Toxicity Test of Protease P "Amano" 6

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Introduction

Protease P "Amano" 6 is prepared by purifying the enzyme from the extract of *Aspergillus sp.* cultivated by a unique method. Protease P "Amano" 6 is a digestive enzyme preparation having a powerful protease activity.

In order to assess the safety of Protease P "Amano" 6, the acute, subacute and chronic toxicity tests of the preparation were performed by using mice and rats, and the results and some aspect are reported here.

Materials and methods

Mice(ddy/S strain) and rats (Wistar strain) were commercially purchased and housed in animal rooms maintained at temperature of 23 ± 2℃ and relative humidity of 55 ± 5%, respectively. After a quarantine period for one week healthy animals were used. Solid food CE-2 (NIPPON CLEA) was given ad libitum in the acute and subacute toxicity test, and powder food CA-1 (NIPPON CLEA) was given in the chronic toxicity test. Tap water was given ad libitum in all tests.

Protease P "Amano" 6 was dissolved in purified water mixed with powder food for the chronic toxicity test.

1. Acute toxicity test

Male and female mice weighing of 20 ± 2g (Mean ± SE) and 19 ± 2g, respectively were assigned to each group consisting of 10 animals.

Three methods of administration, that is, oral, subcutaneous, intraperitoneal were employed. The volume of test substance, that is, oral administration was 5 ml per 100g body weight, subcutaneous and intraperitoneal administration were 1 ml per 100g body weight. In the oral route, the test substance was administered by gavage via a metallic stomach tube after fasting for 16 hours. Subcutaneous and intraperitoneal administrations were performed by injecting the test substance at the dorsal site and the center of hypogastric region. After administration the toxicological and pharmacological symptoms of animals were observed everyday.

The LD50 values and their 95% confidence limits were calculated by Van der Waerden method.

For animals found dead, autopsy was performed with the minimum delay.

All survivors were autopsied at the termination of observation period and grossly

examined into the each organ in the thoracic and intraperitoneal cavity and the site of injection. In addition, histological examinations on the sites of administration were also examined.

2. Subacute toxicity test

In each group 10 rats weighing 80 ± 10g (male) and 90 ± 10g (female) were used. Three to four animals were kept together into a stainless steel net cage (370 x 250 x 170mm) and fed as mentioned above. Based on the results of acute toxicity test, three dose levels, 200 mg/kg, 1,000 mg/kg and 5,000 mg/kg, were employed. The administrative volume of the test substance was 1 ml per 100g body weight. The test substance was administered orally by gavage via a metallic stomach tube for 6 days per week during 5 weeks test period. The control group received 1 ml of purified water per 100g body weight. In both the control and treated groups, animals were fasted 6 hours before the administration and general conditions of the animals were observed daily thereafter. Animals were weighed, food and water consumption were measured, every 5 day during the test. Dead animals were dissected and conducted to microscopical examination of each organ.

Hematological and serum analyses were carried out by conventional methods. The animals were incised at neck under the ether anesthesia and the blood samples were collected from the carotid artery. Blood samples examined as to total erythrocyte and leucocyte counts; hemoglobin contents (Cyanothioglobulin method); hematocrit value (Capillary method after high speed centrifugation); differential leucocyte (Gimsa stains).

Serum was examined in respect of the following parameters; total proteins (Refractometry); total cholesterol (Zurkowski's method); blood glucose (Orthotoluidine method); s-GOT and s-GPT (Reightman-Frankel's method); alkaline phosphatase (Bessey-Lowry's method); urea-nitrogen (Diacetyl-monooxime method); albumin to globulin ratio (Phosphate method); sodium ion and potassium ion (Atomic absorption).

All animals were sacrificed by exsanguination, and gross observations on organs were performed. At autopsy, weights of brain, pituitary, thyroids, thymus, heart, lung, liver, spleen, adrenal, kidney, urinary bladder, testes, prostate, ovary and uterus of each of rats were recorded.

Subsequently, stomach, duodenum, pancreas and bone marrow of the femur as well as the above mentioned organs for recording of weights were fixed in 10% solution of neutral buffered formalin and staining with hematoxylin eosin. Urine specimens collected for 15 hours immediately after the final administration were analyzed in respect of pH, protein, glucose, urobilinogen and bilirubin.

3. Chronic toxicity test

In each group 10 male rats weighing 140 ± 10g were used. Dose levels were determined, based on the results of subacute toxicity test, to be 500 mg/kg, 1,000 mg/kg and 2,000 mg/kg. Oral administration of the test substance was continued for 26 weeks. Test substance was administered with powder food. Throughout the administration period, general conditions and food consumption were measured at every 7 day. Hematological analysis and urinalysis were carried out at the 12th and 26th weeks of the test period. Serum analysis and histopathological analysis were performed at the end of test in the same manner as the case of subacute toxicity test.

Results

1. Acute toxicity test

The LD50 values and its 95% confidence limits of the test substance in mice and rats are shown in Table 1 and Table 2, respectively.

1-1. Oral administration

Mice exhibited sedative state, piloerection and shutting eyes from a few hours after administration. In some cases, a decrease of reaction to stimulus, apathy, crouching and lying were observed. But diarrhea and vomiting were not observed.

The death of mice occurred laying prostrate or lateral position from 24 to 48 hours after administration. Survival mice recovered with 72 hours after administration. Rats showed almost same signs as the case of mice, but the recovery was rather faster than mice. In anatomical findings, in the dead and survival cases of both mice and rats, no inflammation and ulcer were observed in stomach and intestine of the site of administration. Also, in any other organs, no macroscopically abnormal findings were observed.

No histological abnormality was observed on the stomach and duodenum of survival mice and rats (Photo. 1, 2), and there was no sex difference in these findings.

1-2. Subcutaneous administration

Mice exhibited sedative state from a few after administration in the same as the case of oral administration, and were observed piloerection and were shutting the eyes. In the some cases, a decrease of reaction to stimulus, apathy, crouching and lying were observed.

Survival mice almost recovered within 96 hours after final administration.

In the case of the rats almost the same symptoms were observed, but the recover was rather retard. At autopsy of mice and rats, hyperemia, hemorrhage and necrosis were observed at the subcutaneous tissue of injection site (Photo. 3, 4).

In the survival cases of both mice and rats, no histological characteristic findings were observed except the hemorrhage, hyperemia and necrosis at the subcutaneous tissue of the injection site (Photo. 5).

No sex difference was seen in these findings.

1-3. Intraperitoneal administration

In the case of mice, the writhing behaviors were observed about 40 minutes after administration, but not dyspnea and vomiting. Mice got into sedative state from one hour after administration in the same as the case oral and subcutaneous administration. With the time a decrease of response to stimulus, apathy, crouching and lying were observed in some cases.

In the survival cases, mice recovered within 48 hours after administration.

In case of the rats same symptoms were observed, but recovery time was rather retard. At autopsy of mice and rats, congestion and hemorrhage were observed on the serous membranes of large and small intestines and abdominal cavity. Ascites was also observed (Photo. 6, 7).

In the survival cases, a slight congestion was observed on the serous membranes of large and small intestines and abdominal cavity.

There was no sex difference in these findings.

2. Subacute toxicity test

2-1. General symptoms and mortality

No abnormal behavior and symptoms were observed after administration.

2-2. Body weight changes

Body weight changes of rats are shown in Figure 1.

In the latter term of the test, inhibition of body weight gains was observed in the group receiving 5,000 mg/kg of male rats, but no significant difference was found between the control and treated group.

