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Original Article

Safety assessment of isomaltodextrin-producing enzymes from *Paenibacillus alginolyticus*

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ABSTRACT — Isomaltodextrin-producing enzymes (PP enzyme) include two extracellular enzymes (α-glucosyltransferase and α-amylase) produced by *Paenibacillus alginolyticus* PP710. These enzymes are essential for producing isomaltodextrin (IMD), a highly branched α-glucan, from starch. In this study, we evaluated the safety of this PP enzyme. No genotoxicity was observed when the PP enzyme was assayed in standardized bacterial reverse mutation and chromosome aberration tests. An acute toxicity study in rats showed no toxic effects of PP enzyme at 2,000 mg/kg. No animals died, and no effects of enzyme administration were observed in a 14-day repeated oral-dose toxicity study in rats at the maximum dose of 1,000 mg/kg/day. The no observed adverse effect level was determined to be 1,000 mg/kg/day in a 90-day subchronic gavage toxicity study in rats. No animals died, and no abnormal findings due to consumption of the PP enzyme were observed in this study. These safety evaluation results demonstrated that the PP enzyme was safe for application as an ingredient in the manufacturing of the food ingredient IMD.

Key words: α-Glucosyltransferase, α-Amylase, Isomaltodextrin, Genotoxicity, Animal study

INTRODUCTION

Isomaltodextrin (IMD) is a highly branched α-glucan produced enzymatically from starch (Tsusaki *et al.*, 2012). IMD contains only glucose units and alpha bonds, which consist of the following glucosidic linkages: 17% α-1 (nonreducing end groups), 3% α-1,3, 19% α-1,4, 49% α-1,6, 7% α-1,3,6, and 5% α-1,4,6 (Tsusaki *et al.*, 2012). The average molecular weight of IMD is approximately 5,000 (Fig. 1). IMD is a white powder with no odor; it is freely soluble in water and dissolves into a clear solution. Therefore, IMD is unlikely to alter the smell, color, or taste of the product in which it is used. The dietary fiber content of IMD is more than 80% (Tsusaki *et al.*, 2009). Accordingly, IMD is expected to be applicable for widespread use in foods as a new soluble dietary fiber. Various studies have been conducted to evaluate the safety of IMD, including acute toxicity studies, 90-day repeated oral-dose toxicity studies, genotoxicity studies, and studies to determine the no observed adverse effect level (NOAEL) for loose stools; all of these reports have found that IMD is a highly safe substance (Sadakiyo *et al.*, 2017). Moreover, IMD has been evaluated as a food that is generally recognized as safe (GRAS) by experts in the United States of America, and GRAS certification was obtained from the U.S. Food and Drug Administration (FDA) on June 6, 2016.

Among enzymes used in IMD manufacturing, those derived from *Paenibacillus alginolyticus* PP710 (PP enzyme) include α-glucosyltransferase and α-amylase,

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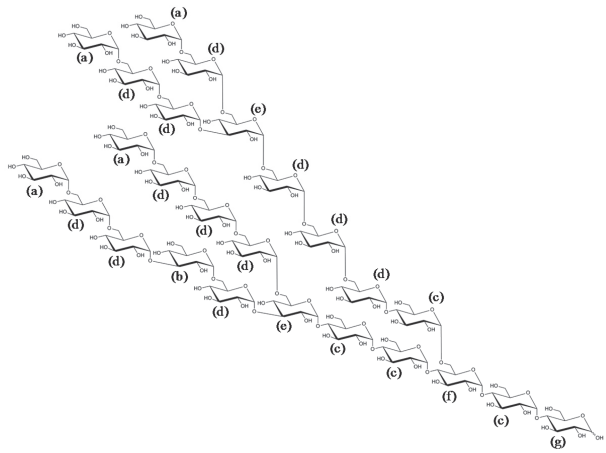

Fig. 1. The putative structure of IMD. (a) Nonreducing-end α-Glcp; (b) 1,3-linked α-Glcp; (c) 1,4-linked α-Glcp; (d) 1,6-linked α-Glcp; (e) 1,3,6-linked α-Glcp; (f) 1,4,6-linked α-Glcp; and (g) reducing-end Glcp.

which play important roles in forming an IMD-like molecular structure from starch. The α-glucosyltransferase is an exo-type enzyme that cleaves glucose units from the nonreducing end of glucan, which is transferred to the 6-OH position at the nonreducing end of glucan as an acceptor. This forms a repeated α-1,6 linkage structure at the nonreducing end (α-1,6-glucan). The α-amylase is an endo-type enzyme that cleaves glucan chains of any length from the nonreducing end of glucan. The cleaved chain is transferred to the 3-OH position at the glucose of the α-1,6-glucan as an acceptor. This enzyme reaction forms an α-1,3-branched α-1,6-glucan. The α-amylase also reacts with a residual long α-1,4-glucan chain at the reducing end; and the nonreducing end, which exhibits a branched structure, is transferred to the 3-OH position at the glucose of the α-1,6-glucan as an acceptor. This reaction forms a more complex branching structure and a new nonreducing end, activating α-glucosyltransferase activity. A continuous joint reaction of these two enzymes produces IMD.

Industrially, PP enzyme is manufactured by removing microbial cells from the culture solution of *P. alginolyticus* PP710 by membrane filtration and condensing the filtrate. Bergey's Manual of Systematic Bacteriology states that the members of the genus *Paenibacillus* are not associated with human or mammalian pathogenicity. The American Type Culture Collection classifies *P. alginolyticus* as biosafety risk group 1, as does the German Government Committee on Biologic Agents (American Type Culture Collection, 2016; German Collection of Microorganisms and Cell Cultures, 1997). This species is not listed in the FDA Bad Bug Book (US Food and Drug Administration, 2012). Accordingly, *P. alginolyticus* PP710 is considered to be highly safe. As a result, PP enzyme is also thought to be safe. However, no studies have reported the safety of PP enzyme to date.

Therefore, in this study, we evaluated the safety of PP enzyme to satisfy the requirements of the guidance of European Food Safety Authority regarding the safety assessment for food enzymes (European Food Safety Authority, 2009).

MATERIALS AND METHODS

All tests were performed at Biosafety Research Center (BSRC; Iwata, Japan).

Test substance

PP enzyme manufactured by Hayashibara Co., Ltd. (Okayama, Japan) was used. PP enzyme was manufactured by culturing *P. alginolyticus* PP710, removing microbial cells through membrane filtration, and condensing the filtrate. The cells resulted in a light yellow to brown solution with a characteristic odor. The samples were cryopreserved under sealed conditions until use. Tests were performed from April to December 2015, using PP enzyme from the same lot in all tests. Measured values at the start and end of the 90-day repeated oral-dose toxicity study were as follows: total organic solids, 2.40 and 2.43%; 1,4-α-glucan 6-α-glucosyltransferase activity, 1,015 and 1,031 U/mL; and α-amylase activity, 79.58 and 80.74 U/mL. The density of PP enzyme at the start of the 90-day repeated oral-dose toxicity study was 1,006 g/mL.

Total organic solids were calculated using the following formula: % total organic solids = 100 – (mean % ash + mean % water + % diluent and/or other additives). Because the test substance did not include the diluent and/or other additives and was not diluted with the diluent, the total organic solids content was calculated by assuming the percent diluent and/or other additives to be 0. One unit (U) of the 1,4-α-glucan 6-α-glucosyltransferase was defined as the amount of enzyme producing 1 μmol maltotriose per minute under the conditions described in Method 7 for α-glucosyltransferase in Japan's Specifications and Standards for Food Additives (JSSFA) 9th Edition. One unit (U) of the α-amylase was defined as the amount of enzyme reducing the blue color of potato starch produced by iodine by 10% per minute under the conditions described in Method 1 for α-amylase in JSSFA 9th Edition.

Animals

In the acute-toxicity study and 14-day and 90-day repeated oral-dose toxicity studies, rats (Crl:CD [SD], SPF) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). Animals were individually housed in wire-mesh cages in an animal room. The room was controlled at a temperature of 20-26°C, a relative humidity of 35-70%, a ventilation frequency of not less than 12 volumes exchanged per hour, and a 12-hr light cycle (lights on from 7:00 AM to 7:00 PM). Animals were allowed access to a pelleted diet CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*.

Ethics

Animal experiments were performed in compliance with the Act on Welfare and Management of Animals (Act No. 105 of October 1, 1973) and Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notice No. 88, Japanese Ministry of the Environment, April 28, 2006).

Bacterial reverse mutation test

A bacterial reverse mutation test was performed according to the Organisation for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals (Organisation for Economic Co-operation and Development, 1997). *Salmonella typhimurium* strains (TA98, TA100, TA1535, and TA1537) were purchased from Prof. Bruce N. Ames (University of California, USA). An *Escherichia coli* strain (WP2*uvrA*) was provided by the National Institute of Hygienic Science (the present National Institute of Health Sciences, Tokyo, Japan). S9 mix was commercial S9 mix (S-9/Cofactor A set; Oriental Yeast Co., Ltd.), prepared by mixing S9 and cofactor A at a ratio of 1:9. This study was conducted according to the pre-incubation method. All treatments were divided into two groups, and each group was evaluated in the absence (-S9 assay) and presence (+S9 assay) of S9 mix. This study was performed twice. In the dose-finding study (doses: 8.19, 20.5, 51.2, 128, 320, 800, 2,000, and 5,000 µg/plate), neither mutagenicity nor bacterial growth inhibition were observed in any of the strains treated with the substance in either the -S9 or +S9 assay. Therefore, the bacterial strains were treated with PP enzyme at doses of 156, 313, 625, 1,250, 2,500, and 5,000 µg/plate. Additionally, positive and negative control substances were tested concurrently. Water for injection, the solvent used to prepare the test substance solutions, was used as a negative control for all experiments. The following compounds were employed as positive controls: sodium azide (NaN_3), 2-aminoanthracene (2-AA), 9-aminoacridine (9-AA), and 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2). The above compounds, except for 9-AA (positive control), were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 9-AA was purchased from Sigma-Aldrich (St. Louis, MO, USA). The following positive control substance solutions that had been prepared at the BSRC were used in this study: AF-2, 9-AA, and 2-AA solutions were prepared using dimethyl sulfoxide (DMSO; Nacalai Tesque Inc., Kyoto, Japan) and NaN_3 solution was prepared using water for injection (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan). The positive controls were set as shown in Table 1.

