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Safety evaluation of nuclease P1 from *Penicillium citrinum*

Nobuo Okado [a, *], Kazushige Hasegawa [b], Fukutaro Mizuhashi [b], Barry S. Lynch [c], Trung D. Vo [c], Ashley S. Roberts [c]

[a] Shin Nihon Chemical Co., Ltd., 19-10 Showa-cho, Anjyo, Aichi 446-0063, Japan
[b] Biosafety Research Center, Foods, Drugs, and Pesticides, 582-2, Shioshinden, Iwata, Shizuoka 437-1213, Japan
[c] Intertek Scientific and Regulatory Consultancy, 2233 Argentia Road, Suite 201, Mississauga, Ontario L5N 2X7, Canada

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ABSTRACT

Nuclease P1 has been widely used in the food industry to enhance or create flavor. One commercial source of this enzyme is *Penicillium citrinum*, an anamorphic mesophilic fungus with a long history of safe use in Europe and Asia as a fermentation organism used in the production of ribonucleases. Given the intended use in food for human consumption, and noting its potential presence at trace levels in finished products, a series of safety studies including an *in vitro* Ames and chromosome aberration assay, an *in vivo* rat erythrocyte micronucleus assay and a 90-day oral toxicity study in rats were conducted. No mutagenic activity was observed in the Ames assay. Equivocal activity in the chromosome aberration assay was not replicated in the micronucleus assay at doses of up to 1007 mg total organic solids (TOS)/kg body weight (bw)/day. Following oral administration of nuclease P1 at dosages of 10.1, 101 or 1007 mg TOS/kg bw/day to Sprague-Dawley rats, no adverse effects on any study parameter were observed. The no-observed-adverse-effect level was considered to be 1007 mg TOS/kg bw/day. The results of the genotoxicity studies and subchronic rat study support the safe use in food production of nuclease P1 produced from *P. citrinum*.

1. Introduction

A number of commercial applications of nuclease P1 exist in the food industry. Nuclease P1 (phosphodiesterase; EC 3.1.30.1) is a well-characterized endogenous enzyme that displays strong 5′-phosphodiesterase activity, hydrolyzing the P-O-β′ linkage to yield the 5'-phosphomononucleotide and the corresponding phosphooligonucleotide end-products (Lahm et al., 1990). The enzyme acts directly on single stranded ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) present in intended food substrates during the manufacture of the food ingredients and does not catalyze the hydrolysis of double-stranded DNA (Sigma-Aldrich, 2015). The intended reaction products of nuclease P1 activity under the intended conditions of use consist of the 5'-nucleotides [*i.e.*, guanosine-5′-monophosphate (GMP), adenosine-5'-monophosphate (AMP), uridine-5′-monophosphate (UMP), and cytidine-5′-monophosphate (CMP)] from RNA. The reaction products of nuclease P1 activity occur naturally in foods of animal and vegetable origin, such as animal meats (*e.g.*, beef and pork) and fish (*e.g.*, bonito), which are rich in inosine-5′-monophosphate (IMP) and AMP, and, therefore, have a long history of safe consumption. Furthermore, natural sources of 5′-nucleotides such as dried bonito and shiitake mushrooms are rich in IMP and GMP nucleotides, respectively, and are traditionally used in Asian cooking to impart or enhance flavor (Burdock et al., 2000). Partially hydrolyzed yeast extracts have also been used as sources of 5′-nucleotides for flavorings.

Nuclease P1 used in commercial applications of food processing is obtained from naturally-producing fungal sources, most notably from non-genetically modified *Penicillium citrinum* or *Leptographium procerum* (Amfep, 2014). Similarly, *P. citrinum* is

Abbreviations: 2-AA, 2-aminoanthracene; 9-AA, 9-aminoacridine hydrochloride; A/G, albumin:globulin; AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; AMP, adenosine-5′-monophosphate; bw, body weight; CBS, Centraalbureau Voor Schimmelcultures; CMP, cytidine-5′-monophosphate; DNA, deoxyribonucleic acid; EDTA-2K, ethylenediaminetetraacetic acid-2K; GLP, Good Laboratory Practice; GMP, guanosine-5′-monophosphate; IE, immature erythrocytes; IMP, inosine-5′-monophosphate; MHLW, Ministry of Health, Labour and Welfare; MI, mitotic index; MNIE, micronucleated immature erythrocytes; NaN_3, sodium azide; NP, *Penicillium citrinum* strain NP 11–15; OECD, Organisation of Economic Co-operation and Development; RNA, ribonucleic acid; TOS, total organic solids; UMP, uridine-5′-monophosphate.

* Corresponding author.
E-mail address: nokado@snc-enzymes.co.jp (N. Okado).

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currently listed as an authorized source of ribonuclease in France, China, and Japan (JORF, 2006; MHLW, 2011; Ministry of Health of the PRC, 2011). The safety of a phosphodiesterase (EC 3.1.26.5) from *P. citrinum* has been previously evaluated in *in vitro* genotoxicity studies and in 5- and 13-week dietary toxicity studies in rats (Kondo et al., 2001); however, this particular enzyme is somewhat different in activity profile relative to nuclease P1 (EC 3.1.30.1). The ribonuclease assessed by Kondo et al. (2001) cleaves off extra or precursor nucleotides from single stranded DNA and transfer RNA (tRNA), while nuclease P1 (EC 3.1.30.1) is active towards both single stranded RNA and single-stranded DNA. Thus, given the vast history of use in humans, key toxicity assessments were conducted in accordance with current study guidelines to help further document the safe history of use. As a result, the objective of the present study was to specifically evaluate the safety of a nuclease P1 enzyme preparation derived from *P. citrinum* strain NP 11–15 (herein referred to as NP) for use in food processing. To this end, NP was subjected to a standard battery of toxicological testing, consisting of a repeated-dose 90-day oral toxicity test conducted in rats and a series of *in vitro* and *in vivo* genotoxicity tests, including a bacterial reverse mutation test, an *in vitro* mammalian chromosomal aberration test, and an *in vivo* mammalian erythrocyte micronucleus test.

