NISIN

Prepared at the 71st JECFA (2009), published in FAO JECFA Monographs 7 (2009), superseding specifications for Nisin Preparation prepared at the 68th JECFA (2007) and published in FAO JECFA Monographs 4 (2007). An ADI of 0-33,000 units/kg bw was established at the 12th JECFA (1968).

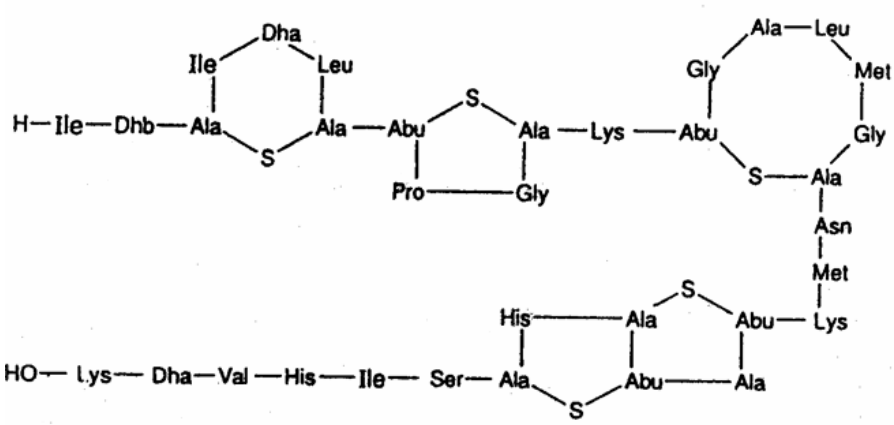
SYNONYMS Nisin preparation; INS No. 234

DEFINITION Nisin is a mixture of closely related antimicrobial polypeptides produced by strains of *Lactococcus lactis* subsp. *lactis*. The major polypeptide is Nisin A. Nisin is produced in a sterilized medium of non-fat milk solids or of a non-milk-based fermentation source, such as yeast extract and carbohydrate solids. Nisin can be recovered from the fermentation medium by various methods, such as injecting sterile, membrane filtration; acidification; salting out; and spray-drying. Non-fat milk solids or solids from other fermentation sources are present in the product. Nisin is available in the commerce as a preparation consisting of Nisin and sodium chloride and is stable at ambient temperatures and upon heating under acid conditions (maximum stability at pH 3).

C.A.S. number 1414-45-5 (Nisin A)

Chemical formula $C_{143}H_{230}N_{42}O_{37}S_7$ (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 and not less than 50% w/w sodium chloride

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

PURITY

Loss on drying (Vol. 4)	Not more than 3.0% (105°, 2 h) (See under “General methods, Inorganic Components.”)
Lead (Vol. 4)	Not more than 1 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the method 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 sample (See Volume 4 under “General Methods, Microbiological Analyses.”)

TESTS

IDENTIFICATION TESTS

Differentiation from other antimicrobial substances

Stability to acid

Suspend a 100-mg sample in 0.02 N hydrochloric acid as described in “Standard stock solution” under Method of Assay. Boil this solution for 5 min and determine the nisin activity as directed under Method of Assay. No significant loss of activity is noted following this heat treatment. The calculated nisin concentration of the boiled sample is 100% (+/- 5%) of the assay value. Adjust the pH of the nisin solution 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. Again determine the nisin concentration as directed under Method of Assay. Complete loss of the antimicrobial activity of nisin is observed following this treatment.

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. *L. lactis* will grow in 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.)

METHOD OF ASSAY **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 units/ml. Immediately before use, dilute the solution further with 0.02 N hydrochloric acid to give 50 units/ml. (NOTE: Nisin containing 2.5% nisin, minimum potency of 10^6 IU/g, obtainable from Sigma, St Louis, USA or Fluka, Buchs, Switzerland, may be used for the Standard stock solution, as well as the preparation under the name of Nisaplin, available from Danisco, Copenhagen, Denmark).

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, the nisin content in the sample and standard is the same. Dilute the sample solution in 0.02 N hydrochloric acid to give an approximate concentration of 50 units of nisin per ml.

Resazurin solution

Prepare a 0.0125% w/v solution of resazurin in water immediately before 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 unit per ml sample and standard solutions into 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 is made in turn across each row of tubes containing the same nominal concentration, 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. Make the addition in the same order as for 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 tubes under fluorescent light in a black matt-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 row of tubes to find the nearest match in colour. Make further matches at the next two lower concentrations of the sample and standard. Interpolation of matches may be made at half dilution steps. As the standard tubes contain known amounts of nisin, calculate the concentration of nisin in the sample solution. Obtain three readings of the solution and average them. Calculate the activity in terms of IU per gram of product.

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. Each ml of reacted 0.1 N silver nitrate is equivalent to 5.844 mg of NaCl. Calculate the percentage of sodium chloride in the sample taken by the equation:

$$\text{Sodium chloride \% (w/w)} = 100 \times 58.44(50 \times A - V \times B)/(W)$$

where

A is the concentration of the silver nitrate solution;
B is the concentration of the ammonium thiocyanate solution;
V is the volume (ml) of the ammonium thiocyanate consumed;
W is the weight of the sample (mg); and
58.44 is the formula weight of sodium chloride.