Food and Agriculture Organization of the United Nations

92nd JECFA - Chemical and Technical Assessment (CTA), 2021

RIBONUCLEASE P ENZYME PREPARATION FROM *PENICILLIUM CITRINUM*

Prepared by Kristie Laurvick, M. S., and reviewed by Jannavi R. Srinivasan, Ph.D.

1. ***Summary***

This Chemical and Technical Assessment summarizes data and information on the Ribonuclease P enzyme preparation from *Penicillium citrinum* (strain designation AE-RP) that was submitted to JECFA. This document also discusses published information relevant to the safety of the ribonuclease P enzyme, including the *P. citrinum* AE-RP production strain and details related to the manufacturing, specifications, use and use levels of the enzyme in food. This document uses the expression "ribonuclease P" to refer to the modified enzyme and its amino acid sequence, and the expression "ribonuclease P enzyme preparation" to refer to the product formulated for commercial use. Data and information related to Ribonuclease P from a second production strain, *P. citrinum* RP-4, was also submitted to JECFA for evaluation. The Committee did not include Ribonuclease P from *P. citrinum* RP- 4 in the current evaluation based, in part, on a lack of specific compositional data for this product.

Ribonuclease P catalyses the hydrolysis of the 5' phosphate group of RNA to form a 5' monophosphate. The ribonuclease P enzyme preparation is used as a processing aid in the manufacture of processed yeast products and flavouring substances and in preparations with naturally occurring RNA. The degradation of RNA to produce free phosphonucleotides, specifically guanine and adenine, enhances the organoleptic properties and consistency of the final food or food ingredient. Ribonuclease P is intended for use at levels up to 1000 milligrams of Total Organic Solids per kilogram of raw material (mg TOS/kg).

The *P. citrinum* production organism has been shown to be non-pathogenic and non-toxigenic. *P. citrinum* strains deposited at public type culture collections have been designated as Safety Level 1. The production strain, *P. citrinum* AE-RP, was obtained from the parent strain *P. citrinum* IAM 7003 using conventional mutation techniques.

The ribonuclease P enzyme preparation is manufactured by controlled fermentation of *P. citrinum* AE-RP in accordance with Good Manufacturing Practices (GMP). The ribonuclease P enzyme is released into the fermentation medium and subsequently recovered and concentrated using multiple filtration techniques. The enzyme is spray dried and standardized with dextrin into a powdered enzyme preparation. The ribonuclease P enzyme preparation complies with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (JECFA, 2006).

Ribonuclease P is not known to be allergenic when used in food processing. The sponsor examined the potential for this enzyme to be a food allergen by comparing its amino acid sequence to sequences of known allergens contained within the AllergenOnline and Allermatch databases using internationally accepted search criteria. No meaningful identity with known allergens was observed. Based on the results obtained, oral exposure of ribonuclease P is not anticipated to pose any risk of allergenicity.

2. Description

White to dark brown powder.

3. Method of manufacture

3.1 P. citrinum

P. citrinum belongs to the genus *Penicillium*, and the family *Trichocomaceae. P. citrinum* is a filamentous fungus which is ubiquitous in the environment. It occurs on various plants, including citrus fruits, wheat and other cereal grains (Schmidt-Heydt, Stoll & Geisen, 2019). *P. citrinum* is also referred to as *Citromyces subtilis.*

The taxonomic classification of this microorganism is as follows:

Kingdom:	Fungi
Phylum:	Ascomycota
Class:	Eurotiomycetes
Order:	Eurotiales
Family:	Trichocomaceae
Genus:	Penicillium
Species:	*Penicillium citrinum*

Penicillium species are recognised for use in food applications (Bourdichon et al., 2012). *P. citrinum* is recognised as a source organism for the production of enzymes intended for use in food processing (Pariza & Johnson, 2001), including ribonuclease P, which is permitted for use in food processing in France, Denmark, China, and Korea (Legifrance, 2006; MfFLoF, 2008; USDA, 2015; MFDS, 2019). Strains of *P. citrinum* that have been deposited at public type culture collections such as the American Type Culture Collection (ATCC) and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) have been designated as Safety Level 1. In Europe, *P. citrinum* is not included on the list of pathogens in Annex III of Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work, nor is it included on the list of pathogens in Belgium (EC, 2000; Belgian Biosafety Server, 2008).