Table 1. Bacterial strains, positive controls, and doses used in the bacterial reverse mutation test.

S9 mix	Strain	Positive control*	Dose (µg/plate)	Concentration (µg/mL)
-	TA98	AF-2	0.1	1.0
	TA100	AF-2	0.01	0.1
	TA1535	NaN_3	0.5	5.0
	TA1537	9-AA	80	800
	WP2*uvrA*	AF-2	0.01	0.1
+	TA98	2-AA	0.5	5.0
	TA100	2-AA	1.0	10
	TA1535	2-AA	2.0	20
	TA1537	2-AA	2.0	20
	WP2*uvrA*	2-AA	10	100

*AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide; NaN_3, sodium azide; 9-AA, 9-aminoacridine hydrochloride; 2-AA, 2-aminoanthracene.

The results were considered to be positive when the mean number of revertant colonies on a plate treated with the test substance increased by 2-fold or more as compared with that in the negative control group; the increase also had to be reproducible or dose dependent. No statistical analysis was performed for the evaluation.

Chromosome aberration test

A mammalian chromosome aberration test was performed according to the OECD Guidelines for the Testing of Chemicals (Organisation for Economic Co-operation and Development, 2014). A fibroblast cell line derived from the lungs of the Chinese hamster (CHL/IU) was used for this study. The CHL/IU cell line was obtained from the National Institute of Hygienic Sciences (the present National Institute of Health Sciences). The cells were preserved in liquid nitrogen after addition of DMSO at a 10% volume ratio. The frozen cells were thawed and cultured for use in this study. Cells at the second passage were used for the cell growth inhibition test, and those at the 11th passage were used for the chromosomal

aberration test.

The same lot of frozen cells was checked for mycoplasma contamination at the National Institute of Biomedical Innovation, and the results revealed no contamination. No abnormalities were found in the doubling time (14.7 hr), number of chromosomes (84% of the cells showed a modal number of 25), or other characteristics.

CHL/IU cells were treated with the test substance or control substances under the following conditions: 1) short-term treatment for 6 hr in the absence of S9 mix, followed by an 18-hr recovery period (-S9 assay); 2) short-term treatment for 6 hr in the presence of S9 mix, followed by an 18-hr recovery period (+S9 assay); 3) continuous treatment for 24 hr in the absence of S9 mix (24-hr assay). Water for injection was used as a negative control, and mitomycin C (MMC; Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) and cyclophosphamide (CP; Shionogi & Co., Ltd., Osaka, Japan) were used as positive controls.

Test substance concentration levels for the chromosomal aberration test were selected based on the results of the cell growth inhibition test. Microscopic examination of cells with chromosomal aberrations was conducted at three concentrations (1,250, 2,500, and 5,000 µg/mL) in -S9, +S9, and 24-hr assays.

If the incidence of cells with chromosomal aberrations in the test substance treatment group met all of the criteria (1, at least one of the concentrations caused a statistically significant increase compared with that in the negative control; 2, the degree of increase showed significant concentration dependence; 3, the incidence was greater than the acceptable range calculated from the historical data at BSRC for the negative control group), the test substance was considered to be positive. However, the biological relevance of the results was taken into consideration for the final judgment.

Acute oral toxicity study in rats

An acute oral toxicity study was conducted according to the OECD Guidelines for the Testing of Chemicals (Organisation for Economic Co-operation and Development, 2001). Ten rats (8 weeks of age) were used for the study. A single dose of 1,000 or 2,000 mg/kg of PP enzyme as an aqueous solution (200 or 400 mg/mL) was administered to the rats by oral gavage following an overnight fast. The dose volume was 0.5 mL per 100 g-body weight (BW). The animals were fasted for an additional 3 hr after dosing and then allowed food and water *ad libitum*. The general condition of the animals was observed once before dosing, within 30 min after dosing, and once at 1, 2, 3, and 4 hr after dosing (day 0). For 14 days from the first day after dosing (from day 1 to day 14), the general condition of the test animals was observed once daily. Viability and signs of toxicity were assessed, and any observed effects were recorded. Body weights were recorded on days 0 (before dosing), 7, and 14. All animals were sacrificed on day 14, and the organs/tissues were examined.

Fourteen-day repeated-oral dose toxicity study in rats

A 14-day repeated-oral dose toxicity study was conducted according to the OECD Guidelines for the Testing of Chemicals (Organisation for Economic Co-operation and Development, 1998). Sixty Crl:CD (SD) rats (5 weeks old; 30 males and 30 females) were quarantined and permitted to acclimate to the environment for 6 days. Fifty rats (6 weeks of age; 25 males and 25 females) were used in the study. The rats were assigned to groups on the date that test substance administration was started. The test substance was administered using a Teflon gastric tube once daily for 14 days at doses of 0, 30, 100, 300, and 1,000 mg/kg/day, at a volume of 0.5 mL/100 g-BW. The administration solution was prepared using distilled water for injection (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan). Animals were observed for general condition twice daily before and after administration (once before animal transport on the day of autopsy). BW and food consumption were measured on days 1, 4, 8, 11, and 14. The BWs of animals scheduled for autopsy were also measured on day 15. Hematology tests were performed for all surviving animals on the day of the scheduled autopsy (day 15). For blood collection, animals were fasted from around 5:00 PM on the previous day of autopsy and underwent laparotomy under isoflurane anesthesia and blood collection through the abdominal aorta. Blood was collected in blood-collecting tubes (INSEPACK II-D; Tokuyama Sekisui Co., Ltd., Osaka, Japan) containing EDTA-2K and was evaluated using an ADVIA120 system (Bayer AG, Leverkusen, Germany) to determine hematocrit (HCT), hemoglobin (HGB), red blood cell count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte ratio/count (Reticulocyte), platelet count (PLT), white blood cell count (WBC), differential leukocyte ratio, neutrophil count (NEUT), lymphocyte count (LYMPH), monocyte count (MONO), eosinophil count (EOSN), basophil count (BASO), and large unstained cell count (LUC). In addition, blood was collected in Venoject II blood-collecting tubes (Terumo Corporation, Tokyo Japan) containing 3.2% sodium citrate solution and

centrifuged at 1,700 × *g* for 13 min at room temperature to obtain plasma. The plasma samples were evaluated to determine the prothrombin time (PT) and active partial thromboplastin time (APTT) using a STA Compact coagulation analyzer (Roche Diagnostics K.K., Tokyo, Japan). Organ weight measurement and macroscopic examination (autopsy) of systemic organs and tissues were performed. Animals scheduled for autopsy were necropsied after blood collection and euthanasia, which was performed by exsanguination under isoflurane anesthesia.

Ninety-day repeated-oral dose toxicity study in rats

A 90-day repeated-oral dose toxicity study was conducted according to the OECD Guidelines for the Testing of Chemicals (Organisation for Economic Co-operation and Development, 1998). Ninety Crl:CD (SD) rats (5 weeks of age; 45 males and 45 females) were purchased, and 80 rats (6 weeks of age; 40 males and 40 females) were used in the study. Each animal was individually caged and allowed to acclimate to the laboratory conditions for 8 days before treatment. PP enzyme (0, 100, 300, or 1,000 mg/kg/day) was administered daily by gavage to Crl:CD (SD) rats (10 males and 10 females for each dose) for 90 days. Solutions of 20, 60, and 200 mg/mL were prepared for the 100, 300, and 1,000 mg/kg groups, respectively. The dose volume was 0.5 mL per 100 g-BW. The general condition of the animals was observed twice a day (before and after dosing) during the administration period, and BWs and food consumption were determined on days 1 (before grouping), 8, 15, 22, 29, 36, 43, 50, 57, 64, 71, 78, 85, and 90. The animals to be necropsied were weighed before necropsy (day 91). The Functional Observational Battery (FOB) was conducted for observation of home cage characteristics (posture, tremor, twitch, tonic convulsion, clonic convulsion) and responses to removal from cages (ease of removal, vocalization, muscle tone, subnormal temperature, piloerection, soiled fur, coarse fur, skin color, lacrimation, exophthalmos, pupillary size, salivation), behaviors in an open field (air-righting reflex, motor activity, respiration, eyelids, abnormal gait, tremor, twitch, tonic convulsion, clonic convulsion, stereotypic behavior, abnormal behavior), sensorimotor reactivity tests (pinna response, approach contact, touch response, pain response, pupillary reflex), grip strength tests (forelimb, hindlimb), and locomotor activity tests (amount of movement in a 10-min interval, total amount of movement [1-hr activity]). Tests for home cage observation, responses to removal from cages, and behaviors in an open field were conducted once during the quarantine period and once a week thereafter. Tests for sensorimotor function, grip strength, and locomotor activity were also conducted during the 13th week of administration. Urinalysis at the 13th week of administration and ophthalmological examinations during the quarantine period (days -3, -2) and administration period (day 86) were conducted. For the urinalysis, fresh urine (within 3 hr of urination) and pooled urine (24 hr) were collected. After confirmation of the volume and color, pooled urine samples were centrifuged at approximately 400 × *g* for 5 min to separate the supernatant and residue (sediment). After completion of the administration period, clinical examinations (examinations of hematology and blood chemistry) and pathological examinations (organ weight measurement and macroscopic and histopathological examinations) were conducted. Hematological and blood chemical examinations were conducted on days 91 and 92. Blood samples were collected from the abdominal aorta under isoflurane anesthesia after overnight fasting. Blood samples were collected into INSEPACK-II-D blood-collecting tubes (Tokuyama Sekisui) containing an anticoagulant (EDTA-2K). Plasma samples were prepared from the blood samples collected into blood-collecting tubes (Venoject II (Terumo Corporation) containing an anticoagulant (3.2% sodium citrate solution) and centrifuged at 1,700 × *g* for 13 min at room temperature. For examination of blood chemistry, serum samples were prepared from blood samples collected into blood-collecting tubes (Venoject II) containing a Gel and Clot activator and centrifuged at 1,700 × *g* for 7 min at room temperature. For pathological examination (organ weight measurement and macroscopic and histopathological examinations), all animals were necropsied after blood sampling and euthanized by exsanguination under isoflurane anesthesia.