2-3. Food and water consumption

Food and water consumption are shown in Figure 2 and 3, respectively. No difference was found in both male and female rats between the control and treated groups.

2-4. Hematological analysis

The results are shown in Table 3. No significant difference was found between the

control and treated groups in both male and female rats.

2-5. Blood biochemical analysis

The results are shown in Table 4. No difference was found in both male and female rats between the control and the treated groups.

2-6. Urinalysis

The volume of both the control and the treated groups were 4 to 7 ml for 15 hours, but no difference was found in both male and female rats between the control and the treated groups. Regarding to glucose, urobilinogen and bilirubin, no difference was found in both male and female rats between the control and the treated groups. The protein positive cases were found in the control and treated group.

2-7. Organ weight

The absolute and relative organ weights are shown in Table 6, 7 and 8. No significant difference was found in both male and female rats between the control and the treated groups.

2-8. Gross pathological findings

No macroscopically toxicological findings were observed in all the male and female rats in the treated groups.

2-9. Histopathological findings

Liver : No cellular in filtration of a small round cell in Glisson's sheath was observed in all groups of male and female. While, slight adhesion of a fatty deposition was observed in all male and female including control groups. Generally, this sign was found naturally. So, slight adhesion of fatty deposition was not considered to be due to the test substance.

Heart : Except a slight cellular infiltration was found in one male rat receiving 200 mg/kg, no significant findings were found in any other animals.

Lungs : Alveolar composition of both control and treated groups were normal, and congestion, pulmonary edema and change of inflammation were not found. No histologically characteristic findings were observed on blood vessel.

Spleen : No histologically characteristic findings were observed in male and female rats of the control and treated groups.

Thyroids : Follicles in male and female rats of the control and treated groups were normal.

Adrenals : Zona glomerulosa, zona fasciculata, zona reticularis and medulla of the suprarenal in male and female rats of the control and treated groups were normal.

Pituitary : No histologically characteristic findings were observed on the anterior lobe, pars intervals and posterior lobe.

3. Chronic toxicity test

3-1. General symptoms and mortality

Throughout the experimental period, no significant general symptoms were observed in all groups. One to two dead cases was observed in the control and the treated groups. These deaths were caused to inadvertent administrations.

3-2. Body weight changes

Body weight changes of rats are shown in Figure 4. In the end of the experiment, no difference of body weights was found in male rats between the control and the treated groups.

3-3. Food and water consumption

Food and water consumption are shown in Figure 5 and 6,respectively. No difference was found in male rats between the control and the treated groups.

3-4. Hematological analysis

The results of hematological analysis at 3 and 6 month administration are shown in Table 9 and 10, respectively. At 3 and 6 month, no significant difference was found between the control and treated groups in male rats.

3-5. Blood biochemical analysis

The results of serum analysis are shown in Table 11. No difference was found in male rats between the control and the treated groups on the s-GOT, s-GPT, alkaline phosphatase, glucose, protein, total cholesterol, blood urea nitrogen, sodium and potassium ion.

3-6. Urinalysis

The results of urinalysis at 3 and 6 months are shown in Table 12 and 13.

At 3 and 6 month after administration of the test substance, in a few cases of both the control and the treated groups protein was positive, but were slight.

Regarding to glucose, bilirubin and urobilinogen, no abnormality was found.

3-7. Organ weight

The absolute organ weight and relative one are shown in Table 14 and 15, respectively. With both absolute and relative weights no significant difference was found between the control and the treated groups.

3-8. Gross pathological findings

No macroscopically abnormal findings were observed in all the male rats in the treated groups and the control.

3-9. Histopathological findings

Liver : Small round cell infiltration in Glisson's sheath and fatty deposition were not

observed in all groups.

Kidney : No cell proliferation and thickening of basement membrance were observed in all groups.

Heart : No histologically characteristic findings were observed in all groups.

Lungs : Alveolar composition of control and treated groups were normal. No congestion, edema and histological change due to inflammation were found.

Spleen : In all male rats of the control and treated groups, no histological toxicological findings were observed.

Thyroids : Follicle of both control and treated groups were normal. No toxicological findings were observed on the other sites.

Adrenal : Zona glomerulosa, zona fasciculata, zona reticularis of adrenal cortex and medulla of the suprarenal in male rats of the control and treated groups were normal. No histologically characteristic findings were observed on reticularis of adrenal cortex and suprarenal medulla.

Pituitary : No histologically characteristic findings were observed on the rostal lobe, par intermediate and posterior lobe.

Brain : No degeneration of neuron and nerve fibers were observed in the control and the treated groups. Also no hydrocephalus was observed.

Thymus : No histologically characteristic findings were observed.

Pancreas : No histologically characteristic findings were observed on islet of Langerhans and on the other sites.

Stomach and duodenum : In all male rats of the control and the treated groups, no histologically characteristic findings were observed on the pars proventricularis and pars glandularis area (Photo. 9, 10) and duodenum.

Bone marrow : No histologically characteristic findings were observed on the erythroblasts, megakaryocytes and granulocytes.

Urinary bladder : In all male rats of the control and the treated groups, no histologically characteristic findings were observed.

Prostate and testes : Prostate and formation of spermatozoon in testes in male rats of the control and treated groups were normal.

Discussion and summary

The acute, subacute and chronic toxicity tests for Protease P "Amano" 6 were carried out the following results were observed.

1. Acute toxicity test

In the oral study, the LD50 values of Protease P "Amano" 6 were 15,900 mg/kg to male mice, 17,300 mg/kg to female mice, 17,800 mg/kg to male rats, 14,400 mg/kg to female rats.

In the subcutaneous study, the LD50 values were 109.5 mg/kg to male and female mice, 82.9 mg/kg to male rats 103.2 mg/kg to female rats.

In the intraperitoneal study, the LD50 values were 64.9 mg/kg to male mice, 64.1 mg/kg to female mice, 62.9 mg/kg to male rats,65.3 mg/kg to female rats.

In these three studies, both rats and mice showed almost the same LD50 values, so it is considered that there is not a great difference due to strain. In the case of oral and subcutaneous administration, both mice and rats got into sedative state from a few hours after administration of the test substance, and in some cases piloerection, shutting the eyes, a decrease of response to stimulus, apathy, crouching, and lying were observed. While, in the case of intraperitoneal administration in both rats and mice, the writhing behaviors were observed about 40 minutes after administration of the test substance, and the same symptoms as the case of oral and subcutaneous administration observed after 1 hour after administration.

Gross pathological findings were as follows ; Necrosis, loss of hair, erosion around the site of subcutaneous injection, and hyperemia and hemorrhage of intestinal duct in the case of intraperitoneal administration.

While, no abnormal findings were observed in the case of oral administration. Findings on the subcutaneous and intraperitoneal administration were considered to be due to the action of protease in the Protease P "Amano" 6.

2. Subacute toxicity test

A slight inhibition of body weight gains was observed in the group receiving 5,000 mg/kg of male. No difference was found between the control and the treated groups of both male and female regarding general symptoms and food and water consumption.

On the hematological analysis, no significant difference was found between the control and the treated groups in both male and female rats. In aspects of the serum analysis, urinalysis and organ weights, no difference was found between the control and the treated groups. No histologically characteristic findings due to the test substance were observed.

