Safety Assessment of Protease P "Amano" 6 derived from *Aspergillus melleus*

- Reverse mutation test in bacteria -

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Introduction

Protease P "Amano" 6 is a preparation of the enzyme extracted and purified from a culture broth of *Aspergillus melleus*. This preparation is mainly used for protein hydrolysis in the food industry.

This report presents the results of a reverse mutation test of Protease P "Amano" 6 in bacteria, according to the Guidelines for In vitro Mutagenicity Testing[1], issued by the Ministry of Labor, Japan.

Materials and Methods

1. Test article

Protease P "Amano" 6 (Lot No.PZQ05520, protease activity: 759,000u/g (Unified method, pH 8.0)) was subjected to this study.

2. Positive control agents

Mutagenic agents used as positive controls were as follows: 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2, Lot No.STJO738, Wako Pure Chemicals Industries, Co., Ltd.), N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG; Lot No. VIR6328, Nacalai Tesque, Inc.), 9-aminoacridine hydrochloride (9AA, Lot No. MIK4279, Nacalai Tesque, Inc.), 2-aminoanthracene (2AA; Lot No. M5H7547, Nacalai Tesque, Inc.).

3. Preparation of test article and positive control solutions

In the preliminary test, 4 test article solutions (5,000, 1,250, 313 and 78 μ g/plate (ratio:1/4)) were prepared by serial dilution using distilled water. In the final test, 5 test article solutions (5,000, 2,500, 1,250, 625 and 313 μ g/plate (ratio:1/2)) were employed according to the results of the preliminary test.

Positive control agents were dissolved in dimethylsulfoxide (DMSO for absorption spectrometry; Dojin Chemicals Co., Ltd.) in accordance with the Guidebook to In Vitro Mutagenicity Testing[2] and stored at -80℃ before use. Frozen positive control solutions were melted when in use.

Each solution except positive control solutions was prepared before use and the procedures stated above were carried out under sterile environment.

4. Bacterial strains

Four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and a strain of *Escherichia coli* (WP2uvrA) were obtained from Hashima Laboratory, Nihon Bioresearch Center Inc., on December 1, 1987.

A 0.5ml culture fluid of each strain was dispensed in sterile screw capped test tubes containing 0.044ml of DMSO. These aliquots were rapidly frozen in dry ice-acetone and then stored at -80 ℃.

5. Preparation of test culture fluid

2.5 g of Nutrient broth No.2 (Oxoid Ltd.) was dissolved in distilled water for injection (Otsuka Pharmacological industries Co. Ltd.) and measured up to 100ml. Each 10ml of the media was dispensed in screw capped L-shape test tubes and then sterilized by autoclaving (121℃, 20min.). Frozen and stored tester bacterial suspensions were thawed and 20 μ l of each suspension was added into a nutrient broth with the sterilized micro pipette and then incubated at 37℃ for 8hrs using a shaking incubator (LH-1000, Advantec-Toyo Co., Ltd., shaking frequency: 44 times/min, shaking width: 30mm). These cultures provided suspensions of from 1.28 to 1.85 × 109 organisms/ml which were measured photometrically (660nm).

6. Preparation of the supplemented liver fraction (S9 mix)

S9 (Lot No. 92022103 for the preliminary test, Lot No. 92022103 and 92061208 for the final test) and cofactor (Lot No. 999201) were purchased from Oriental Yeast Co., Ltd. S9 mix with the composition shown in the following table was sterilized by filtration through a membrane filter (0.45 μ m porosity) and kept in an ice-water bath before use.

Table Composition of S9 mix

Component		Volume (ml)
S9		0.1
Cofactor		0.9
	Component of cofactor	Final conc. (μ mol/ml)
	$MgCl_2$	8
	KCl	33
	Glucose-6-phosphate	5
	NADPH	4
	NADH	4
	Na_2HPO_4	84
	NaH_2PO_4	16

7. Media

Minimal glucose agar medium (Tesmedia ATM, Lot No.AM050FH) was purchased from Nisshin Flour Milling Co., Ltd. Top agar medium was prepared as follows; one part of a solution containing 0.5mM L-histidine and 0.5mM D-biotin for the test with *Salmonella typhimurium* or 0.5mM L-tryptophan for the test with *Echerichia coli* was sterilized by filtration and then mixed with 10 parts of a solution containing 0.6% Bacto Agar (Difco Co., Ltd.) and 0.5% NaCl that had been sterilized by autoclaving.

8. Procedure

The test was performed by the preincubation method in accordance with the Guidelines[1)] issued by the Ministry of Labor, Japan. An aliquot (0.1ml) of the test article solution and 0.1ml of bacterial suspension were mixed well with 0.5ml of 0.1M phosphate buffer (0.1M, pH 7.4) and then preincubated at 37 ℃ for 20 minutes while shaking. The mixture was then added to 2ml of the top agar medium maintained at 45 ℃ and then transferred to each petri dish containing minimal glucose agar (two dishes used for the preliminary test, three for the final test). This process was also carried out replacing the 0.1M phosphate buffer with the S9 mix. After incubation at 37 ℃ for 48 hours, the number of colonies of prototrophic revertants was counted on each plate on a colony counter (Colony Analyzer Systems CA-7II, System Science Co., Ltd). At the same time, each plate was grossly observed for growth inhibition of the tester bacteria, contamination by foreign micro organisms, sedimentation of the solid body and other findings on the plate.