P. citrinum is known to produce mycotoxins, including citrinin (Park et al., 2008), and is an occasional opportunistic human pathogen. It has been known to cause pneumonia in immunocompromised individuals, including in patients receiving chemotherapy for acute leukaemia (Mok, et al., 1997; Hesse, et al. 2017).

3.2 P. citrinum production strain

The *P. citrinum* AE-RP production strain was obtained by conventional mutation using N-methyl-N'-nitro-N-nitroso-guanidine, exposure to ultra-violet light, and monospore isolation of the parental strain, *P. citrinum* IAM7003 (originally housed at IMA Culture Collection; presently, held at Japan Collection of Microorganisms under *P. citrinum* JCM22500). The identity of the production strain has been confirmed by BLAST analysis of the 28S rDNA-D1/D2 sequence with the APOLLON DB-FU ver.1.0 database that includes all sequences in the International Nucleotide Sequence Database (GenBank/DBK/EMBL). The 28S rDNA-D1/D2 sequence of *P. citrinum* AE-RP was reported to align with 100% similarly to several strains of *P. citrinum*, including *P. citrinum* NRRL 1984 (Accession No. AF033422). *P. citrinum* AE-RP does not produce mycotoxins, including citrinin.

3.3 Fermentation, recovery, and formulation

Ribonuclease P is produced by a controlled aerobic submerged batch-fed fermentation of a pure culture of *P. citrinum* AE-RP. The manufacture of the ribonuclease P enzyme preparation consists of three steps: fermentation (pre-, seed and main fermentation), recovery, and formulation. Control measures are in place for physical and chemical quality control during fermentation and downstream processing. Enzyme activity during production is also tested periodically. All raw materials used in the manufacture of ribonuclease P enzyme preparation are food-grade.

Production of the enzyme begins with the cultivation of an inoculum that is used for the seed fermentation. The product of the seed fermentation is used to initiate the main fermentation. The main fermentation is carried out under specific pH, temperature, and aeration conditions. The fermentation process continues for a predetermined time or until the desired level of the enzyme has been produced. Following fermentation, the culture broth containing the enzyme is separated from the biomass consisting of the production organism, other microbials, and spent fermentation medium by a series of filtration steps. This is followed by concentration and purification of the enzyme-containing liquid. The resulting enzyme concentrate is subsequently spray dried and standardized with dextrin. The entire process is performed in accordance with current Good Manufacturing Practices using raw materials of food grade quality. The enzyme concentrate was tested for the chemical and microbiological purity requirements described in the 'General Specifications and Considerations for Enzyme Preparations Used in Food Processing' (JECFA, 2006). The enzyme concentrate was tested to demonstrate the absence of mycotoxins.

4. Identity and Characterization

4.1 Ribonuclease P

Ribonuclease P is a zinc metalloenzyme which catalyses endonucleolytic cleavage of RNA, removing 5'-nucleotides. It is classified by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology (IUBMB) as follows:

Accepted name:	Ribonuclease P
Other name(s):	RNase P
Reaction:	Endonucleolytic cleavage of RNA, removing 5'-extranucleotides from tRNA precursor
Systematic name:	Ribonuclease P
EC No.:	3.1.26.5
CAS No.:	71427-00-4

Ribonuclease P produced by *P. citrinum* is not known to have any subsidiary or secondary enzymatic activities. The primary sequence of ribonuclease P has been determined to consist of 342 amino acids; its molecular weight by calculation from the determined amino acid sequence is 35 kDa.

Ribonuclease P activity is determined spectrophotometrically by measuring the production of phosphate resulting from hydrolysis of adenosine 3'-monophosphate by ribonuclease P at 750 nm; 1 unit of activity is defined as the quantity of enzyme required to liberate 1 µmol of phosphate (as phosphoric acid) per minute under the conditions of the assay. The mean activity of ribonuclease P from three batches of the powder enzyme concentrate is 112 600 U/g.