Hematological tests consisted of HCT, HGB, RBC, MCV, MCH, MCHC, Reticulocyte, PLT, WBC, differential leukocyte ratio, NEUT, LYMPH, MONO, EOSN, BASO, LUC, PT, APTT, and fibrinogen analyses. Hematology was performed using an ADVIA120 system (Bayer AG) and STA Compact coagulation analyzer (Roche Diagnostics K.K.), and an STA Compact coagulation analyzer was used to measure blood PT, APTT, and fibrinogen levels. Blood samples containing EDTA-2K were analyzed with an ADVIA120 system, and plasma samples containing 3.2% sodium citrate solution were analyzed with an STA Compact analyzer. Blood chemistries consisted of total protein (T-Protein), glucose, triglyceride (TG), total cholesterol (T-Cho), blood urea nitrogen (BUN), creatinine, total bilirubin (T-Bilirubin), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase

(γ-GTP), calcium (Ca), inorganic phosphorous (iP), sodium (Na), potassium (K), chloride (Cl), albumin ratio, α_1-globulin ratio, α_2-globulin ratio, β-globulin ratio, γ-globulin ratio, albumin/total globulin ratio (A/G), albumin concentration, α_1-globulin concentration, α_2-globulin concentration, β-globulin concentration, and γ-globulin concentration. Sodium, K, and Cl were measured using an EA06R electrolyte analyzer (A&T Corporation, Kanagawa, Japan). Albumin, α_1-globulin, α_2-globulin, β-globulin, γ-globulin, and A/G were measured using an Epalyzer 2 Plus electrophoresis analyzer (Helena Laboratories, Beaumont, TX, USA). Other blood chemistry values were determined with an automatic multi-analyzer Hitachi 7170 (Hitachi, Ltd., Tokyo, Japan). Urinalysis was performed to measure fresh urine (pH, occult blood, ketone bodies, glucose, protein, bilirubin, and urobilinogen) and pooled urine (urinary volume, color, osmotic pressure, sediment, Na concentration, K concentration, Cl concentration, total Na excretion, total K excretion, and total Cl excretion). pH, occult blood, ketone bodies, glucose, protein, bilirubin, and urobilinogen were measured with an N-Multistix SG-L system (Siemens Healthcare Diagnostics K.K., Tokyo, Japan) and CLINITEK Advantus system (Siemens Healthcare Diagnostics K.K.). The supernatant was used for determination of osmotic pressure, Na, K, and Cl. Osmotic pressure was measured with an AUTO&STAT OM-6030 (ARKRAY, Inc., Kyoto, Japan). Sodium, K, and Cl were measured using an EA06R electrolyte analyzer. Sediment was measured by microscopic examination to determine levels of erythrocytes, leukocytes, squamous cells, transitional epithelial cells, renal tubular epithelial cells, casts, fat globules, mucous threads, and crystals.

Statistical analysis

Statistical analyses were performed by the following methods. For chromosome aberration tests, the evaluation was based on the total incidence of aberrant cells minus the number of cells with only gaps (-gaps). The incidence of aberrant cells was analyzed by Fisher's exact test (one-sided significance level: 2.5%). If significant differences were observed in the test substance group, concentration dependency was analyzed by the Cochran-Armitage trend test (one-sided significance level: 2.5%). For the 14-day repeated-oral dose toxicity study in rats, BW, food consumption, hematological values, organ weight, and organ-to-body weight ratios in each group were initially analyzed by Bartlett's tests for equality of variances with a two-sided significance level of 20%. When the test showed homoscedasticity (insignificant), the data were analyzed by Dunnet's multiple comparison tests based on the exact probability calculation method extended for unbalanced data. When the test showed heteroscedasticity (significant), the data were analyzed by Dunnett's step-down multiple comparison with Satterthwaite's approximation. In both tests, significance differences between the control and each administration group were determined at two-sided significance levels of 5% and 1%. No statistical analyses were performed for the results of general conditions and autopsy findings. For 90-day repeated-oral dose toxicity studies in rats, the quantitative data were initially analyzed by Bartlett's tests for equality of variances at a two-sided significance level of 20%. When Bartlett's tests showed homoscedasticity, the data were analyzed by Dunnett's multiple comparison tests based on the exact probability calculation for imbalanced data. When Bartlett's tests showed heteroscedasticity, the data were analyzed by Dunnett fashion tests with Satterthwaite approximation and step down. In both of these tests, the significance levels versus the control group were two-sided at 5% or 1%. Counting FOB data were analyzed by Steel's tests at two-sided significance levels of 5% or 1%. Data from the examinations for ophthalmology and histopathology were analyzed by paired comparisons based on the step-down permutated multiplicity adjustment calculated by Fisher's exact tests at two-sided significance levels of 5% and 1%.

RESULTS

Bacterial reverse mutation test

In the groups treated with the test substance, the number of revertant colonies did not increase by 2-fold or more compared with that in the negative control group for any of the strains in either the -S9 or the +S9 assay. No growth inhibition was observed for any test strain. The positive control substances induced a marked increase in the number of revertant colonies for each test strain. At the start of exposure and at the time of colony counting, no precipitation was observed. The reproducibility of the negative result was confirmed between the dose-finding and main study.

Chromosome aberration test

Cell growth inhibition test

Cell growth was not inhibited by 50% or more in the -S9, +S9, or 24-hr assays. At the start and end of the treatments, no precipitation was observed at any concentration.

Short-term treatment: -S9 assay

The incidences of cells with structural chromosomal aberrations in groups treated with PP enzyme were 0.3%,

1.0%, and 0.0% at 1,250, 2,500, and 5,000 μg/mL, respectively; these values were not significantly different compared with that in the negative control group (1.0%). The incidences of polyploid cells in the groups treated with PP enzyme were 0.3%, 0.0%, and 0.0% at 1,250, 2,500, and 5,000 μg/mL; these values were also not significantly different compared with that in the negative control group (0.0%). No inhibition of cell growth was observed at any concentration. The relative cell growth rate at the highest concentration (5,000 μg/mL) was 125.7%. At the start and end of treatment, no precipitation was observed at any concentration. In the group treated with the positive control substance (MMC), a high incidence of cells with structural chromosomal aberrations (52.0%) was observed; this difference was significantly higher than that in the negative control group.

Short-term treatment: +S9 assay

The incidence of cells with structural chromosomal aberrations in the groups treated with PP enzyme was 0.0% at all concentrations tested (1,250, 2,500, and 5,000 μg/mL); these values were not significantly different compared with that in the negative control group (0.0%). The incidence of polyploid cells in the groups treated with PP enzyme was 0.0% at all concentrations tested (1,250, 2,500, and 5,000 μg/mL). These values were also not significantly different compared with that in the negative control group (0.7%). No inhibition of cell growth was observed at any concentration. At either the start or the end of the treatments, no precipitation was observed at any concentration. The relative cell growth rate at the highest concentration (5,000 μg/mL) was 94.1%. At the start and end of the treatment, no precipitation was observed at any concentration. In the group treated with the positive control substance (CP), a high incidence of cells with structural chromosomal aberrations (56.3%) was observed; this value was significantly higher compared with that in the negative control group.

Continuous treatment: 24-hr assay

The incidences of cells with structural chromosomal aberrations in the groups treated with PP enzyme were 0.0%, 0.7%, and 0.0% at 1,250, 2,500, and 5,000 μg/mL, respectively; these values were not significantly different compared with that in the negative control group (1.3%). The incidence of polyploid cells in the groups treated with PP enzyme was 0.0% at all concentrations tested (1,250, 2,500, and 5,000 μg/mL), which was the same as that in the negative control group (0.0%). No inhibition of cell growth was observed at any concentration. The relative cell growth rate at highest concentration (5,000 μg/mL) was 113.2%. At the start and end of the treatment, no precipitation was observed at any concentration. In the group treated with the positive control substance (MMC), a high incidence of cells with structural chromosomal aberrations (33.7%) was observed; this value was significantly higher than that in the negative control group.

Acute oral toxicity study in rats

All animals survived, and no animals showed abnormal clinical signs during the observation period. The rats showed normal BW gain during the observation period and showed no abnormal macroscopic findings in the examined organs and tissues.

Fourteen-day repeated-oral dose toxicity study in rats

The results of hematology and tissue weight per BW are shown in Tables 2 and 3, respectively. During the administration period, no animals died, and no abnormalities in general condition likely to be associated with the test substance were observed in either administration group. Head injury was observed on days 14 and 15 in one female in the 1,000 mg/kg/day group. However, this finding was often observed in untreated rats of the same strain and was observed in a single case in this study. BW, food consumption, and hematological tests showed no statistically significant differences between the control group and each administration group. For organ weights, the absolute weight of the epididymis was significantly lower in males in the 30 mg/kg/day group than the control group; however, this change was not associated with the dose. Other organ weights showed no statistically significant differences between the control group and each administration group. For macroscopic findings of organs and tissues, no organs or tissues showed evidence of any macroscopic changes likely to be associated with the test substance in males or females.