3. Chronic toxicity test

No difference was found between the control and the treated groups of male with regard to general conditions, body weight, food and water consumption. Hematological analysis and urinalysis were carried out at 3 month and at the end of experimental period, and no difference was found between the control and treated groups on all points of tested.

Blood biochemical analysis was carried out at the end of experimental period, and no difference was found between the control and treated groups in terms of s-GOT, s-GPT, alkalinephosphatase, glucose, total cholesterol, blood urea nitrogen, sodium and potassium ion.

No difference of organ weights and no histologically abnormal findings due to the test substance were observed.

The maximum safety dose of Protease P "Amano" 6 was considered to be 2,000 mg/kg/day, which dose is 200 - 750 times than that of clinical dose (50 - 200 mg/day).

Therefore, the safety of Protease P "Amano" 6 can be assured as long as it is administered orally.

Acknowledgement

We would like to thank [redacted] Tokyo dental college for the histopathological examinations.

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Table 1 LD_{50} value and its confidence limit of Protease P "Amano" 6 in mice.

Route	Dose (mg/kg)	Mortality No. of death / No. of treat.		LD_{50} mg/kg (95% confidence limit)	
		Male	Female	Male	Female
p.o.	10,000	0/10	—	15,900 (15,000 ~ 16.800)	17,300 (16,500 ~ 18,200)
	12,000	1/10	0/10		
	14,500	4/10	2/10		
	17,300	7/10	6/10		
	20,800	8/10	7/10		
	25,000	10/10	10/10		
s.c.	76.9	0/10	0/10	109.5 (105.4 ~ 113.8)	109.5 (105.1 ~ 114.2)
	87.8	1/10	2/10		
	100.0	3/10	3/10		
	113.9	7/10	7/10		
	130.0	8/10	8/10		
	148.2	9/10	8/10		
	169.0	10/10	10/10		
i.p.	34.4	0/10	0/10	64.9 (61.8 ~68.2)	64.1 (61.0 ~67.4)
	43.9	1/10	1/10		
	50.0	3/10	2/10		
	57.1	4/10	5/10		
	65.0	4/10	5/10		
	74.1	6/10	7/10		
	84.5	9/10	8/10		
	96.4	9/10	9/10		
	109.8	9/10	9/10		
	125.4	10/10	10/10		

Table 2 LD_{50} value and its confidence limit of Protease P "Amano" 6 in rats.

Route	Dose (mg/kg)	Mortality No. of death / No. of treat.		LD_{50} mg/kg (95% confidence limit)	
		Male	Female	Male	Female
p.o.	13,100	0/10	0/10	17,800 (17,600 ~ 18,000)	14,400 (14,100 ~ 14,700)
	14,500	1/10	4/10		
	15,900	2/10	6/10		
	17,300	3/10	8/10		
	19,000	8/10	9/10		
	20,800	9/10	10/10		
	22,800	10/10	—		
s.c.	37.0	0/10	0/10	82.9 (73.6 ~93.5)	103.2 (92.0 ~115.7)
	53.0	4/10	1/10		
	77.0	6/10	4/10		
	111.0	9/10	6/10		
	160.0	8/10	8/10		
	230.0	6/10	8/10		
	320.0	10/10	10/10		
i.p.	25.3	0/10	0/10	62.9 (55.8~ ~71.0)	65.3 (57.9 ~73.6)
	36.4	2/10	2/10		
	52.5	7/10	4/10		
	75.5	6/10	7/10		
	108.8	7/10	7/10		
	156.6	8/10	9/10		
	225.6	10/10	10/10		

Photo.1 Glandular stomach of a male rat treated with 25,000 mg/kg Protease P "Amano" 6 (× 100. H.E.)

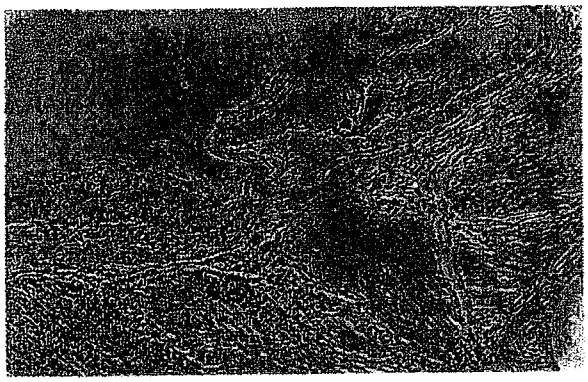


Photo.2 Fore stomach of a male rat treated with 25,000 mg/kg Protease P "Amano" 6 (× 100. H.E.)

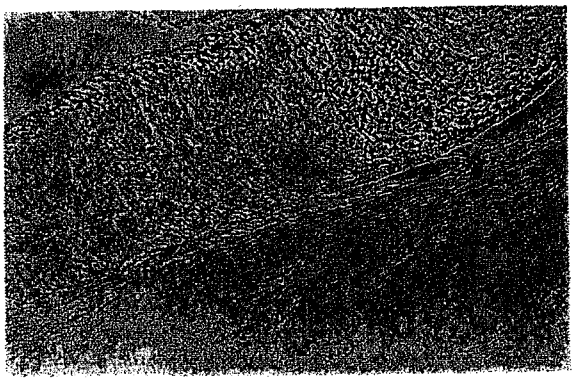


Photo.3 Autopsy of a male mouse treated with 169.0 mg/kg Protease P "Amano" 6 (s.c.)

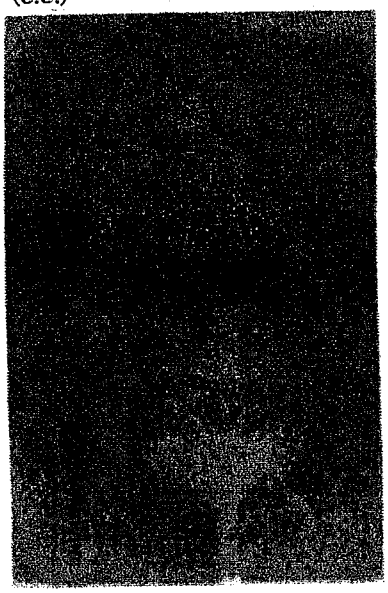


Photo.4 Autopsy of a male rat treated with 320.0 mg/kg Protease P "Amano" 6 P (s.c

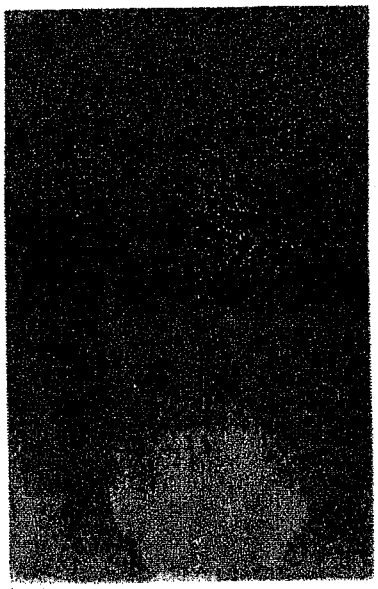


Photo.5 Skin of a male rat treated with 111.0 mg/kg Protease P "Amano" 6 (× 100. H.E.)