2. Materials and methods

2.1. Enzyme preparation

The Sumizyme NP enzyme preparation (Lot No. 040522T), produced as described below, was provided by Shin Nihon Chemical (Anjyo, Aichi, Japan) as a liquid. The same manufacturing lot was used in all studies.[1] The test article used in all studies met the established product specifications based on analytical testing. The stock solution contained 56,400 unit (U)/mL with the amount of test article per unit of enzyme activity providing 100.7 mg total organic solids (TOS)/mL (the dose and concentration described below were shown as TOS/mL). The enzyme preparation was stored in an airtight container under frozen conditions (−28.0 to −13.2 °C), and analytical testing indicated that the test article was enzymatically stable throughout the experimental period for all studies.

Briefly, the NP enzyme preparation was prepared by standard culture methods using the production strain *P. citrinum* NP 11–15. *P. citrinum* NP 11–15 was originally isolated from rice based on its ability to produce high ribonuclease activity, its viability, and its suitability for industrial production. The strain has been deposited in the Centraalbureau Voor Schimmelcultures (CBS) Fungal Biodiversity Centre collection (CBS No. 117107). Following cultivation, the *P. citrinum* culture was subjected to multiple filtration and ultrafiltration steps to remove the production strain and other materials (*e.g.,* proteins, saccharides, lipids, salts and other compounds less than 5000 molecular weight). Alternatively, concentration of the filtrate can be performed by vacuum evaporation. The concentrated filtrate is then heat-treated at 60 °C for 30 min. A final series of filtration steps is then applied to remove insoluble materials and any potential contaminating microorganisms and residual amounts of the production strain.

The liquid concentrate, in addition to the vacuum-dried concentrate, may be formulated with dextrin and spray-dried or formulated with glycerol to produce a liquid enzyme preparation. The final NP enzyme preparation was analytically determined to be free from any chemical (*e.g.,* heavy metals and mycotoxins) or microbiological contamination (*e.g.,* bacteria, including coliforms, *Escherichia coli* and *Salmonella* species, and mold).

2.2. 90-day oral toxicity study

This study was conducted in accordance with OECD Test Guideline No. 408 (OECD, 1998b) and the Guideline for Designation of Food Additives and for Revision of Standards for use of Food Additives (Ministry of Health, Labor and Welfare of Japan, 1996). Further methodological details are provided below.

2.2.1. Preparation of dosing formulations

NP dosing formulations were prepared on each administration day by serially diluting the thawed stock solution (100.7 mg total organic solids [TOS]/mL) 10-fold with distilled water (Japanese Pharmacopoeia grade) (Otsuka Pharmaceutical Factory, Tokushima, Japan) to obtain the mid- and low-dose formulation concentrations of 10.1 and 1.01 mg TOS/mL, respectively. The undiluted stock solution served as the high-dose concentration. Dose formulations were stored on ice until administration.

2.2.2. Animals and treatment

Fifty (50) male and 50 female four-week-old Crl:CD(SD) [SPF] rats were obtained from Charles River Laboratories Japan (Atsugi, Kanagawa, Japan). Upon receipt, animals were quarantined and acclimatized to the experimental environment for seven days. Animals were housed in individual wire mesh cages hung in an automatic water flushing breeding rack (Tokyo Giken Service, Tokyo, Japan). The laboratory conditions were maintained at a temperature of 23 ± 3 °C (actual value: 22.2–24.1 °C) and at a relative humidity of 55 ± 20% (actual value: 42–84%), with a fresh air ventilation rate of 10 or more changes per hour and a 12-h light/dark cycle. The cages were exchanged every two weeks and the feeders were changed once per week. Animals were provided *ad libitum* a commercial diet (CRF-1; Lot Nos. 050805 and 050908; Oriental Yeast, Tokyo, Japan) that was sterilized by irradiation and tap water (Iwata municipal tap water) from an automated water supply system. All levels of contaminants in the feed and water were analytically determined to be within the acceptable limits.

During the acclimatization period, animals were observed daily for signs of general health and body weights were measured on the day of receipt (day −7) and on the last day of the quarantine and acclimation period (day −1). Ophthalmological examinations were conducted for all animals prior to initiation of dosing. Forty (40) animals of each sex that were in good general health were selected and randomly assigned to one of four groups (10/sex/group) based on their body weights on the first experimental day (day 1) using a stratified body weight procedure based on the body weight just before the initiation of dosing. On the first day of dosing, animals were five weeks of age, and body weights ranged from 132 to 146 g for males and 108–126 g for females.

Doses of NP to be administered in the 90-day study were selected based on the results of a two-week repeated oral dose range-finding study, conducted in the same lab. In the two-week study, animals were provided NP at 10.1, 101 or 1007 mg TOS/kg body weight (bw)/day. Control animals were administered vehicle control (distilled water). Since no compound-related effects were observed following administration of NP, 1007 mg TOS/kg bw/day was selected as the high-dose to be administered in the 90-day study.

[1] All studies were conducted at Biosafety Research Center (formally, Biosafety Research Center, Foods, Drugs, and Pesticides), 582-2, Shioshinden, Iwata, Shizuoka 437–1213, Japan. Biosafety Research Center is a GLP-compliant testing facility. All studies were performed in compliance with the Organisation of Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (GLP) (OECD, 1998a).