[Chem. Pharm. Bull. 34(8)3312—3319(1986)]

Purification and Characterization of a New Peptide-Mannan, SP-I, as a Gastric Secretion-Inhibitory Principle from Autolysate of Brewer's Yeast

TSUTOMU MIMURA,*,[a] YASUTAKA MAKINO,[a] HIROSHI NAKAJIMA,[a] MASARU OKABE,[a] YASUHIRO KOHAMA,[a] TETSUO KATO[b] and MASUCHIKA TAKEUCHI[b]

Faculty of Pharmaceutical Sciences, Osaka University,[a] Yamadakami 1–6, Suita, Osaka 565, Japan and Asahi Beer Co., Ltd.,[b] Ohmori-kita 2–13–1, Ohta-ku, Tokyo 143, Japan

(Received January 16, 1986)

A substance showing gastric secretion-inhibitory activity in rats was purified from defatted cell wall of brewer's yeast cells through extraction by autoclaving in distilled water, pepsin digestion, fractionation with ethanol, diethyl aminoethyl-Sephadex A-50 column chromatography and gel filtration on a Sepharose 6B column. The substance, named SP-I, markedly decreased gastric juice secretion in pylorus-ligated rats when administered intraperitoneally or intravenously at a dose of 2.5 mg/kg. SP-I reduced Shay ulceration by 81% (25 mg/kg × 2, i.p.), aspirin ulceration by 56% (25 mg/kg, i.p.) and phenylbutazone ulceration by 82% (25 mg/kg, i.p.). It was also shown that the decreases of hexosamine and sialic acid contents in gastric mucosa caused by aspirin administration in pylorus-ligated rats were significantly recovered by the administration of SP-I. SP-I, a new peptide-mannan (95% mannan, 3% peptide), showed a homogeneous pattern in ultracentrifugal analysis, and its molecular weight was estimated to be several hundred thousand daltons.

Keywords——brewer's yeast; gastric juice inhibitory substance; peptide-mannan; ulceration; gastric secretion

Since microorganisms produce such a wide variety of metabolites, it seems reasonable to assume that they include potentially useful pharmacologically active compounds. In connection with our studies on microbial products with inhibitory action on gastric secretion, we have demonstrated several inhibitory principles including melanoprotein from *Streptomyces bottropensis*,[1] glycoprotein from *Bacillus subtilis*[2] and a family of fatty acids from dried brewer's yeast.[3] The present study was undertaken to seek a new metabolite with inhibitory action on gastric secretion using autolysate of brewer's yeast as a source. We describe here the isolation and characterization of a new peptide-mannan as an active principle.

Experimental

Extraction and Purification——The procedure is shown in Fig. 1. Twenty kilograms of brewer's yeast (Asahi Beer Co., Tokyo, *Saccharomyces cerevisiae*) were autolysated in 20 l at pH 2.5 at 52±2 °C for 17 h, and the autolysate was centrifuged at 10000 rpm for 20 min to obtain the yeast cell wall (YCW). The YCW was suspended in 3 volumes of ethanol, stirred at 80—83 °C for 2 h, and allowed to stand at room temperature. The mixture was filtered through a Toyo filter paper No. 50, and the residue was washed with hot ethanol in the same manner to leave the defatted YCW (DF-YCW). The DF-YCW was suspended in 5 volumes of distilled water, autoclaved at 120 °C for 20 min, cooled, and centrifuged at 10000 rpm for 20 min. The supernatant (autoclaved DF-YCW: ADF-YCW) was concentrated to 1/3 of the initial volume at 50 °C in a vacuum. The concentrated fluid was digested with 1/5000 amount of pepsin at pH 1.8 for 20 h at 37 °C. The digestion was terminated by adjusting the pH to 7.0 and boiling for 5 min to leave pepsin-digested ADF-YCW (PADF-YCW). The ADF-YCW solution was added to chilled ethanol to give the ethanol 0—50% precipitate, which was further fractionated to yield the ethanol 30—40% precipitate. The ethanol precipitate was purified by means of successive column chromatographies on a diethyl aminoethyl (DEAE)-

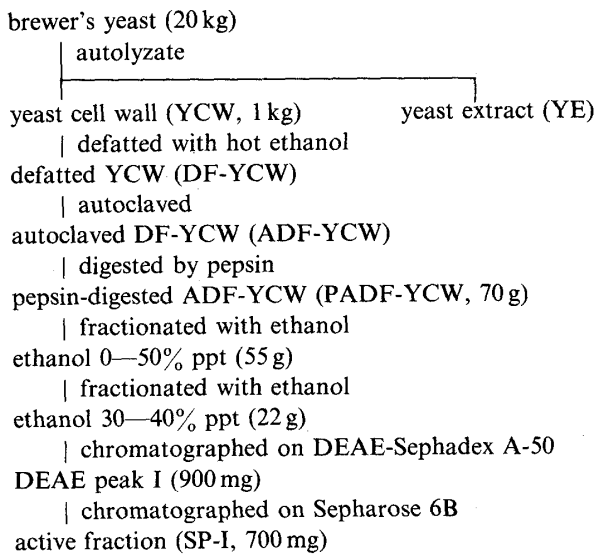

Fig. 1. Extraction and Purification of Gastric Secretion-Inhibitory Principle, SP-1, from Brewer's Yeast

Sephadex A-50 and Sepharose 6B to give an active fraction, SP-I (yield: 700 mg). Portions of the extracts and fractions from the extraction and purification stages were desalted, lyophilized and subjected to bioassay.