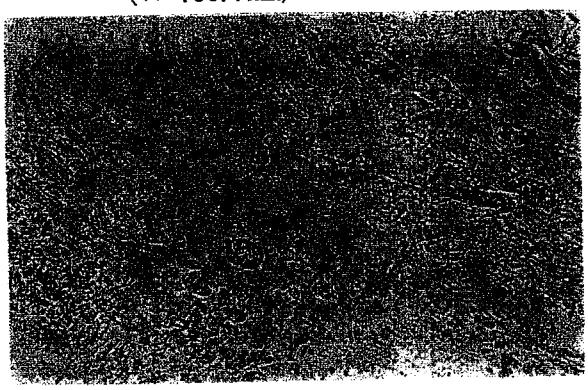


Photo.6 Autopsy of a male mouse treated with 125.4 mg/kg Protease P "Amano" 6 (i.p.)

Photo.7 Autopsy of a male rat treated with 225.6 mg/kg Protease P "Amano" 6 (i.p.)

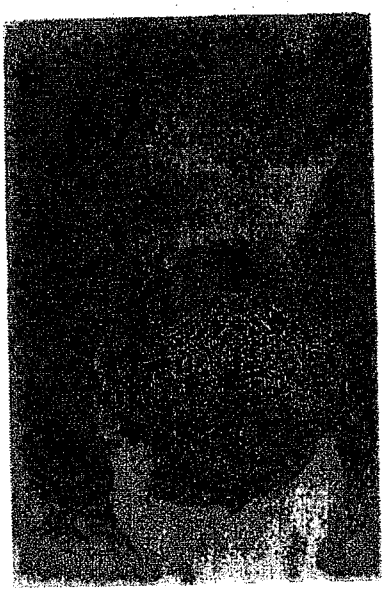


Fig.1 Body weight changes of male and female rats treated with Protease P "Amano" 6 for 30 days (Mean± SD)

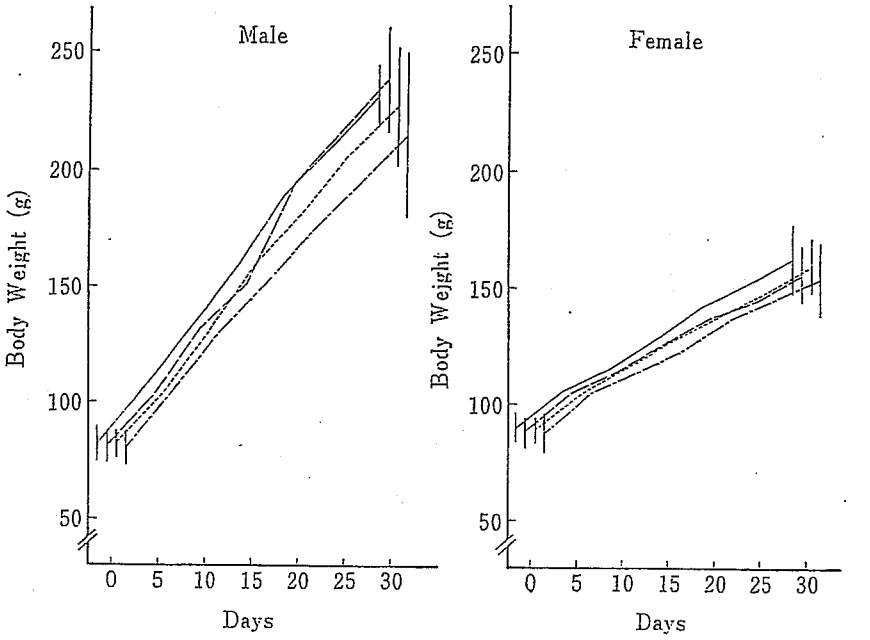


Fig.2 Food consumption of male and female rats treated with Protease P "Amano" 6 for 30 days

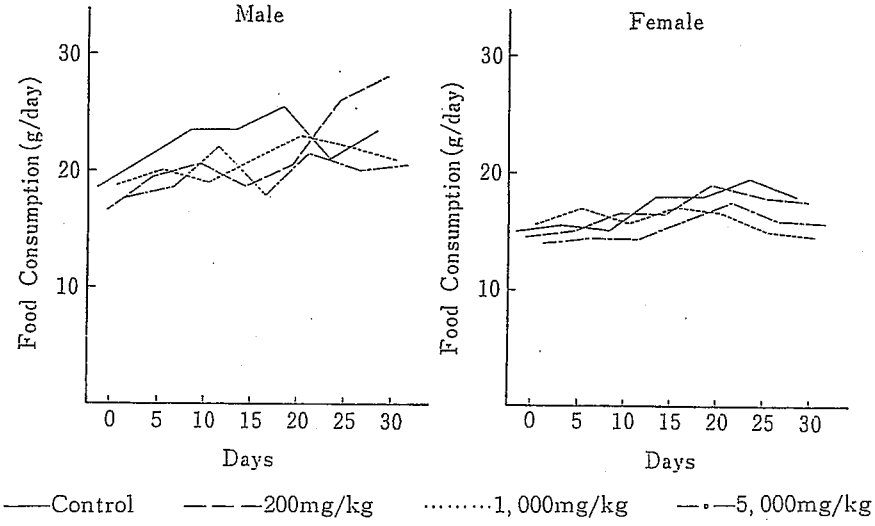


Table 3 Hematological findings in male and female rats treated with Protease P "Amano" 6 for 30 days

Sex	Dose (mg/kg)	No. of rats	Red blood cell ($\times 10^4$/mm^3)	Hemoglobin (g/dl)	Hematocrit (%)	White blood cell ($\times 10^3$/mm^3)	Differentiation of White blood cell (%)			
							Eos-sino.	Neutro.	Lymph.	Mono.
Male	Control	10	851.3±67.5	16.8±0.6	47.0±4.2	8.78±0.54	0.7	24.2±2.8	74.0±2.0	1.1
	200	10	847.4±88.2	16.5±0.8	48.7±1.8	8.94±0.69	0.7	24.9±3.0	73.5±2.4	0.9
	1,000	10	844.7±55.8	16.8±2.1	49.3±4.7	8.43±0.37	0.9	24.0±2.4	74.3±2.4	0.8
	5,000	10	812.6±59.2	16.3±1.3	45.1±8.1	8.97±0.56	0.8	24.5±2.0	73.8±1.8	0.9
Female	Control	10	801.1±65.2	16.9±0.9	44.4±2.2	7.95±0.68	0.6	25.0±1.8	73.4±2.5	1.0
	200	10	744.8±73.7	16.6±1.5	43.7±2.1	8.32±0.62	0.7	24.1±2.0	74.1±2.0	1.1
	1,000	10	756.2±71.9	16.8±1.0	46.1±1.1	8.16±0.55	0.6	25.3±2.4	73.2±1.5	0.9
	5,000	10	747.8±63.2	16.5±0.5	44.8±3.0	7.92±0.38	0.8	24.8±1.2	73.5±1.6	0.9

Mean±S.D.