Ninety-day repeated-oral dose toxicity study in rats

The results of hematology, blood chemistry, urine analysis, and tissue weight per BW are shown in Tables 4-7.

Clinical signs

One male in the 100 mg/kg/day was found dead on day 80. No abnormalities were observed in this animal until death. In surviving animals, no abnormal signs were observed during the administration period.

FOB

There were no toxicologically significant abnormalities with regard to response to removal from the cage, condition in hand-held observations, behaviors in an open-field, or sensorimotor reactivity for each experimental day. In addition, there were no significant

Table 2. Hematology data from the 14-day repeated-oral dose toxicity study in rats administered PP enzyme.

Sex		Male					Female				
Dose (mg/kg/day)		0	30	100	300	1000	0	30	100	300	1000
Number of animals		5	5	5	5	5	5	5	5	5	5
HCT	(%)	41.8 ± 0.8	40.8 ± 1.7	40.7 ± 2.4	41.8 ± 1.6	41.4 ± 1.2	41.3 ± 2.0	40.7 ± 1.3	40.8 ± 1.4	41.5 ± 1.7	39.7 ± 1.8
HGB	(g/dL)	14.7 ± 0.3	14.3 ± 0.6	14.3 ± 0.8	14.6 ± 0.6	14.5 ± 0.5	14.9 ± 0.5	14.5 ± 0.4	14.4 ± 0.5	14.6 ± 0.7	14.1 ± 0.7
RBC	($\times 10^6$/mm^3)	6.84 ± 0.16	6.75 ± 0.27	6.64 ± 0.37	6.95 ± 0.27	6.80 ± 0.28	7.12 ± 0.34	6.86 ± 0.33	6.99 ± 0.29	7.12 ± 0.34	6.96 ± 0.38
MCV	(μm^3)	61.2 ± 1.3	60.5 ± 1.5	61.3 ± 2.1	60.1 ± 1.6	61.0 ± 1.0	58.0 ± 0.6	59.4 ± 1.7	58.5 ± 2.3	58.3 ± 1.6	57.1 ± 1.1
MCH	(pg)	21.5 ± 0.3	21.2 ± 0.4	21.4 ± 0.8	21.0 ± 0.5	21.3 ± 0.4	20.9 ± 0.6	21.2 ± 0.5	20.7 ± 0.8	20.6 ± 0.7	20.3 ± 0.5
MCHC	(%)	35.2 ± 0.3	35.1 ± 0.3	34.9 ± 0.4	34.9 ± 0.3	35.0 ± 0.4	36.0 ± 0.8	35.7 ± 0.5	35.4 ± 0.4	35.4 ± 0.4	35.5 ± 0.4
Reticulocyte	(%)	4.7 ± 0.7	4.8 ± 0.7	4.9 ± 0.3	4.7 ± 0.4	4.3 ± 0.4	2.7 ± 0.7	2.7 ± 0.8	2.6 ± 0.8	2.6 ± 0.4	3.0 ± 0.6
	($\times 10^9$/L)	319.3 ± 42.6	321.2 ± 36.3	328.1 ± 20.7	326.6 ± 20.8	290.5 ± 21.6	191.3 ± 42.7	185.0 ± 56.8	180.8 ± 48.9	186.1 ± 23.5	206.2 ± 32.3
PLT	($\times 10^3$/mm^3)	1198 ± 61	1153 ± 67	1273 ± 189	1139 ± 73	1125 ± 122	1231 ± 65	1140 ± 104	1273 ± 330	1166 ± 173	1134 ± 144
WBC	($\times 10^3$/mm^3)	7.72 ± 1.96	8.55 ± 1.09	7.66 ± 1.89	8.80 ± 2.77	8.79 ± 1.99	5.34 ± 2.27	5.36 ± 1.45	5.84 ± 2.52	4.40 ± 0.79	4.26 ± 1.27
Differential leukocyte ratios											
NEUT	(%)	16.5 ± 3.5	12.0 ± 2.8	15.6 ± 6.5	15.7 ± 2.1	17.9 ± 5.9	15.4 ± 7.3	14.9 ± 5.1	18.4 ± 6.9	16.1 ± 3.7	19.8 ± 3.8
LYMPH	(%)	79.7 ± 3.7	84.2 ± 2.8	80.5 ± 5.5	79.6 ± 2.8	78.6 ± 6.2	80.8 ± 7.4	80.9 ± 5.1	77.1 ± 7.2	79.0 ± 4.4	75.1 ± 3.4
MONO	(%)	2.3 ± 0.4	2.1 ± 0.8	2.6 ± 0.9	3.0 ± 1.1	2.5 ± 0.7	2.1 ± 0.8	2.1 ± 0.8	2.5 ± 1.2	2.7 ± 1.1	2.7 ± 0.2
EOSN	(%)	0.8 ± 0.2	0.7 ± 0.2	0.7 ± 0.3	0.7 ± 0.3	0.5 ± 0.2	1.0 ± 0.3	1.5 ± 0.3	1.5 ± 0.8	1.6 ± 0.5	1.6 ± 0.5
BASO	(%)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1
LUC	(%)	0.7 ± 0.1	0.8 ± 0.5	0.5 ± 0.2	0.9 ± 0.3	0.5 ± 0.1	0.6 ± 0.2	0.6 ± 0.2	0.5 ± 0.1	0.5 ± 0.2	0.7 ± 0.2
NEUT	($\times 10^3$/mm^3)	1.27 ± 0.48	1.01 ± 0.26	1.14 ± 0.29	1.40 ± 0.55	1.57 ± 0.63	0.76 ± 0.31	0.81 ± 0.41	1.13 ± 0.85	0.72 ± 0.26	0.85 ± 0.29
LYMPH	($\times 10^3$/mm^3)	6.15 ± 1.49	7.20 ± 0.99	6.22 ± 1.81	6.96 ± 2.07	6.91 ± 1.71	4.39 ± 2.19	4.33 ± 1.17	4.47 ± 1.90	3.46 ± 0.55	3.19 ± 0.98
MONO	($\times 10^3$/mm^3)	0.18 ± 0.06	0.19 ± 0.08	0.20 ± 0.08	0.28 ± 0.14	0.21 ± 0.06	0.10 ± 0.04	0.12 ± 0.07	0.12 ± 0.03	0.12 ± 0.05	0.12 ± 0.04
EOSN	($\times 10^3$/mm^3)	0.07 ± 0.04	0.07 ± 0.02	0.05 ± 0.03	0.06 ± 0.04	0.04 ± 0.02	0.05 ± 0.01	0.08 ± 0.02	0.07 ± 0.03	0.07 ± 0.03	0.07 ± 0.02
BASO	($\times 10^3$/mm^3)	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.00
LUC	($\times 10^3$/mm^3)	0.05 ± 0.01	0.07 ± 0.05	0.04 ± 0.02	0.08 ± 0.04	0.04 ± 0.02	0.03 ± 0.02	0.03 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.03 ± 0.00
PT	(s)	13.2 ± 2.2	13.7 ± 2.0	13.4 ± 1.7	13.3 ± 1.3	14.5 ± 2.0	8.6 ± 0.1	8.6 ± 0.4	8.8 ± 0.2	8.7 ± 0.3	9.0 ± 0.4
APTT	(s)	25.3 ± 1.7	24.0 ± 2.8	24.5 ± 1.3	24.9 ± 1.2	25.3 ± 2.3	15.9 ± 1.6	18.0 ± 1.5	15.9 ± 2.6	16.8 ± 2.0	17.3 ± 1.6

Values are means ± SDs. There were no significant differences compared with the control group.

Table 3. Relative organ weight data from the 14-day repeated-oral dose toxicity study in rats administered PP enzyme.