4.2 Ribonuclease P Enzyme Preparation

The ribonuclease P enzyme preparation consists of the enzyme, ribonuclease P, and substances from the fermentation process; these constitute proteins, peptides, amino acids, carbohydrates, lipids and salts. The components of fermentation are referred to as Total Organic Solids (TOS).

The TOS content of an enzyme preparation is calculated according to the following equation (NAS/NRC, 1981; JECFA, 2006):

$$TOS\ (\%) = 100 - (A + W + D)$$

where

A is the % ash,
W is the % water and
D is the % diluents and/or other formulation ingredients.

The Ribonuclease P enzyme preparation is marketed as a powder formulation. A representative composition of the Ribonuclease P enzyme concentrate is provided below:

Enzyme TOS:	44.4 %
Ash:	1.87 %
Water:	6.5 %
Dextrin:	47.2 %

The specifications for the ribonuclease P preparation include activity (approximately 13 800 U/g), lead (≤ 5 mg/kg), coliforms (≤ 30 CFU/g), *Salmonella* (absent in 25 g), *E. coli* (absent in 25 g), antimicrobial activity (absent by test), and loss on drying.

Ribonuclease P enzyme preparation complies with the General Specifications for Enzyme Preparations used in Food Processing as established by the 67th meeting of the Joint Expert Committee on Food Additives (JECFA, 2006).

5. Functional Uses

The ribonuclease P enzyme preparation is intended to be used as a processing aid in the production of processed yeast products and flavouring substances and preparations with naturally occurring RNA. The degradation of the RNA substrate in raw materials to produce free phosphonucleotides, specifically guanine and adenine, enhances the consistency and organoleptic properties of the final food or food ingredient.The ribonuclease P enzyme preparation is used at a maximum level of 1000 mg TOS/kg raw material.

6. Fate in food

Ribonuclease P is a ubiquitous enzyme responsible for generating mature 5’ ends of transfer RNA. It has been identified in representatives of the Archaea, Bacteria, and Eucarya domains, as well as in mitochondria and chloroplasts (Frank & Pace, 1998); as such it is naturally occurring in materials that are regularly ingested by humans. In addition to ribonuclease P, the enzyme preparation will contain proteins, peptides, carbohydrates and salts from the fermentation process that are common to the human diet.

Ribonuclease P enzyme preparation is intended to be used in the manufacture of processed yeast products and other flavouring substances with naturally occurring RNA. While it is assumed that the

ribonuclease P is carried over to finals foods, the enzyme is inactivated and denatured during processing yeast products by treatment at high temperatures and is not expected to have any technical effect on the final food. When used in the production of other flavouring substances, steps are taken to denature or remove the enzyme or to ensure that any residual enzyme activity is minimal. Lack of RNA substrate or water activity in the final product, or treatment at specific pH and temperature conditions are examples of conditions that may be applied to minimize residual enzyme activity.
If present, ribonuclease P will be digested, as would any other protein occurring in food. Therefore, use of ribonuclease P in the processing of food categories described will not have a significant effect on the human body.

7. References

Scientific Institute of Public Health Division of Biosafety and Biotechnology, Belgian Biosafety Server (2008). List of bacteria and similar organisms presenting at the wild stata a biological risk for immunocompetent humans and/or animals and corresponding maximum biological risk. (https://www.biosafety.be/sites/default/files/h_a_bacteries.pdf).

Bourdichon F, Casaregola S, Farrokh C, Frisvad JC, Gerds ML, Hammes WP et al. (2012). Food fermentations: microorganisms with technological beneficial use. Int J Food Microbiol. 154(3):87–97.

EC (2000). Directive 2000/54/EC of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work (seventh individual Directive within the meaning of Article 16(1) of Directive 89/391/EEC). Off J Eur Communities. 43(L262):21–45. (http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=celex:32000L0054).

Frank, D. N., & Pace, N. R. (1998). Ribonuclease P: unity and diversity in a tRNA processing ribozyme. *Annual review of biochemistry*, *67*, 153–180. (https://doi.org/10.1146/annurev.biochem.67.1.153).