Fig.3 Water consumption of male and female rats treated with Protease P "Amano" 6 for 30 days

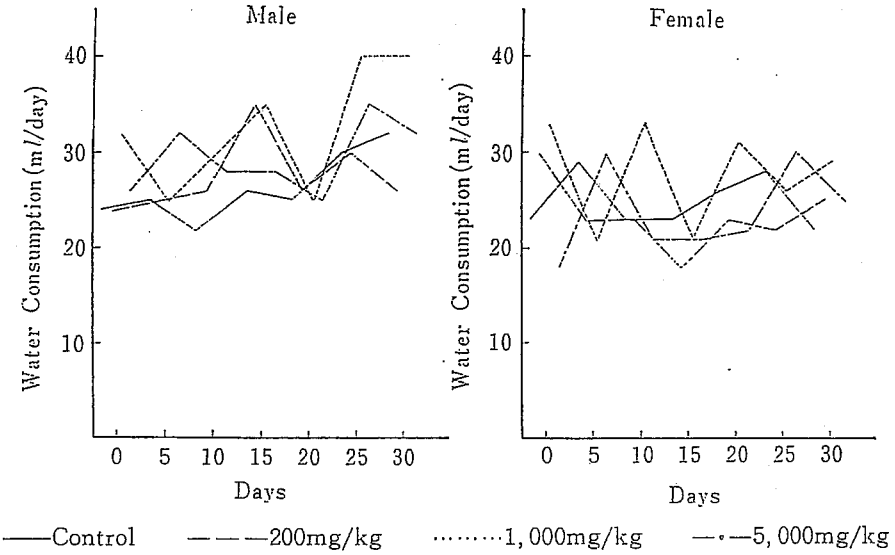


Table 4 Serum analysis of male and female rats treated with Protease P "Amano" 6 for 30 days

Sex	Dose (mg/kg)	No. of rats	S-GOT (Karmen unit)	S-GPT (Karmen unit)	Al-pase (B-L unit)	Glucose (mg/dl)	Protein (g/dl)	Total cholesterol (mg/dl)	Blood urea nitrogen (mg/dl)	Sodium) ion (mEq/l)	Potassium ion (mEq/l)
Male	Control	10	115.3±18.2	33.0±1.2	4.7±0.3	142±13	6.5±0.4	53.2±4.4	16.4±1.0	130.0±8.9	6.2±0.4
	200	10	116.5±21.4	33.0±3.1	4.4±0.5	156±18	6.5±0.3	54.1±3.8	17.2±1.2	124.3±7.2	6.5±0.4
	1,000	10	104.8±17.4	32.6±1.0	4.7±0.5	141±11	6.4±0.2	53.8±2.2	16.0±0.8	133.3±7.2	6.1±0.5
	5,000	10	105.2±17.0	32.4±1.4	4.5±0.3	131±16	6.3±0.5	50.1±3.9	16.8±1.1	131.8±4.3	6.4±0.4
Female	Control	10	104.9±21.2	35.7±2.3	3.4±0.2	157±13	6.7±0.8	50.4±1.4	15.8±2.1	124.8±4.7	6.5±0.3
	200	10	106.8±14.4	34.3±1.5	3.4±0.1	167±11	6.6±0.4	49.0±1.1	16.1±1.5	128.4±7.3	6.7±0.4
	1,000	10	115.4±15.8	36.0±1.5	3.3±0.3	160±11	6.4±0.9	51.3±1.5	15.7±1.3	127.0±6.5	6.4±0.4
	5,000	10	105.9±11.5	35.6±1.0	3.4±0.2	149±20	6.2±0.6	50.6±0.9	16.8±1.9	126.7±4.4	6.5±0.3

Mean±S.D.

Table 5 Urinalysis of male and female rats treated with Protease P "Amano" 6 for 30 days

Sex	Dose (mg/kg)	No. of rats	pH	Protein		Glucose		Bilirubin		Urobilinogen	
				−	±	−	+	−	±	−	+
Male	Control	10	6.4±0.5	9	1	10	0	10	0	10	0
	200	10	6.3±0.4	10	0	10	0	10	0	10	0
	1,000	10	6.8±0.4	10	0	10	0	10	0	10	0
	5,000	10	6.5±0.5	9	1	10	0	10	0	10	0
Female	Control	10	6.5±0.4	10	0	10	0	10	0	10	0
	200	10	6.1±0.3	9	1	10	0	10	0	10	0
	1,000	10	6.3±0.2	9	1	10	0	10	0	10	0
	5,000	10	6.3±0.3	10	0	10	0	10	0	10	0

pH : Mean±S.D.

Table 6 Absolute organ weight of male rats treated with Protease P "Amano" 6 for 30 days

Sex	Dose (mg/kg)	No. of rats	Initial body weight (g)	Final body weight (g)	Liver (g)	Kidneys (g)	Heart (g)	Lungs (g)	Spleen (g)	Thyroids (mg)	Adrenals (mg)	Pituitary (mg)	Brain (g)	Thymus (g)	Urinary bladder (mg)	Testes (g)	Prostate (g)
Male	Control	10	82±8	230±14	6.31±0.64	1.54±0.33	0.71±0.07	0.78±0.10	0.37±0.03	17.9±2.6	34.2±8.5	7.9±0.7	1.26±0.08	0.27±0.04	64.6±13.3	2.57±0.36	0.16±0.06
	200	10	81±7	238±22	6.34±0.28	1.62±0.41	0.73±0.10	0.82±0.08	0.36±0.02	18.3±1.9	40.3±7.6	8.0±0.9	1.24±0.05	0.29±0.03	69.2± 9.6	2.60±0.28	0.19±0.05
	1,000	10	82±6	227±25	6.15±0.74	1.56±0.21	0.73±0.05	0.83±0.11	0.37±0.04	18.5±1.4	31.0±4.1	7.7±0.6	1.22±0.06	0.26±0.05	72.9±11.8	2.38±0.41	0.16±0.06
	5,000	10	80±8	215±35	6.04±0.82	1.56±0.16	0.68±0.09	0.80±0.05	0.35±0.05	16.1±2.3	36.1±7.0	7.4±0.7	1.26±0.04	0.25±0.05	61.6±10.5	2.52±0.33	0.17±0.04

Mean±S.D.

Table 7 Absolute organ weight of female rats treated with Protease P "Amano" 6 for 30 days

Sex	Dose (mg/kg)	No. of rats	Initial body weight (g)	Final body weight (g)	Liver (g)	Kidneys (g)	Heart (g)	Lungs (g)	Spleen (g)	Thyroids (mg)	Adrenals (mg)	Pituitary (mg)	Brain (g)	Thymus (g)	Urinary bladder (mg)	Uterus (g)	Ovariums (mg)
Female	Control	10	90±6	162±15	4.15±0.33	1.08±0.14	0.51±0.66	0.67±0.05	0.31±0.04	13.3±2.1	51.5±6.6	9.7±0.8	1.16±0.08	0.24±0.07	57.9±9.2	0.28±0.08	87.8±7.7
	200	10	88±6	155±13	4.03±0.42	1.07±0.21	0.47±0.09	0.65±0.08	0.30±0.03	13.1±1.4	50.8±7.2	9.4±0.5	1.12±0.07	0.21±0.05	53.9±7.4	0.26±0.04	84.0±5.0
	1,000	10	89±5	159±13	4.11±0.18	1.11±0.24	0.49±0.05	0.66±0.07	0.30±0.04	13.9±1.9	52.9±3.3	9.9±0.6	1.15±0.07	0.23±0.06	54.6±8.8	0.29±0.07	84.1±6.9
	5,000	10	87±9	154±16	4.04±0.40	1.11±0.18	0.48±0.08	0.67±0.08	0.29±0.04	12.9±1.9	56.5±6.5	9.2±0.9	1.17±0.08	0.20±0.07	51.5±10.4	0.24±0.07	83.9±6.1

Mean±S.D.