Sex		Male					Female				
Dose (mg/kg/day)		0	30	100	300	1000	0	30	100	300	1000
Number of animals		5	5	5	5	5	5	5	5	5	5
Body weight	(g)	264 ± 14	266 ± 14	271 ± 24	260 ± 12	258 ± 17	184 ± 10	188 ± 8	191 ± 9	180 ± 10	188 ± 11
Brain	(g)	1.98 ± 0.07	1.97 ± 0.10	2.04 ± 0.16	2.01 ± 0.03	2.02 ± 0.07	1.87 ± 0.04	1.89 ± 0.07	1.92 ± 0.04	1.86 ± 0.07	1.89 ± 0.07
per body weight	(%)	0.751 ± 0.019	0.739 ± 0.012	0.756 ± 0.091	0.776 ± 0.037	0.785 ± 0.053	1.021 ± 0.076	1.007 ± 0.073	1.006 ± 0.034	1.033 ± 0.083	1.003 ± 0.065
Heart	(g)	1.02 ± 0.11	1.04 ± 0.15	1.05 ± 0.09	0.98 ± 0.08	0.98 ± 0.13	0.69 ± 0.05	0.74 ± 0.06	0.75 ± 0.07	0.72 ± 0.05	0.76 ± 0.06
per body weight	(%)	0.384 ± 0.022	0.391 ± 0.041	0.390 ± 0.024	0.377 ± 0.025	0.378 ± 0.029	0.376 ± 0.014	0.396 ± 0.022	0.391 ± 0.031	0.397 ± 0.015	0.401 ± 0.016
Lungs	(g)	1.17 ± 0.11	1.09 ± 0.07	1.18 ± 0.03	1.13 ± 0.10	1.12 ± 0.10	0.90 ± 0.08	0.94 ± 0.08	0.94 ± 0.07	0.94 ± 0.05	0.92 ± 0.05
per body weight	(%)	0.442 ± 0.037	0.408 ± 0.017	0.437 ± 0.028	0.434 ± 0.029	0.432 ± 0.028	0.491 ± 0.030	0.498 ± 0.040	0.492 ± 0.033	0.522 ± 0.008	0.488 ± 0.021
Liver	(g)	8.69 ± 0.93	8.84 ± 0.67	9.02 ± 1.31	8.47 ± 0.82	7.82 ± 0.68	5.66 ± 0.41	5.94 ± 0.36	6.00 ± 0.32	5.76 ± 0.36	5.86 ± 0.27
per body weight	(%)	3.290 ± 0.263	3.320 ± 0.080	3.319 ± 0.291	3.252 ± 0.177	3.024 ± 0.082	3.074 ± 0.085	3.161 ± 0.147	3.142 ± 0.104	3.199 ± 0.091	3.111 ± 0.071
Kidneys	(g)	2.38 ± 0.26	2.34 ± 0.27	2.33 ± 0.19	2.29 ± 0.22	2.25 ± 0.18	1.59 ± 0.13	1.66 ± 0.04	1.65 ± 0.14	1.57 ± 0.12	1.70 ± 0.12
per body weight	(%)	0.900 ± 0.058	0.879 ± 0.061	0.860 ± 0.037	0.880 ± 0.056	0.869 ± 0.044	0.865 ± 0.044	0.882 ± 0.026	0.863 ± 0.043	0.869 ± 0.045	0.903 ± 0.070
Spleen	(g)	0.58 ± 0.01	0.60 ± 0.05	0.59 ± 0.12	0.59 ± 0.14	0.58 ± 0.08	0.43 ± 0.04	0.40 ± 0.03	0.41 ± 0.07	0.37 ± 0.05	0.40 ± 0.05
per body weight	(%)	0.219 ± 0.008	0.227 ± 0.018	0.216 ± 0.028	0.227 ± 0.045	0.224 ± 0.024	0.234 ± 0.015	0.215 ± 0.014	0.216 ± 0.029	0.206 ± 0.019	0.211 ± 0.018
Adrenal glands	(mg)	51 ± 3	49 ± 3	46 ± 2	50 ± 9	44 ± 5	52 ± 10	53 ± 5	54 ± 9	58 ± 3	55 ± 3
per body weight	(%)	0.020 ± 0.002	0.018 ± 0.001	0.017 ± 0.001	0.019 ± 0.003	0.017 ± 0.002	0.028 ± 0.004	0.028 ± 0.002	0.028 ± 0.004	0.032 ± 0.003	0.029 ± 0.002
Testes	(g)	2.74 ± 0.13	2.51 ± 0.21	2.56 ± 0.19	2.66 ± 0.17	2.68 ± 0.25	-	-	-	-	-
per body weight	(%)	1.041 ± 0.054	0.946 ± 0.102	0.951 ± 0.126	1.022 ± 0.044	1.041 ± 0.137	-	-	-	-	-
Ovaries	(mg)	-	-	-	-	-	73 ± 9	79 ± 8	76 ± 11	83 ± 4	78 ± 9
per body weight	(%)	-	-	-	-	-	0.040 ± 0.003	0.042 ± 0.003	0.040 ± 0.006	0.046 ± 0.003	0.042 ± 0.007
Thymus	(mg)	567 ± 178	672 ± 118	573 ± 63	510 ± 116	509 ± 85	526 ± 88	484 ± 85	538 ± 135	465 ± 51	503 ± 173
per body weight	(%)	0.214 ± 0.062	0.252 ± 0.038	0.211 ± 0.010	0.196 ± 0.041	0.197 ± 0.030	0.286 ± 0.049	0.257 ± 0.043	0.280 ± 0.061	0.258 ± 0.025	0.264 ± 0.079
Uterus	(mg)	-	-	-	-	-	332 ± 113	412 ± 193	374 ± 84	411 ± 138	363 ± 104
per body weight	(%)	-	-	-	-	-	0.182 ± 0.070	0.217 ± 0.093	0.196 ± 0.043	0.226 ± 0.064	0.194 ± 0.062
Epididymides	(mg)	513 ± 60	436 ± 36*	453 ± 56	469 ± 30	477 ± 36	-	-	-	-	-
per body weight	(%)	0.195 ± 0.024	0.164 ± 0.016	0.169 ± 0.030	0.180 ± 0.008	0.186 ± 0.027	-	-	-	-	-

Values are means ± SDs. Significant difference from control group: *$p < 0.05$ (Dunnett's multiple comparison test).

Table 4. Hematology data from the 90-day repeated-oral dose toxicity study in rats administered PP enzyme.

Sex		Male				Female			
Dose (mg/kg/day)		0	100	300	1000	0	100	300	1000
Number of animals		10	9	10	10	10	10	10	10
HCT	(%)	43.9 ± 1.3	43.7 ± 1.2	44.0 ± 1.8	43.7 ± 1.0	41.6 ± 2.3	41.9 ± 2.0	41.9 ± 1.9	42.2 ± 1.5
HGB	(g/dL)	15.1 ± 0.4	15.0 ± 0.4	15.1 ± 0.6	14.9 ± 0.4	14.5 ± 0.9	14.7 ± 0.6	14.6 ± 0.7	14.8 ± 0.6
RBC	($\times 10^6$/mm^3)	8.63 ± 0.35	8.73 ± 0.42	8.80 ± 0.25	8.67 ± 0.28	7.96 ± 0.63	8.10 ± 0.44	7.69 ± 0.41	8.17 ± 0.36
MCV	(μm^3)	50.9 ± 2.0	50.1 ± 1.8	50.1 ± 1.5	50.5 ± 1.8	52.4 ± 1.7	51.7 ± 1.7	54.6 ± 2.0*	51.7 ± 1.7
MCH	(pg)	17.6 ± 0.7	17.2 ± 0.7	17.1 ± 0.5	17.2 ± 0.6	18.3 ± 0.5	18.1 ± 0.5	19.0 ± 0.7**	18.2 ± 0.6
MCHC	(%)	34.5 ± 0.4	34.4 ± 0.4	34.3 ± 0.5	34.1 ± 0.3	34.8 ± 0.4N	35.0 ± 0.3	34.9 ± 0.6	35.1 ± 0.3
Reticulocyte	(%)	2.2 ± 0.2N	2.1 ± 0.4	2.1 ± 0.4	2.1 ± 0.2	1.9 ± 0.4	1.8 ± 0.3	2.1 ± 0.4	1.9 ± 0.3
	($\times 10^9$/L)	187.1 ± 22.6	179.9 ± 29.6	187.3 ± 33.9	182.8 ± 17.9	151.4 ± 26.1	147.0 ± 25.2	164.4 ± 31.9	157.3 ± 23.2
PLT	($\times 10^3$/mm^3)	944 ± 114	956 ± 99	978 ± 126	1014 ± 176	1022 ± 179	960 ± 116	981 ± 117	922 ± 88
WBC	($\times 10^3$/mm^3)	8.07 ± 2.47	8.47 ± 1.75	8.41 ± 2.60	8.40 ± 1.58	4.60 ± 1.58	4.87 ± 1.40	4.24 ± 0.90	5.42 ± 1.54
Differential leukocyte ratios									
NEUT	(%)	19.9 ± 6.4	16.0 ± 6.3	20.0 ± 4.7	17.0 ± 5.7	15.7 ± 5.7	17.6 ± 7.8	16.6 ± 5.1	13.1 ± 4.7
LYMPH	(%)	74.4 ± 6.9	78.9 ± 6.9	74.9 ± 5.1	76.9 ± 6.4	78.5 ± 6.4	76.7 ± 7.5	77.2 ± 5.7	81.2 ± 4.9
MONO	(%)	3.2 ± 0.8	2.6 ± 1.0	2.9 ± 0.7	3.1 ± 1.2	3.0 ± 0.8	3.0 ± 0.8	3.3 ± 0.8	2.7 ± 1.0
EOSN	(%)	1.7 ± 0.5N	1.7 ± 1.0	1.7 ± 0.5	2.2 ± 0.9	1.9 ± 0.6	2.0 ± 0.7	2.3 ± 0.8	2.1 ± 0.8
BASO	(%)	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0*
LUC	(%)	0.8 ± 0.3	0.7 ± 0.3	0.5 ± 0.1	0.6 ± 0.2	0.8 ± 0.4	0.7 ± 0.3	0.6 ± 0.2	0.9 ± 0.3
NEUT	($\times 10^3$/mm^3)	1.52 ± 0.42N	1.44 ± 0.85	1.70 ± 0.77	1.42 ± 0.46	0.68 ± 0.23	0.82 ± 0.39	0.71 ± 0.28	0.68 ± 0.23
LYMPH	($\times 10^3$/mm^3)	6.10 ± 2.23N	6.60 ± 0.93	6.28 ± 1.90	6.49 ± 1.55	3.66 ± 1.46	3.77 ± 1.26	3.26 ± 0.72	4.44 ± 1.43
MONO	($\times 10^3$/mm^3)	0.25 ± 0.08	0.22 ± 0.09	0.24 ± 0.09	0.26 ± 0.09	0.13 ± 0.04	0.14 ± 0.05	0.14 ± 0.05	0.14 ± 0.05
EOSN	($\times 10^3$/mm^3)	0.13 ± 0.03N	0.14 ± 0.07	0.14 ± 0.04	0.18 ± 0.06	0.08 ± 0.03	0.09 ± 0.03	0.10 ± 0.04	0.12 ± 0.05
BASO	($\times 10^3$/mm^3)	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01
LUC	($\times 10^3$/mm^3)	0.06 ± 0.04N	0.05 ± 0.02	0.04 ± 0.02	0.05 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	0.03 ± 0.01	0.05 ± 0.02
PT	(s)	12.5 ± 1.3	12.0 ± 1.6	11.8 ± 2.2	11.7 ± 1.8	8.7 ± 0.2	8.7 ± 0.2	8.7 ± 0.2	8.7 ± 0.2
APTT	(s)	24.7 ± 2.2	23.8 ± 2.8	23.2 ± 2.4	23.4 ± 2.4	17.2 ± 1.8	16.6 ± 1.3	16.2 ± 1.6	16.1 ± 0.9
Fibrinogen	(mg/dL)	295 ± 26N	292 ± 12	316 ± 39	313 ± 27	215 ± 31	223 ± 28	219 ± 18	232 ± 17

Values are means ± SDs. Nonparametric analysis: N. Significant difference from the control group: *$p < 0.05$, **$p < 0.01$ (Dunnett's multiple comparison test).

