume 4 (under “General Methods, Metallic Impurities”).
Microbiological criteria (Vol. 4)	*Salmonella* species: Absent in 25 g of sample Total coliforms: Not more than 30 per gram *Escherichia coli*: Absent in 25 g of sample

TESTS

IDENTIFICATION TESTS

Differentiation from other antimicrobial substances

Stability to acid

Sample stock solution: Suspend 1 g of sample in 1 L of 0.02 N hydrochloric acid to give a solution containing 1000 IU/mL.

Sample Preparation: Make a dilution of the Sample stock solution with 0.02 N hydrochloric acid to arrive at a concentration of 50 IU/ml. Boil this solution for 5 min and measure the nisin activity as directed under ‘Determination of Nisin Activity’, in METHOD OF ASSAY.
The calculated nisin concentration of the boiled sample should be 100% (+/- 5%) of the assay value indicating no significant loss of activity following this heat treatment.

Instability to Alkali

Adjust the pH of the unused portion of the boiled nisin solution from ‘Stability to acid’ to 11.0 by adding 5 N sodium hydroxide. Heat the

solution at 65° for 30 min, and then cool. Adjust the pH to 2.0 by adding hydrochloric acid dropwise. Measure the nisin activity as directed under 'Determination of Nisin Activity' in METHOD OF ASSAY. Record loss of the antimicrobial activity of nisin following this treatment. Complete loss of the antimicrobial activity should be observed following the treatment described.

Tolerance of *Lactococcus lactis* to high concentrations of Nisin
Prepare cultures of *L. lactis* (ATCC 11454, NCIMB 8586) in sterile skim (<1% fat) milk by incubating for 18 h at 30°. Prepare one or more flasks containing 100 ml of litmus milk, and sterilize at 121° for 15 min. Suspend 0.1 g of sample in the sterilized litmus milk, and allow to stand at room temperature for 2 h. Add 0.1 ml of the *L. lactis* culture, and incubate at 30° for 24 h. Record *L. lactis* growth. *L. lactis* will grow at this concentration of sample (about 1000 IU/ml); however, it will not grow in similar concentrations of other antimicrobial substances. (NOTE: This test will not differentiate nisin from subtilin.)

PURITY TESTS

Determination of sodium chloride
Transfer about 200 mg of the sample, accurately weighed, into a glass-stoppered flask containing 50 ml of water. Agitate the flask to dissolve the sample while adding 3 ml of nitric acid, 5 ml of nitrobenzene, 50.0 ml of standardized 0.1 N silver nitrate, and 2 ml of ferric ammonium sulfate TS. Shake the solution well, and titrate the excess silver nitrate with 0.1 N ammonium thiocyanate. The titration endpoint is indicated by the appearance of a red colour. Calculate the percentage of sodium chloride in the sample taken by the equation:

$$\text{Sodium chloride, \%} = \frac{100 \times 58.44 \times [(50 \times A) - (V \times B)]}{W}$$

where
A is the concentration of the silver nitrate solution;
B is the concentration of the ammonium thiocyanate solution;
V is the volume of the ammonium thiocyanate in ml; and
W is the weight of the sample in mg

METHOD OF ASSAY

Principle
Nisin activity, expressed in International Units (IU), refers to the amount of nisin required to inhibit growth of 1 bacterial cell in 1 millilitre of broth. 1 IU of nisin is equivalent to 0.025 µg. Commercial nisin preparations consist typically of 2.5% w/w of nisin along with sodium chloride and milk-fat solids.

Determination of Nisin Activity

Preparation of the test organism
*Lactococcus lactis subsp. cremoris (*ATCC 14365, NCDO 495) is subcultured daily in sterile separated milk by transferring one loopful to a McCartney bottle of litmus milk and incubating at 30°. Prepare inoculated milk for the assay by inoculating a suitable quantity of sterile skim milk with 2 percent of a 24 h culture, and place it in a water-bath at 30° for 90 min. Use immediately.

Standard stock solution
Dissolve an accurately weighed quantity of standard nisin in 0.02 N hydrochloric acid to give a solution containing 5,000 IU/ml. Immediately before use, dilute the solution further with 0.02 N hydrochloric acid to give

50 units/ml. (NOTE: Nisin containing 2.5% w/w nisin, at a minimum potency of 10^6 IU nisin per gram (IU/g) is obtainable from Sigma, St. Louis, USA or Fluka, Buchs, Switzerland. A preparation under the name of Nisaplin, containing at a minimum potency of $3x10^6$ IU/g, of nisin available from DuPont Nutrition Biosciences, Copenhagen, Denmark, may also be used for the Standard stock solution).

Sample solution

Weigh an amount of sample sufficient to ensure that corresponding tubes of the sample and standard series match, i.e., within close limits, so that the nisin content in the sample and standard are similar. Dilute the sample solution in 0.02 N hydrochloric acid to obtain an approximate concentration of 50 IU per ml (IU/ml).

Resazurin solution

Prepare a 0.0125% w/v solution of resazurin in water immediately prior to use.

Procedure

Pipet graded volumes (0.60, 0.55, 0.50, 0.45, 0.41, 0.38, 0.34, 0.31, 0.28, 0.26 ml) of the 50 IU/ml sample and standard solutions into two rows of 10 dry 6-inches x 5/8-inch bacteriological test tubes. Add 4.6 ml of the inoculated milk to each by means of an automatic pipetting device. (NOTE: The addition of inoculated milk should be made in turn across each duplicate row of tubes containing the same nominal concentration, and not along each row of ten tubes). Place the tubes in a water-bath at 30° for 15 min, then cool in an ice-water bath while adding 1 ml resazurin solution to each. Add the resazurin solution in the same order as the addition of inoculated milk, using an automatic pipetting device. Thoroughly mix the contents of the tubes by shaking. Continue incubation at 30° in a water bath for a further 3-5 min.

Examine the standard and sample tubes under fluorescent light in a black matte-finish cabinet. Compare the sample tube of the highest concentration that shows the first clear difference in colour (i.e., has changed from blue to mauve) with tubes of the standard to find the nearest match in colour. Make further matches at the next two lower concentrations of the sample with the standard. Interpolation of matches may be made at half dilution steps. Obtain three readings of the sample solution and average them. Calculate the activity of nisin in the sample from the standard nisin activities.

Convert nisin activity from IU to µg nisin, using the conversion factor 1 IU = 0.025µg