Table 8 Relative organ weight of male and female rats treated with Protease P "Amano" 6 for 30 days

Sex	Dose (mg/kg)	No. of rats	Liver (g/100g)	Kidneys (g/100g)	Heart (g/100g)	Lungs (g/100g)	Spleen (g/100g)	Thyroids (mg/100g)	Adrenals (mg/100g)	Pituitary (mg/100g)	Brain (g/100g)	Thymus (g/100g)	Urinary bladder (mg/100g)	Testes (g/100g)	Prostate (g/100g)	Uterus (g/100g)	Ovariums (mg/100g)
Male	Control	10	2.73±0.24	0.67±0.08	0.30±0.03	0.34±0.04	0.16±0.02	7.7±0.7	14.8±1.5	3.4±0.2	0.55±0.05	0.12±0.02	28.0±4.6	1.11±0.12	0.07±0.02		
	200	10	2.66±0.31	0.68±0.04	0.31±0.02	0.35±0.02	0.15±0.02	7.7±0.5	16.9±2.8	3.3±0.2	0.52±0.04	0.12±0.03	29.1±5.1	1.09±0.08	0.08±0.02		
	1,000	10	2.71±0.19	0.69±0.07	0.30±0.03	0.32±0.04	0.16±0.01	8.0±0.8	13.7±1.9	3.4±0.3	0.54±0.05	0.12±0.01	32.1±6.2	1.05±0.11	0.07±0.03		
	5,000	10	2.81±0.21	0.72±0.07	0.32±0.03	0.37±0.04	0.16±0.02	7.5±0.6	16.8±2.6	3.4±0.1	0.59±0.05	0.12±0.03	28.6±4.8	1.17±0.10	0.08±0.02		
Female	Control	10	2.58±0.18	0.67±0.08	0.32±0.03	0.42±0.03	0.19±0.02	8.3±0.7	32.0±5.2	6.0±0.4	0.72±0.04	0.35±0.04	54.6±3.0			0.17±0.03	54.6±2.9
	200	10	2.60±0.09	0.69±0.07	0.30±0.02	0.42±0.04	0.19±0.01	8.5±0.6	32.8±3.8	6.1±0.2	0.72±0.03	0.35±0.03	54.2±1.8			0.17±0.02	54.2±3.1
	1,000	10	2.58±0.02	0.70±0.08	0.31±0.03	0.42±0.02	0.19±0.02	8.8±0.8	33.3±6.0	6.3±0.4	0.72±0.04	0.34±0.04	52.9±2.9			0.18±0.02	52.9±4.4
	5,000	10	2.62±0.25	0.72±0.09	0.31±0.03	0.44±0.04	0.19±0.02	8.4±0.7	36.7±5.5	6.0±0.4	0.72±0.05	0.33±0.04	54.5±3.2			0.16±0.02	54.5±3.0

Mean±S.D.

Fig.4 Body weight changes of male rats treated with Protease P "Amano" 6 for 26 weeks (Mean± SE)

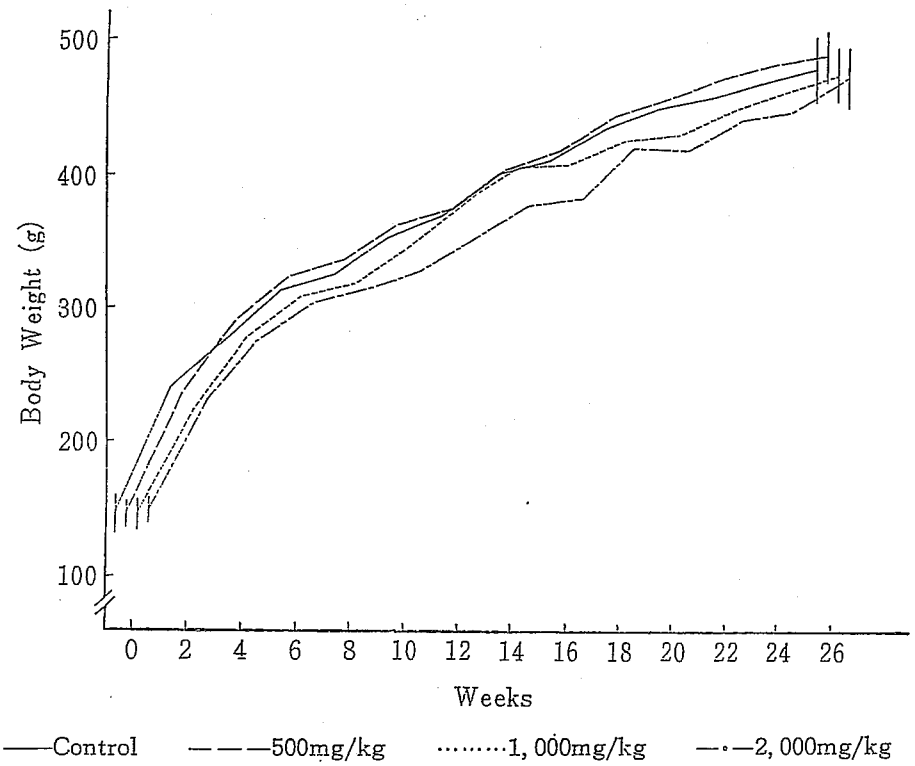


Fig.5 Food consumption of male rats treated with Protease P "Amano" 6 for 26 weeks

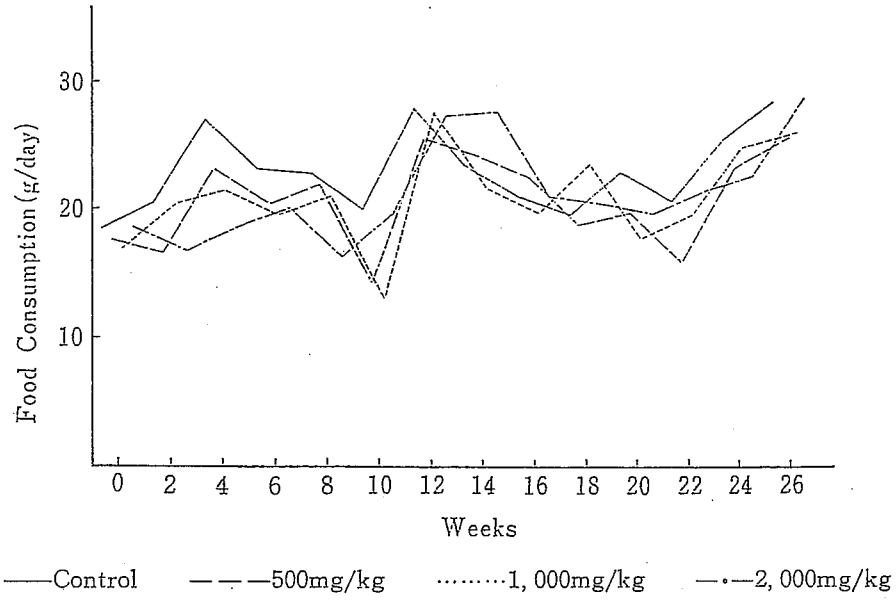
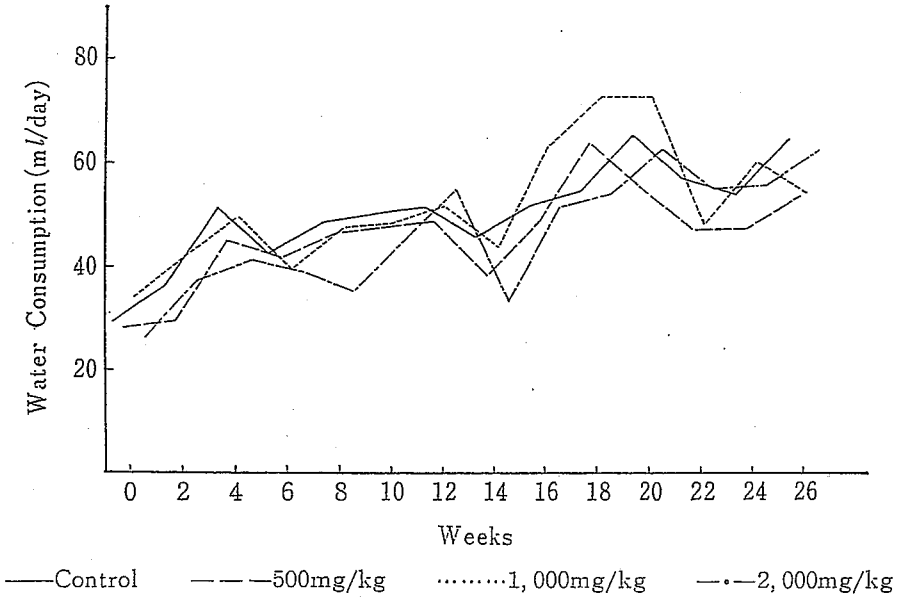