Table 5. Blood chemistry and serum protein electrophoresis data from the 90-day repeated-oral dose toxicity study in rats administered PP enzyme.

Sex		Male				Female			
Dose (mg/kg/day)		0	100	300	1000	0	100	300	1000
Number of animals		10	9	10	10	10	10	10	10
Glucose	(mg/dL)	173 ± 19N	187 ± 37	171 ± 18	187 ± 33	161 ± 24	160 ± 16	163 ± 20	164 ± 20
TG	(mg/dL)	57 ± 21	64 ± 30	68 ± 25	64 ± 22	25 ± 9	26 ± 10	26 ± 9	25 ± 8
T-Cho	(mg/dL)	64 ± 11	70 ± 9	65 ± 12	60 ± 7	73 ± 9N	78 ± 9	82 ± 24	72 ± 11
BUN	(mg/dL)	11.6 ± 1.5	12.0 ± 1.4	12.1 ± 2.4	13.4 ± 1.7	13.2 ± 1.4	12.7 ± 1.3	13.5 ± 1.9	13.4 ± 1.8
Creatinine	(mg/dL)	0.29 ± 0.03	0.31 ± 0.04	0.30 ± 0.05	0.32 ± 0.04	0.33 ± 0.04	0.34 ± 0.03	0.35 ± 0.04	0.36 ± 0.04
T-Bilirubin	(mg/dL)	0.06 ± 0.01	0.06 ± 0.02	0.06 ± 0.02	0.05 ± 0.02	0.10 ± 0.03N	0.09 ± 0.02	0.10 ± 0.02	0.07 ± 0.01#
AST	(U/L)	122 ± 36	99 ± 28	141 ± 45	109 ± 35	82 ± 23N	88 ± 55	109 ± 122	70 ± 11
ALT	(U/L)	34 ± 9	31 ± 11	33 ± 7	31 ± 6	26 ± 6N	32 ± 27	44 ± 65	20 ± 4
ALP	(U/L)	283 ± 65	327 ± 57	305 ± 67	303 ± 43	139 ± 41	143 ± 22	116 ± 29	142 ± 23
γ-GTP	(U/L)	0.6 ± 0.1	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.8 ± 0.3N	0.8 ± 0.3	1.0 ± 0.6	0.8 ± 0.2
Ca	(mg/dL)	9.69 ± 0.25	9.67 ± 0.35	9.73 ± 0.19	9.61 ± 0.29	9.82 ± 0.28	9.64 ± 0.26	9.91 ± 0.22	9.73 ± 0.27
iP	(mg/dL)	6.10 ± 0.43	6.19 ± 0.35	6.18 ± 0.34	6.21 ± 0.38	5.03 ± 0.48N	4.71 ± 0.61	4.78 ± 0.65	5.33 ± 0.27
Na	(mmol/L)	144.0 ± 1.0	143.9 ± 0.8	144.2 ± 1.1	144.1 ± 0.7	143.0 ± 1.1N	142.8 ± 1.2	142.7 ± 1.7	142.7 ± 0.7
K	(mmol/L)	4.72 ± 0.38	4.61 ± 0.20	4.75 ± 0.28	4.73 ± 0.24	4.40 ± 0.36	4.29 ± 0.28	4.45 ± 0.27	4.43 ± 0.28
Cl	(mmol/L)	105.7 ± 1.1	105.1 ± 1.4	105.7 ± 1.1	105.8 ± 1.2	106.3 ± 1.5	106.4 ± 1.1	106.3 ± 2.1	106.4 ± 1.3
Serum protein electrophoresis									
Albumin	(%)	49.4 ± 2.3N	49.9 ± 2.0	49.6 ± 3.4	48.8 ± 1.6	58.6 ± 2.4	57.9 ± 1.3	57.8 ± 2.5	57.4 ± 1.9
α_1-Globulin	(%)	22.7 ± 2.5	22.6 ± 2.1	21.1 ± 3.8	23.2 ± 2.1	17.0 ± 2.3	16.0 ± 1.5	17.0 ± 1.6	16.2 ± 1.9
α_2-Globulin	(%)	7.2 ± 0.9	7.0 ± 0.8	7.3 ± 0.8	7.5 ± 0.6	5.8 ± 0.9	6.1 ± 0.5	6.0 ± 0.6	5.9 ± 0.7
β-Globulin	(%)	16.8 ± 1.1	16.6 ± 1.3	17.7 ± 1.6	17.0 ± 1.1	14.1 ± 1.3	15.0 ± 1.1	14.2 ± 1.1	15.4 ± 1.0*
γ-Globulin	(%)	4.0 ± 1.1N	3.8 ± 1.0	4.3 ± 0.6	3.6 ± 0.4	4.5 ± 0.9	5.2 ± 0.8	5.0 ± 0.7	5.2 ± 1.0
A/G		0.98 ± 0.09N	1.00 ± 0.08	0.99 ± 0.13	0.95 ± 0.06	1.42 ± 0.14	1.38 ± 0.07	1.38 ± 0.14	1.35 ± 0.11
T-protein	(g/dL)	5.74 ± 0.25	5.70 ± 0.24	5.80 ± 0.18	5.61 ± 0.32	6.40 ± 0.23N	6.18 ± 0.46	6.31 ± 0.25	6.01 ± 0.34#
Albumin	(g/dL)	2.83 ± 0.14	2.84 ± 0.14	2.87 ± 0.14	2.73 ± 0.13	3.76 ± 0.26	3.58 ± 0.26	3.65 ± 0.26	3.45 ± 0.25*
α_1-Globulin	(g/dL)	1.31 ± 0.17	1.29 ± 0.16	1.23 ± 0.25	1.30 ± 0.15	1.09 ± 0.15	0.99 ± 0.15	1.07 ± 0.10	0.97 ± 0.13
α_2-Globulin	(g/dL)	0.41 ± 0.06	0.40 ± 0.05	0.42 ± 0.04	0.42 ± 0.04	0.37 ± 0.05	0.37 ± 0.03	0.38 ± 0.04	0.35 ± 0.03
β-Globulin	(g/dL)	0.96 ± 0.08	0.95 ± 0.08	1.03 ± 0.10	0.95 ± 0.09	0.90 ± 0.07	0.92 ± 0.07	0.90 ± 0.07	0.93 ± 0.07
γ-Globulin	(g/dL)	0.23 ± 0.07N	0.22 ± 0.05	0.25 ± 0.03	0.20 ± 0.03	0.29 ± 0.05	0.32 ± 0.06	0.31 ± 0.04	0.31 ± 0.06

Values are means ± SDs. Nonparametric analysis: N. Significant difference from the control group: #$p < 0.05$ (Dunnett's fashion test with Satterthwaite approximation and step down). Significant difference from the control group: *$p < 0.05$ (Dunnett's multiple comparison test).

Table 6. Urinalysis data from the 90-day repeated-oral dose toxicity study in rats administered PP enzyme.