Hesse, S. E., Luethy, P. M., Beigel, J. H., & Zelazny, A. M. (2017). *Penicillium citrinum*: Opportunistic pathogen or idle bystander? A case analysis with demonstration of galactomannan cross-reactivity. *Medical mycology case reports*, *17*, 8–10. (https://doi.org/10.1016/j.mmcr.2017.05.003).

JECFA (2006). General specifications and considerations for enzymes used in food processing [prepared by the Committee at its sixty-seventh meeting (2006)]. In: Combined compendium of food additive specifications, Joint FAO/WHO Expert Committee on Food Additives, 67th Meeting, June 20-29, 2006, Rome. Rome: Food and Agriculture Organization of the United Nations (FAO) / Geneva: Joint FAO/WHO Expert Committee on Food Additives (JECFA); 63-67. (FAO JECFA Monograph 3; http://www.fao.org/3/a-a0675e.pdf).

Legifrance (2006). Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires NOR: ECOC0600115A [Annex IB]. J Off Repub Fr. (Oct.). (https://www.legifrance.gouv.fr/affichTexte.do?dateTexte=20090916&cidTexte=JORFTEXT000000271061&fastPos=3&fastReqId=464085344&oldAction=rechExpTexteCode#LEGIARTI000020676815 [Dernière modification : 4 novembre 2018]).

MFDS (2019). [Food Additives. B. Phosphodiesterase]. In: Korea food additives code. Chungcheongbuk-do, South Korea: Ministry of Food and Drug Safety (MFDS); 1606–8. (https://www.foodsafetykorea.go.kr/foodcode/04_00.jsp).

MfFLoF (2008). Afgørelse: Enzympoduktet RP-IG accepters anvendt ved fremstilling af gaerekstrakt i en maengde pa hojest 0,5 % enzymprodukt. Gaerekstraktet anvendes I snacks og suppe in maengde

pa højest 0,5% … *Penicillum citrinum*. Soborg, Denmark: Ministeriet for Fodevarer, Landbrug og Fiskerie Fodevarestyrelsen, Kontor for Fødevarekvalitet, Teknologii og Markedsføring (MfFLoF) [Danish Food Administration, Office of Food Quality, Technology and Marketing] (J.nr.: 2007-20-5406-00113/BICB; 16.10.2008).

Mok, T., Koehler, A. P., Yu, M. Y., Ellis, D. H., Johnson, P. J., & Wickham, N. W. (1997). Fatal Penicillium citrinum pneumonia with pericarditis in a patient with acute leukemia. *Journal of clinical microbiology*, *35*(10), 2654–2656. https://doi.org/10.1128/jcm.35.10.2654-2656.1997.

NAS/NRC (1981). [Total organic solids]. In: Food chemicals codex, third edition. National Academy of Sciences (NAS) / National Research Council (NRC). Washington, DC: National Academies Press (NAP).

Pariza MW, Johnson EA (2001). Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Regul Toxicol Pharmacol. 33(2):173–86.

Park SY, Kim R, Ryu CM, Choi SK, Lee CH, Kim JG et al. (2008). Citrinin, a mycotoxin from Penicillium citrinum, plays a role in inducing motility of *Paenibacillus polymyxa*. FEMS Microbiol Ecol. 65(2):229–37.

Schmidt-Heydt, M., Stoll, D., & Geisen, R. (2019). Whole-Genome Sequencing of the Fungus Penicillium citrinum Reveals the Biosynthesis Gene Cluster for the Mycotoxin Citrinin. *Microbiology resource announcements*, *8*(4), e01419-18. https://doi.org/10.1128/MRA.01419-18.

USDA (2015). China - Peoples Republic of - Chinese standards for food additives - GB2760-2014 [Unofficial Translation by USDA]. (GAIN Report no. 15013). Washington (DC): U.S. Department of Agriculture (USDA), Foreign Agricultural Service, Global Agricultural Information Network (GAIN). (https://apps.fas.usda.gov/newgainapi/api/report/downloadreportbyfilename?filename=Standard%20for%20Food%20Additive%20Use%20-%20GB2760-2015_Beijing_China%20-%20Peoples%20Republic%20of_4-28-2015.pdf