Fig.6 Water consumption of male rats treated with Protease P "Amano" 6 for 26 weeks



Number of rats and dead day in chronic toxicity

Group	Num. of dead rats	dead day
Control	1	131th day
500mg/kg	1	128th day
1, 000mg/kg	2	98th, 143th day
2, 000mg/kg	1	105th day

Table 9 Hematological findings in male rats treated with Protease P "Amano" 6 for 12 weeks

Sex	Dose (mg/kg)	No. of rats	Red blood cell ($\times 10^4$/mm^3)	Hemoglobin (g/dl)	Hematocrit (%)	White blood cell ($\times 10^3$/mm^3)
Male	Control	10	811.7±7.4	16.1±0.2	49.0±2.5	8.63±0.53
	500	10	807.7±5.8	15.8±0.5	49.1±2.6	9.03±0.61
	1, 000	10	817.8±9.3	15.9±0.4	48.2±2.5	8.61±0.25
	2, 000	10	830.3±12.8	16.1±0.5	48.2±3.2	8.18±0.70

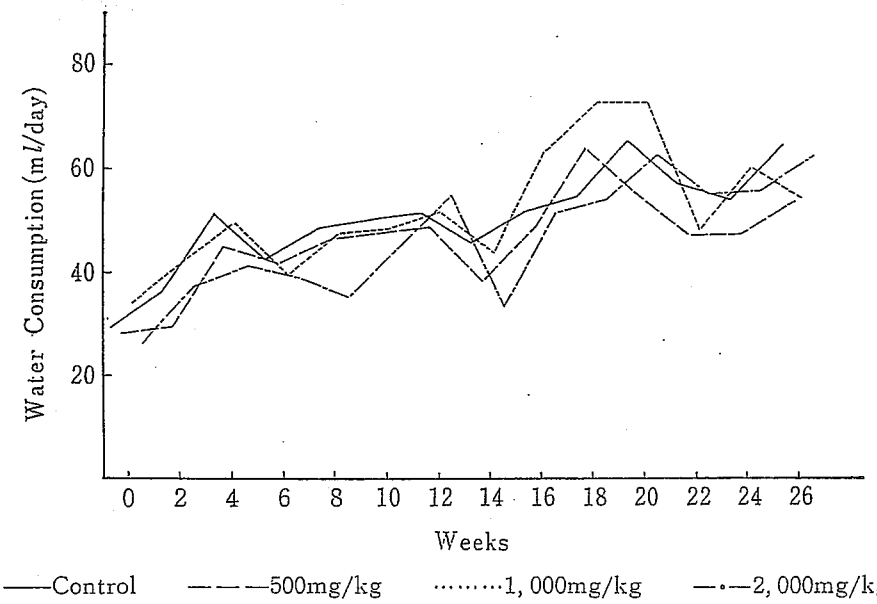
Mean±S.E.

Table 10 Hematological findings in male rats treated with Protease P "Amano" 6 for 26 weeks

Sex	Dose (mg/kg)	No. of rats	Red blood cell ($\times 10^4$/mm^3)	Hemoglobin (g/dl)	Hematocrit (%)	White blood cell ($\times 10^3$/mm^3)	Differentiation of white blood cell (%)			
							Eossino.	Neutro.	Lymph.	Mono.
Male	Control	9	687.3± 6.9	15.8±0.3	50.5±1.0	7.02±0.94	1.1	22.1±3.1	74.6±6.8	2.2
	500	9	686.0±10.2	15.3±0.2	51.3±1.2	6.70±0.13	1.6	27.7±4.6	68.7±6.2	2.0
	1, 000	8	673.5±16.1	15.0±0.3	50.7±1.1	6.81±0.36	2.4	23.9±2.2	71.4±4.4	2.3
	2, 000	9	678.9±13.0	15.4±0.4	50.6±0.9	7.91±0.86	2.2	25.7±3.7	69.7±5.1	2.4

Mean±S.E.

Fig.6 Water consumption of male rats treated with Protease P "Amano" 6 for 26 weeks



Number of rats and dead day in chronic toxicity

Group	Num. of dead rats	dead day
Control	1	131th day
500mg/kg	1	128th day
1, 000mg/kg	2	98th, 143th day
2, 000mg/kg	1	105th day

Table 9 Hematological findings in male rats treated with Protease P "Amano" 6 for 12 weeks

Sex	Dose (mg/kg)	No. of rats	Red blood cell ($\times 10^4$/mm^3)	Hemoglobin (g/dl)	Hematocrit (%)	White blood cell ($\times 10^3$/mm^3)
Male	Control	10	811.7±7.4	16.1±0.2	49.0±2.5	8.63±0.53
	500	10	807.7±5.8	15.8±0.5	49.1±2.6	9.03±0.61
	1,000	10	817.8±9.3	15.9±0.4	48.2±2.5	8.61±0.25
	2,000	10	830.3±12.8	16.1±0.5	48.2±3.2	8.18±0.70

Mean±S.E.

Table 10 Hematological findings in male rats treated with Protease P "Amano" 6 for 26 weeks

Sex	Dose (mg/kg)	No. of rats	Red blood cell ($\times 10^4$/mm^3)	Hemoglobin (g/dl)	Hematocrit (%)	White blood cell ($\times 10^3$/mm^3)	Differentiation of white blood cell (%)			
							Eossino.	Neutro.	Lymph.	Mono.
Male	Control	9	687.3± 6.9	15.8±0.3	50.5±1.0	7.02±0.94	1.1	22.1±3.1	74.6±6.8	2.2
	500	9	686.0±10.2	15.3±0.2	51.3±1.2	6.70±0.13	1.6	27.7±4.6	68.7±6.2	2.0
	1,000	8	673.5±16.1	15.0±0.3	50.7±1.1	6.81±0.36	2.4	23.9±2.2	71.4±4.4	2.3
	2,000	9	678.9±13.0	15.4±0.4	50.6±0.9	7.91±0.86	2.2	25.7±3.7	69.7±5.1	2.4

Mean±S.E.