Sex		Male				Female			
Dose (mg/kg/day)		0	100	300	1000	0	100	300	1000
Number of animals		10	9	10	10 (9)	10	10	10	10
Volume	(mL)	15.5 ± 7.6	18.8 ± 6.6	13.9 ± 3.8	13.4 ± 6.2	14.8 ± 7.2	14.4 ± 5.4	13.5 ± 4.5	11.9 ± 5.1
Osmotic pressure	(mOsm/kg)	1271 ± 430N	1070 ± 252	1433 ± 447	1581 ± 598	1242 ± 633	1271 ± 383	1325 ± 410	1295 ± 461
Na	(mmol/L)	49.5 ± 25.5	50.5 ± 23.3	64.2 ± 28.6	79.3 ± 42.0	83.8 ± 52.9	82.2 ± 28.3	90.5 ± 33.5	83.6 ± 30.6
K	(mmol/L)	166.0 ± 60.2N	140.4 ± 40.7	186.2 ± 68.7	221.1 ± 96.2	179.2 ± 97.7	180.3 ± 56.2	190.9 ± 68.5	181.3 ± 69.4
Cl	(mmol/L)	72.0 ± 31.9N	66.9 ± 34.0	88.7 ± 43.6	105.3 ± 64.2	115.0 ± 68.4	113.3 ± 37.7	123.3 ± 45.6	113.1 ± 50.7
Total excretion									
Na	(mmol/day)	0.72 ± 0.37	0.92 ± 0.44	0.84 ± 0.35	0.89 ± 0.31	0.98 ± 0.27	1.08 ± 0.24	1.13 ± 0.29	0.89 ± 0.29
K	(mmol/day)	2.27 ± 0.48	2.47 ± 0.65	2.39 ± 0.47	2.50 ± 0.54	2.14 ± 0.55	2.36 ± 0.45	2.35 ± 0.45	1.89 ± 0.43
Cl	(mmol/day)	1.04 ± 0.46	1.21 ± 0.65	1.13 ± 0.41	1.18 ± 0.51	1.35 ± 0.34	1.48 ± 0.38	1.52 ± 0.36	1.17 ± 0.39
Color	SY	10	9	10	10	10	10	10	10
pH	6.5	0	0	0	0	0	0	0	1
	7.0	0	0	0	0	1	0	0	0
	7.5	0	0	0	0	1	0	0	0
	8.0	0	0	0	0	1	1	0	0
	8.5	2	2	4	1	3	2	4	4
	≥9.0	8	7	6	8	4	7	6	5
Occult blood	-	7	7	7	8	9	10	10	10
	+/-	2	1	3	1	1	0	0	0
	1+	1	0	0	0	0	0	0	0
	2+	0	1	0	0	0	0	0	0
Ketone bodies	-	3	6	4	2	8	9	9	8
	+/-	3	2	6	5	2	1	1	2
	1+	4	1	0	1	0	0	0	0
	2+	0	0	0	1	0	0	0	0
Glucose (g/dL)	-	10	9	10	9	10	10	10	10
Protein (mg/dL)	-	1	4	1	0	7	7	8	8
	+/-	3	3	6	4	2	3	1	1
	30	3	1	2	3	1	0	1	1
	100	3	1	1	2	0	0	0	0
Bilirubin	-	10	9	10	9	10	10	10	10
Urobilinogen (EU/dL)	0.1	6	9	9	6	7	6	7	8
	1.0	4	0	1	3	3	4	3	2
Erythrocytes	-	10	9	10	10	10	10	10	10
Leukocytes	-	9	9	9	10	8	10	9	9
	1+	1	0	1	0	2	0	1	1
Squamous cells	-	10	9	10	10	10	10	10	10
Transitional epithelial cells	-	10	9	10	10	10	10	10	10
Renal tubular epithelial cells	-	10	9	10	10	10	10	10	10

Table 6. (Continued).

Sex		Male				Female			
Dose (mg/kg/day)		0	100	300	1000	0	100	300	1000
Number of animals		10	9	10	10 (9)	10	10	10	10
Casts	-	10	9	10	10	10	10	10	10
Fat globules	-	10	9	10	10	10	10	10	10
Mucous threads	-	9	7	10	10	9	10	10	10
	+	1	2	0	0	1	0	0	0
Crystals	-	0	0	0	0	0	0	1	0
	+	10	9	10	10	10	10	9	10

Values are means ± SDs. Nonparametric analysis: N. There were no significant differences from the control group in volume, osmotic pressure, and electrolytes. Numbers in parentheses indicate the number of animals used for analysis of pH, occult blood, ketone bodies, glucose, protein, bilirubin, and urobilinogen. Color: SY, slight yellow. Occult blood: -, negative; +/-, 1+, 2+, or 3+. Ketone bodies: -, negative; +/-, 5 mg/dL; 1+, 15 mg/dL; 2+, 40 mg/dL. Glucose: -, negative. Protein: -, negative; +/-, +/-. Bilirubin: -, negative. Erythrocytes: -, 0-4/ μL. Leukocytes: -, 0-4/ μL; 1+, 5-14/ μL. Squamous cells: -, 0-4/ μL. Transitional epithelial: -, 0-4/ μL. Renal tubular epithelial: -, 0-4/ μL. Casts: -, no cells. Fat globules: -, no cells. Mucous threads: -, no cells; +, cells present. Crystals: -, no cells; +, cells present.

Table 7. Relative organ weight data from the 90-day repeated-oral dose toxicity study in rats administered PP enzyme.

Sex		Male				Female			
Dose (mg/kg/day)		0	100	300	1000	0	100	300	1000
Number of animals		10	9	10	10	10 (9)	10	10	10
Body weight	(g)	525 ± 55	530 ± 29	533 ± 37	511 ± 41	288 ± 33	290 ± 23	289 ± 29	282 ± 22
Brain	(g)	2.31 ± 0.09	2.28 ± 0.10	2.27 ± 0.08	2.29 ± 0.09	2.08 ± 0.08	2.09 ± 0.02	2.10 ± 0.10	2.07 ± 0.07
per body weight	(%)	0.443 ± 0.044	0.432 ± 0.027	0.426 ± 0.026	0.450 ± 0.038	0.731 ± 0.078	0.726 ± 0.059	0.729 ± 0.053	0.739 ± 0.059
Heart	(g)	1.58 ± 0.18	1.47 ± 0.12	1.52 ± 0.12	1.52 ± 0.14	0.93 ± 0.12	0.90 ± 0.09	0.93 ± 0.10	0.91 ± 0.10
per body weight	(%)	0.301 ± 0.016	0.278 ± 0.021	0.285 ± 0.019	0.299 ± 0.026	0.322 ± 0.023	0.313 ± 0.028	0.324 ± 0.026	0.324 ± 0.024
Lungs	(g)	1.52 ± 0.14	1.52 ± 0.11	1.52 ± 0.09	1.51 ± 0.12	1.13 ± 0.11	1.12 ± 0.05	1.16 ± 0.08	1.15 ± 0.09
per body weight	(%)	0.290 ± 0.009	0.288 ± 0.024	0.286 ± 0.024	0.295 ± 0.018	0.395 ± 0.019	0.387 ± 0.028	0.402 ± 0.023	0.410 ± 0.022
Liver	(g)	13.18 ± 2.10	13.19 ± 1.40	13.66 ± 1.87	13.09 ± 0.80	6.97 ± 1.00	6.74 ± 0.84	7.08 ± 0.81	6.58 ± 0.70
per body weight	(%)	2.497 ± 0.202	2.488 ± 0.202	2.553 ± 0.203	2.572 ± 0.172	2.417 ± 0.141	2.324 ± 0.229	2.451 ± 0.142	2.335 ± 0.141
Kidneys	(g)	3.19 ± 0.37	3.13 ± 0.24	3.24 ± 0.29	3.18 ± 0.30	1.94 ± 0.25	1.92 ± 0.32	1.91 ± 0.20	1.79 ± 0.18
per body weight	(%)	0.608 ± 0.025	0.592 ± 0.043	0.609 ± 0.041	0.623 ± 0.048	0.674 ± 0.048	0.663 ± 0.106	0.664 ± 0.074	0.635 ± 0.044
Spleen	(g)	0.75 ± 0.15	0.79 ± 0.09	0.76 ± 0.08	0.77 ± 0.08	0.46 ± 0.08	0.50 ± 0.04	0.48 ± 0.05	0.49 ± 0.05
per body weight	(%)	0.143 ± 0.018	0.149 ± 0.018	0.143 ± 0.019	0.152 ± 0.016	0.160 ± 0.021	0.174 ± 0.017	0.168 ± 0.012	0.174 ± 0.019
Adrenal glands	(mg)	65 ± 13	62 ± 7	62 ± 10	64 ± 11	69 ± 10	67 ± 10	70 ± 7	67 ± 10
per body weight	(%)	0.012 ± 0.002	0.012 ± 0.002	0.012 ± 0.002	0.013 ± 0.002	0.024 ± 0.003	0.023 ± 0.003	0.024 ± 0.002	0.024 ± 0.004
Testes	(g)	3.39 ± 0.38	3.39 ± 0.45	3.32 ± 0.22	3.36 ± 0.22	-	-	-	-
per body weight	(%)	0.647 ± 0.068	0.641 ± 0.079	0.625 ± 0.057	0.662 ± 0.057	-	-	-	-
Ovaries	(mg)	-	-	-	-	85 ± 14	88 ± 8	90 ± 20	81 ± 18
per body weight	(%)	-	-	-	-	0.030 ± 0.006	0.030 ± 0.003	0.031 ± 0.007	0.029 ± 0.006
Thyroid glands	(mg)	30 ± 3	22 ± 6##	29 ± 6	29 ± 3	20 ± 4	21 ± 5	20 ± 7	22 ± 3
per body weight	(%)	0.006 ± 0.001	0.004 ± 0.001**	0.005 ± 0.001	0.006 ± 0.001	0.007 ± 0.001	0.007 ± 0.002	0.007 ± 0.002	0.008 ± 0.001
Pituitary	(mg)	15 ± 2	14 ± 1	14 ± 1	14 ± 2	19 ± 5	17 ± 4	17 ± 3	16 ± 2
per body weight	(%)	0.003 ± 0.000	0.003 ± 0.000	0.003 ± 0.000	0.003 ± 0.000	0.007 ± 0.001	0.006 ± 0.001	0.006 ± 0.001	0.005 ± 0.001
Thymus	(mg)	275 ± 43	360 ± 59##	277 ± 67	320 ± 91	261 ± 66	286 ± 58	294 ± 90	272 ± 42
per body weight	(%)	0.053 ± 0.007	0.068 ± 0.010#	0.052 ± 0.014	0.063 ± 0.018	0.091 ± 0.020	0.099 ± 0.023	0.104 ± 0.038	0.096 ± 0.014
Prostate	(mg)	1487 ± 208	1438 ± 238	1491 ± 237	1542 ± 225	-	-	-	-
per body weight	(%)	0.287 ± 0.056	0.272 ± 0.045	0.281 ± 0.052	0.302 ± 0.039	-	-	-	-
Uterus	(mg)	-	-	-	-	696 ± 296	559 ± 175	575 ± 175	569 ± 211
per body weight	(%)	-	-	-	-	0.244 ± 0.113	0.193 ± 0.058	0.201 ± 0.070	0.205 ± 0.089
Epididymides	(mg)	1327 ± 133	1323 ± 121	1251 ± 67	1307 ± 79	-	-	-	-
per body weight	(%)	0.254 ± 0.026	0.250 ± 0.026	0.236 ± 0.025	0.257 ± 0.023	-	-	-	-
Salivary glands	(g)	0.75 ± 0.07	0.77 ± 0.08	0.78 ± 0.10	0.75 ± 0.10	0.45 ± 0.05	0.47 ± 0.04	0.47 ± 0.07	0.48 ± 0.05
	(%)	0.143 ± 0.013	0.145 ± 0.015	0.146 ± 0.016	0.148 ± 0.021	0.157 ± 0.011	0.161 ± 0.015	0.162 ± 0.023	0.171 ± 0.018
Seminal vesicle	(mg)	1588 ± 176	1515 ± 139	1583 ± 322	1553 ± 287	-	-	-	-
per body weight	(%)	0.304 ± 0.037	0.287 ± 0.033	0.299 ± 0.069	0.304 ± 0.050	-	-	-	-