The test article was judged to be mutagenic when the mean number of revertant colonies on the plates containing the test article was not less than twice that of revertant colonies on the solvent control plates and increased in a dose-related manner.

Results and Discussion

The results of the reverse mutation test of Protease P "Amano" 6 consisted of the preliminary and final tests using four types of *Salmonella typhimurium* strains and *Echerichia coli*. These results are shown in Tables 1 to 3.

In the preliminary test for finding the highest amount of the test article to be used in the final test, there was not shown a growth inhibition effect on all tester strains at the highest amount, 5000 μ g/plate.

With Protease P "Amano" 6, the number of revertant colonies was the same as that with the concurrent corresponding solvent control, whether the employed strains were activated metabolically or not. On the other hand, all positive control agents caused a distinct increase in the number of revertant colonies. Thus it is concluded from these results that Protease P "Amano" 6 has no mutagenic activity.

Summary

Mutagenicity of Protease P "Amano" 6 was studied by the reverse mutation tests using bacteria. Protease P "Amano" 6 did not increase the number of revertant colonies over background level in the tester strains, *Salmonella typhimurium*, TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2uvrA at various amounts from 313 to 5000 μ g/plate.

These results indicate that Protease P "Amano" 6 is not mutagenic, nor is it metabolized to a mutagenic substance under an exogenous metabolic activation system with S9 mix.

References

1) Office of the Labor Standard Bureau: "Guidelines for In Vitro Mutagenicity Testing", Notification No. 261 of the Labor Standard Bureau, Ministry of Labor, Japan, May 18, 1985.

2) Chemical Substance Research Section, Industrial Safety & Health Division, Ministry of Labor, Japan: "Guidebook to In Vitro Mutagenicity Testing" (in Japanese), pp53-79,1986

Table 1 Preliminary reversion test of Protease P "Amano" 6 (Lot No. PZQ05520)

With or without S-9 mix	Concentration of sample (μg/plate)	Number of revertant colonies (revertant colonies/plate)															
		Base-pair substitutional type									Frameshifting type						
		TA100			TA1535			WP2uvrA			TA98			TA1537			
S-9 mix (−)	0	171	173	(172)	23	20	(22)	22	25	(24)	26	29	(28)	17	14	(16)	
	78	176	160	(168)	21	27	(24)	22	25	(24)	30	31	(31)	14	16	(15)	
	313	172	164	(168)	24	23	(24)	28	28	(28)	32	29	(31)	24	13	(19)	
	1,250	167	163	(165)	27	33	(30)	24	26	(25)	37	32	(35)	13	13	(13)	
	5,000	181	183	(182)	30	29	(30)	27	24	(26)	36	36	(36)	15	13	(14)	
S-9 mix (+)	0	150	158	(154)	16	21	(19)	16	21	(19)	35	32	(34)	19	20	(20)	
	78	180	178	(179)	23	26	(25)	23	26	(25)	33	33	(33)	18	19	(19)	
	313	190	184	(187)	26	33	(30)	26	33	(30)	27	32	(30)	17	11	(14)	
	1,250	186	183	(185)	26	19	(23)	27	19	(23)	35	38	(37)	18	12	(15)	
	5,000	184	183	(184)	27	23	(25)	27	23	(25)	29	34	(32)	12	13	(13)	
Positive control not requiring S-9 mix	Substance	AF-2			ENNG			AF-2			AF-2			9AA			
	Concentration (μg/plate)	0.01			5			0.01			0.1			80			
	R. colonies	725	735	(730)	429	392	(411)	244	246	(245)	654	646	(650)	1411	1414	(1413)	
Positive control requiring S-9 mix	Substance	2AA			2AA			2AA			2AA			2AA			
	Concentration (μg/plate)	1			2			20			0.5			2			
	R. colonies	768	762	(765)	408	413	(411)	691	691	(691)	466	465	(466)	251	209	(230)	

( ) : Average

Table 2 Final reversion test of Protease P "Amano" 6 (Lot No. PZQ05520)