Table 11 Serum analysis of male rats treated with Protease P "Amano" 6 for 26 weeks

Sex	Dose (mg/kg)	No. of rats	S-GOT (Karmen unit)	S-GPT (Karmen unit)	Al-pase (B-L unit)	Glucose (mg/dl)	Protein (g/dl)	Total cholesterol (mg/dl)	Blood urea nitrogen (mg/dl)	Sodium ion (mEq/l)	Potassium ion (mEq/l)
Male	Control	9	99.6±12.0	34.4±6.1	3.2±0.3	153±18	6.8±0.2	51.7±4.5	16.6±1.8	138.5±3.1	4.9±0.1
	500	9	91.4±10.2	30.1±3.9	3.1±0.2	161±15	6.7±0.2	48.6±5.7	15.6±2.8	138.2±2.1	4.9±0.1
	1,000	8	93.6± 8.9	32.6±4.5	3.3±0.2	142±16	7.1±0.4	52.1±3.6	16.7±1.4	139.1±2.2	5.0±0.2
	2,000	9	93.6±10.9	30.3±5.1	3.5±0.5	151±10	6.7±0.2	52.1±4.0	17.3±1.7	140.0±2.5	4.8±0.1

Mean±S.E.

Table 12 Urinalysis of male rats treated with Protease P "Amano" 6 for 12 weeks

Sex	Dose (mg/kg)	No. of rats	pH	Protein		Glucose		Bilirubin		Urobilinogen	
				−	+	−	+	−	+	−	+
Male	Control	10	6.1±0.4	10	0	10	0	10	0	10	0
	500	10	6.5±0.6	9	1	10	0	10	0	10	0
	1,000	10	6.3±0.5	9	1	10	0	10	0	10	0
	2,000	10	5.9±0.3	10	0	10	0	10	0	10	0

pH : Mean±S.E.

Table 13 Urinalysis of male rats treated with Protease P "Amano" 6 for 26 weeks

Sex	Dose (mg/kg)	No. of rats	pH	Protein		Glucose		Bilirubin		Urobilinogen	
				−	+	−	+	−	+	−	+
Male	Control	9	6.9±0.5	8	1	9	0	9	0	9	0
	500	9	6.4±0.6	8	1	9	0	9	0	9	0
	1,000	8	6.8±0.3	6	2	8	0	8	0	8	0
	2,000	9	6.3±0.6	8	1	9	0	9	0	9	0

pH : Mean±S.E.

Table 14 Absolute organ weight of male rats treated with Protease P "Amano" 6 for 26 weeks

Sex	Dose (mg/kg)	No. of rats	Initial body weight (g)	Final boby weight (g)	Liver (g)	Kidneys (g)	Heart (g)	Lungs (g)	Spleen (g)
Male	Control	9	144±16	477±23	10.58±0.56	2.83±0.10	1.54±0.06	1.84±0.09	0.68±0.03
	500	9	146±11	485±20	10.37±0.53	2.78±0.12	1.49±0.08	1.85±0.07	0.70±0.05
	1,000	8	145±13	471±21	10.45±0.30	2.88±0.04	1.47±0.03	1.84±0.23	0.71±0.03
	2,000	9	148±11	473±18	10.82±0.51	2.85±0.15	1.60±0.12	1.92±0.24	0.71±0.04

Thyroids (mg)	Adrenals (mg)	Pituitary (mg)	Brain (g)	Thymus (g)	Urinary bladder (mg)	Testes (g)	Prostate (g)
31.8±3.4	52.0±3.0	9.3±0.4	1.78±0.05	0.23±0.03	133±12	2.52±0.08	0.46±0.07
31.4±1.9	50.9±3.1	9.1±0.3	1.85±0.04	0.22±0.02	144±18	2.55±0.09	0.49±0.03
30.2±1.7	50.6±2.8	9.3±0.2	1.81±0.04	0.21±0.02	133± 8	2.50±0.09	0.48±0.03
28.5±2.0	51.0±4.6	9.7±0.6	1.80±0.05	0.23±0.02	120±14	2.56±0.07	0.40±0.05

Mean±S.E.

Table 15 Relative organ weight of male rats treated with Protease P "Amano" 6 for 26 weeks

Sex	Dose (mg/kg)	No. of rats	Liver (g/100 g)	Kidneys (g/100 g)	Heart (g/100 g)	Lungs (g/100 g)	Spleen (g/100 g)	Thyroids (mg/100 g)
Male	Control	9	2.22±0.08	0.59±0.02	0.32±0.02	0.39±0.01	0.14±0.02	6.8±0.6
	500	9	2.12±0.05	0.57±0.02	0.31±0.01	0.38±0.02	0.14±0.01	6.5±0.3
	1,000	8	2.22±0.26	0.61±0.03	0.31±0.09	0.39±0.04	0.15±0.01	6.4±0.6
	2,000	9	2.29±0.09	0.60±0.02	0.34±0.02	0.41±0.05	0.15±0.01	6.1±0.5

Adrenals (mg/100 g)	Pituitary (mg/100 g)	Brain (g/100 g)	Thymus (g/100 g)	Urinary bladder (mg/100 g)	Testes (g/100 g)	Prostate (g/100 g)
10.9±0.5	1.9±0.1	0.37±0.01	0.05±0.01	27.8±1.7	0.51±0.03	0.09±0.01
10.5±0.7	1.9±0.1	0.38±0.02	0.05±0.01	29.7±1.9	0.53±0.04	0.10±0.01
10.8±0.7	1.9±0.1	0.38±0.01	0.05±0.01	28.4±1.7	0.54±0.03	0.10±0.01
10.9±1.1	2.0±0.1	0.38±0.02	0.05±0.01	25.6±1.9	0.54±0.03	0.08±0.01

Mean±S.E.

Photo.8 Liver of a male rat treated with 5,000 mg/kg P Protease P "Amano" 6 for 30 days (× 100. H.E.)

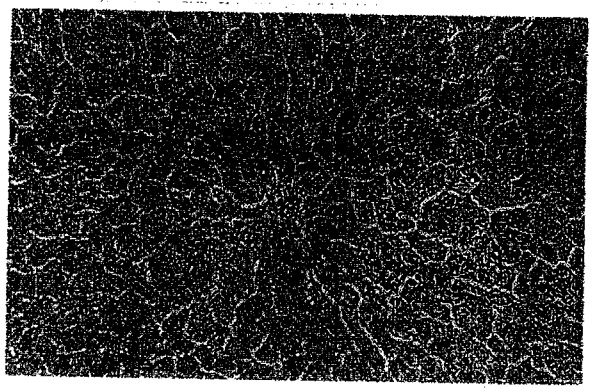


Photo.9 Grandular stomach of a male rat treated with 2,000 mg/kg Protease P "Amano" 6 for 26 weeks (× 100. H.E.)

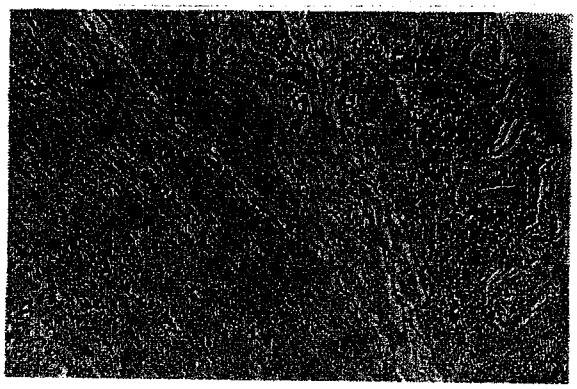


Photo.10 Fore stomach of a male rat treated with 2,000 mg/kg Protease P "Amano" 6 for 26 weeks (× 100. H.E.)