Values are means ± SDs. Numbers in parentheses indicate the number of animals used for analysis of the adrenal glands. Significant difference from the control group: #$p < 0.05$, ##$p < 0.01$ (Dunnett fashion test with Satterthwaite approximation and step down). Significant difference from the control group: **$p < 0.01$ (Dunnett's multiple comparison test).

differences in the frequencies of defecation and urination, and mean grip strength values for both limbs in either sex between the control group and each test substance-treated group. In males, the locomotor activity at 40-50 min after the start of measurement in the 1,000 mg/kg/day group was significantly lower than that in the control group. In females, the locomotor activity at 10-20 min after the start of measurement and total (within 0-60 min of the measurement period) amount of movement in the 1,000 mg/kg/day group were significantly lower than those in the control group.

BWs

There were no significant differences in the BWs of animals of either sex between the control group and each of the test substance-treated groups. Moreover, in BW gains from day 1 to day 90, there were no significant differences between the control group and each of the test substance-treated groups.

Food consumption

There were no significant differences in the food consumption of animals of either sex between the control group and each of the test substance-treated groups during the administration period.

Hematology

In females, the ratio of basophils was significantly higher in the 1,000 mg/kg/day group than in the control group. The MCV and MCH in the 300 mg/kg/day group were significantly higher than those in the control group. However, the latter changes were not considered to be treatment-related effects because there were no changes in the 1,000 mg/kg/day group. In males, there were no significant differences in any parameters between the control group and each of the test substance-treated groups.

Blood chemistry

In females, in the 1,000 mg/kg/day group, the β-globulin ratio was significantly higher than that in the control group, and the total bilirubin, total protein, and albumin concentrations were lower than those in the control group. In males, there were no significant differences in any parameters between the control group and each of the test substance-treated groups.

Urinalysis

There were no changes in any parameters for both sexes in all treatment groups.

Ophthalmology

There were no toxicologically significant abnormalities in the 1,000 mg/kg/day group for either sex. All the observed findings were judged to be spontaneous based on their types, grades, and incidences.

Organ weights

There were no significant changes attributable to the dosing of the test substance for both sexes. The absolute and relative weights of the thyroid glands in males in the 100 mg/kg/day group were significantly lower than those in the control group, and the absolute and relative weights of the thymus were higher in the same dose group. However, these changes were considered to be incidental because there was no dose relationship.

Gross necropsy findings

Dead animal showed red patches in the lungs at necropsy. In the animals who underwent scheduled sacrifice, gross necropsy findings included brown patches in the lungs, white patches in the stomach, renal cysts, focal depression in the kidneys, yellow patches in the prostate, dilatation of the uterus lumen, and pituitary cysts in a few animals. These necropsy findings were not attributable to the dosing of the test substance because all lesions were sporadic and focal and were often observed incidentally in normal rats.

Histopathological findings

In the dead animal, the main histopathological changes consisted of congestion and edema in the lungs, swelling of pancreatic islet cells, and glycogen accumulation in the renal tubular epithelium (Armanni-Ebstein lesions). Adrenal cortical hypertrophy owing to stress overload was also observed. Other changes were incidental, such as cardiomyopathy, hyaline droplets in the renal tubular epithelium, regeneration of renal tubules, and inflammatory changes in the epididymides, prostate, and pancreatic islets. In animals subjected to scheduled sacrifice, there were no findings related to test substance treatment in either sex. All lesions observed were sporadic and often observed in this strain; thus, these lesions were judged to be incidental.

DISCUSSION

As part of the safety evaluation of PP enzyme, we conducted bacterial reverse mutation tests, chromosome aberration tests, acute oral toxicity studies, and 14- and 90-day repeated-oral dose toxicity studies.

In our bacterial reverse mutation study, the highest dose was set at 5,000 μg/plate, the maximum dose prescribed in the guidelines. PP enzyme did not induce increases in the numbers of revertant colonies compared with that in the negative control group, either with or without rat liver S9. The reproducibility of these negative results was confirmed between the dose-finding study and the main study. The mean numbers of revertant colonies in the negative and positive control groups were within the reference values obtained from historical data at the BSRC. Overall, PP enzyme was judged not to induce gene

mutations in bacteria (negative) under the conditions examined in this study.

Based on the results of the cell growth inhibition test, chromosomal aberration tests were conducted at three concentrations (1,250-5,000 μg/mL) in -S9, +S9, and 24-hr assays. The results revealed no significant increase in the incidence of cells with structural chromosomal aberrations or the incidence of polyploid cells induced by PP enzyme in any of the assays. The incidences of cells with chromosomal aberrations in both the negative and positive control groups were within the acceptable range calculated from the historical data at the BSRC. Thus, PP enzyme did not induce chromosomal aberrations in cultured mammalian cells under the conditions in this study.

A review of bacterial reverse mutation and chromosomal aberration tests indicated that there were no effects at any dose and under any conditions. The negative and positive controls demonstrated that the assays performed as designed. Accordingly, under the standard conditions used in these assays, PP enzyme was not genotoxic.

An acute toxicity study was conducted by treating rats (n = 5/group) with 1,000 or 2,000 mg/kg/day. At both doses, no animals died, and no abnormalities were observed in general condition or BW during the observation period. No abnormal findings were noted at autopsy. Under the conditions used in this study, no toxicity of single-dose administration of PP enzyme was observed at 2,000 mg/kg.

In the 14-day repeated oral-dose toxicity study, no animals died, and no toxicity of the test substance was observed in terms of general condition, BW, food consumption, hematology tests, and pathological examinations in females or males for all test groups. Head injury was observed in one female animal on days 14 and 15 in the 1,000 mg/kg/day. However, this observation was not determined to be caused by the test substance because the finding was often observed in untreated rats of the same strain and was observed in a single case in this study. The absolute weight of the epididymis was significantly lower in males in the 30 mg/kg/day group than in the control group. However, because this change was not associated with dose, it was not determined to be caused by the test substance. Based on the type, number, severity, and morphological characteristics of the lesions, all findings noted in the macroscopic observation of organs and tissues were considered to be naturally occurring lesions.

In the 90-day repeated oral-dose toxicity study, one male animal in the 100 mg/kg/day group was found dead on day 80. During the administration period, there were no abnormal changes in the general condition, BW change, or food consumption in this dead animal. Histopathological examination of the dead animal revealed no treatment-related changes or lung lesions due to administration errors. The main histopathological changes in this dead animal consisted of congestion and edema in the lungs, swelling of pancreatic islet cells, and glycogen accumulation in the renal tubular epithelium (Armanni-Ebstein lesions). The direct cause of death was judged to be dyspnea due to pulmonary edema and was though to not be related to administration of the test substance. Glycogen accumulation in the tubular epithelium accompanied with nuclear glycogen, together with swelling of pancreatic islet cell were similar to those changes in spontaneously diabetic SDT rats or WBN/Kob rats; however, the relationship of diabetic condition with pulmonary edema was not clear in this animal. In the surviving animals, there were no adverse effects related to test substance treatment with regard to clinical signs, BW, BW gain, food consumption, ophthalmology, organ weight, necropsy, or histopathological examination. In FOB, the lower locomotor activity at 40-50 min after the start of measurement in the 1,000 mg/kg/day group in males and the locomotor activity at 10-20 min after the start of measurement and total (within 0-60 min of the measurement period) amount of movement in the 1,000 mg/kg/day group in females were judged not to be toxicologically significant because the degree of change was very low. Additionally, no abnormalities to the nerve system were observed in any observation, test, or examination. In hematological examinations, the higher ratio of basophils in females in the 1,000 mg/kg/day group was judged not to be toxicologically significant because the degree of this change was extremely low. In blood chemistry analysis, the higher β-globulin ratio in females in the 1,000 mg/kg/day group and the lower total bilirubin, total protein, and albumin concentrations were judged not to be toxicologically significant because the degrees of these changes were very low and the respective values were within the background reference ranges of the testing facility. In addition, the β-globulin concentration was not significantly higher than that in the control group, and total bilirubin decreased (which was not considered toxicologically significant). No other changes indicating decreased liver function were observed in any examination.

For evaluation of the subchronic safety of PP enzyme, 14- and 90-day repeated-oral dose toxicity studies in rats were conducted at 1,000 mg/kg/day as the highest dose. Based on the results of these studies, the NOAEL of the PP enzyme was set at 1,000 mg/kg/day for both male and female rats under the conditions of this study, which was the highest dose. This would be equivalent to 60 g/day consumed by a 60-kg human.

In summary, PP enzyme was shown to not possess mutagenic or clastogenic activity *in vitro*. In repeated oral-dose toxicity studies, no abnormalities attributed to PP enzyme were observed in any animal. From the data presented in this paper, PP enzyme was shown to have no toxicity, suggesting that PP enzyme may be a safe ingredient for use in the manufacturing of food ingredients.

Conflicts of interest----Hayashibara Co., Ltd. was the sponsor of this study.

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