With or without S-9 mix	Concentration of sample (μg/plate)	Number of revertant colonies (revertant colonies/plate)																	
		Base-pair substitutional type												Frameshifting type					
		TA100				TA1535				WP2uvrA				TA98				TA1537			
S-9 mix (−)	0	171	174	179	(175)	22	19	24	(22)	26	18	19	(21)	19	25	22	(22)	26	16	18	(20)
	313	165	146	155	(155)	26	32	25	(28)	30	20	27	(26)	22	24	19	(22)	23	21	18	(21)
	625	164	167	160	(164)	29	29	28	(29)	22	29	20	(24)	22	23	29	(25)	21	22	13	(19)
	1,250	164	154	160	(159)	29	27	23	(26)	30	22	29	(27)	24	34	27	(28)	27	18	28	(24)
	2,500	182	195	182	(186)	30	29	25	(28)	29	35	34	(33)	26	38	24	(29)	20	15	20	(18)
	5,000	212	213	213	(213)	23	29	34	(29)	27	21	28	(25)	28	34	28	(30)	25	16	24	(22)
S-9 mix (+)	0	147	144	147	(146)	28	22	33	(28)	21	22	20	(21)	35	33	32	(33)	26	27	22	(25)
	313	172	183	173	(176)	31	33	26	(30)	23	26	23	(24)	36	29	39	(35)	23	21	27	(24)
	625	171	184	170	(175)	33	39	33	(35)	27	33	31	(30)	30	35	32	(32)	19	26	22	(22)
	1,250	195	188	193	(192)	35	31	30	(32)	28	36	29	(31)	33	37	33	(34)	23	21	27	(24)
	2,500	199	215	210	(208)	36	35	33	(35)	31	32	24	(29)	36	30	29	(32)	24	18	19	(20)
	5,000	222	219	218	(220)	35	41	39	(38)	23	29	26	(26)	36	37	36	(36)	21	19	20	(20)
Positive control not requiring S-9 mix	Substance	AF-2				ENNG				AF-2				AF-2				9AA			
	Concentration (μg/plate)	0.01				5				0.01				0.1				80			
	R. colonies	547	568	497	(537)	408	391	399	(399)	191	187	190	(189)	646	558	627	(610)	1684	1770	1628	(1694)
Positive control requiring S-9 mix	Substance	2AA				2AA				2AA				2AA				2AA			
	Concentration (μg/plate)	1				2				20				0.5				2			
	R. colonies	773	784	743	(767)	420	434	423	(426)	707	733	698	(713)	471	466	464	(467)	250	297	228	(258)

( ) : Average

Table 3 Final reversion test of Protease P "Amano" 6 (Lot No. PZQ05520)

With or without S-9 mix	Concentration of sample (μg/plate)	Number of revertant colonies (revertant colonies/plate)																	
		Base-pair substitutional type												Frameshifting type					
		TA100				TA1535				WP2uvrA				TA98				TA1537			
S-9 mix (−)	0	167	168	160	(165)	22	23	22	(22)	19	26	19	(21)	23	24	20	(22)	22	18	20	(20)
	313	175	175	170	(173)	24	22	22	(23)	22	23	23	(23)	23	21	21	(22)	16	16	18	(17)
	625	176	174	163	(171)	25	22	25	(24)	22	31	25	(26)	22	24	23	(23)	22	22	23	(22)
	1,250	164	170	167	(167)	24	22	26	(24)	23	20	33	(25)	25	25	21	(24)	25	17	26	(23)
	2,500	177	179	177	(178)	21	23	23	(22)	31	28	29	(29)	23	27	25	(25)	18	25	24	(22)
	5,000	184	189	188	(187)	28	33	28	(30)	27	33	27	(29)	29	31	30	(30)	19	19	20	(19)
S-9 mix (+)	0	159	148	150	(152)	27	20	23	(23)	23	19	24	(22)	28	33	35	(32)	20	17	14	(17)
	313	171	161	169	(167)	23	23	23	(23)	22	18	21	(20)	34	32	36	(34)	15	16	16	(16)
	625	174	172	171	(172)	28	29	28	(28)	21	20	19	(20)	30	35	35	(33)	13	17	15	(15)
	1,250	181	188	181	(183)	27	27	25	(26)	20	20	23	(21)	32	31	27	(30)	14	20	16	(17)
	2,500	203	200	200	(201)	27	26	26	(26)	23	21	21	(22)	35	35	35	(35)	19	18	18	(18)
	5,000	192	196	191	(193)	32	29	32	(31)	26	22	25	(24)	37	35	40	(37)	17	16	15	(16)
Positive control not requiring S-9 mix	Substance	AF-2				ENNG				AF-2				AF-2				9AA			
	Concentration (μg/plate)	0.01				5				0.01				0.1				80			
	R. colonies	640	646	639	(642)	444	420	434	(433)	195	196	194	(195)	594	615	665	(625)	1594	1523	1562	(1560)
Positive control requiring S-9 mix	Substance	2AA				2AA				2AA				2AA				2AA			
	Concentration (μg/plate)	1				2				20				0.5				2			
	R. colonies	729	737	733	(733)	442	451	439	(444)	552	552	552	(552)	497	449	490	(479)	225	247	233	(235)

( ) : Average

STUDY NO.: 45-998-05

TITLE OF STUDY :

Safety Assessment of Protease P "Amano" 6
Derived from *Aspergillus melleus*
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DEPARTMENT OF QUALITY ASSURANCE
REPORT AUDIT STATEMENT

The following report has been audit by Amano Pharmaceutical Co., Ltd. Quality Assurance Units. It is considered to be an accurate description of the procedures and practices employed during the course of the study and an accurate presentation of the findings.

Title : **Safety Assessment of Protease P "Amano" 6**
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