Measurement of Biological Activities——The animals used were Wistar male rats weighing 150—200 g. Samples, from the extraction and purification stages (YCW to PADF-YCW) were suspended in saline and were intraduodenally or intraperitoneally administered to rats. Other samples were also dissolved in saline and were intraperitoneally, intravenously or intraduodenally administered to rats. Saline was used as the control.

Gastric Secretion-Inhibitory Activity: The rats were fasted for 48 h before the experiment. Under anesthesia, the pylorus was ligated according to the method described by Shay *et al.*[4] After 4 h, the animals were sacrificed and the stomachs were removed. The gastric juice volume, total output and total peptic activity were determined as described in our previous paper.[5]

Anti-ulcerogenic Activity: i) Gastric ulcer in pylorus-ligated rats: Rats, previously fasted for 24 h and pylorus-ligated as described above, were used. After 16 h, the animals were sacrificed and the stomachs removed. The degree of gastric ulceration that had developed in the forestomach was estimated by the method of Narumi *et al.*[6] Ulceration was given an ulcer index from 0—5 according to its severity. ii) Aspirin-induced gastric ulcer: Rats were fasted for 24 h. According to the method of Okabe *et al.*,[7] rats were orally given 100 mg/kg of aspirin suspended in 5% Gum Arabic solution immediately after pylorus-ligation. Seven hours later, the rats were sacrificed and their stomachs removed to examine the lesion in the glandular portion. The sum of the length of all lesions was used as an ulcer index. iii) Phenylbutazone-induced gastric ulcer: According to the method of Suzuki *et al.*,[8] rats fasted for 24 h were orally given 200 mg/kg of phenylbutazone suspended in 5% Gum Arabic solution. Five hours later, rats were sacrificed and their stomachs removed to examine the lesion in the glandular stomach area. The sum of the diameter of each lesion was used as an ulcer index.

Determination of Mucopolysaccharide: Removed stomachs were incised at the greater curvature and rinsed with saline. The mucosal layer was scraped off from the glandular portion of the stomach. Determinations of mucopolysaccharide, hexosamine,[9] sialic acid[10] and uronic acid[11] were carried out as previously described in detail.[12]

Measurement of Rectal Temperature: Rats showing a rectal temperature of 37 to 38 °C were used. The temperature was taken before and at 1 h intervals after sample injection. Lipopolysaccharide from *Escherichia coli* (Serotype 0127: BB, Sigma) was used as a positive control.

Measurement of Inflammatory Activity: The right hind paws of rats were inoculated with 0.1 ml of sample, while the left hind paws were inoculated with saline. At the indicated time, the volume of the paws was measured by dipping the paws in mercury. The inflammatory activity was calculated based on the difference in volume between sample-inoculated and saline-injected paws. As a positive control, carrageenin (Picnin-A, Zushikagaku) was used.

Analytical Procedures——Carbohydrate content was determined by the phenol–H_2SO_4 method using glucose as a standard. Carbohydrate composition was obtained by analyzing a hydrolysate with 2 N HCl at 100 °C for 6 h for neutral sugar and a hydrolysate with 4 N HCl at 100 °C for 8 h for amino sugar using a liquid chromatograph (Hitachi 034). Protein content was determined by the Folin–Lowry method[13] using bovine albumin as a standard. Amino acid composition was obtained by analyzing a hydrolysate with 6 N HCl at 110 °C for 24 h using the liquid chromatograph. The ultraviolet (UV) spectrum was measured with a spectrophotometer (Shimadzu MPS-2000). Ultra-centrifugal analysis was carried out using an ultracentrifuge (Hitachi model 282).

Results

Purification of the Active Principle

As shown in Table I, gastric secretion-inhibitory activity during the extraction and purification of the active principle from the autolysate of brewer's yeast was recovered in YCW, ADF-YCW and PADF-YCW, successively. These fractions, when intraduodenally administered, showed a significant inhibitory effect on three parameters (gastric volume, total acid output and total peptic activity) of gastric secretion in pylorus-ligated rats. When PADF-YCW was intraperitoneally administered, the inhibitory action was approximately 5-fold

TABLE I. Gastric Secretion-Inhibitory Activities of Fractions Obtained during the Extraction in Pylorus-Ligated Rats (4 h)

Sample[a]	Dose (mg/kg)	Route	Gastric volume (ml)	Total acid output (μeq)	Total peptic activity (mg as tyrosine)
Control	—	i.d.	3.11±0.40	317.6±62.0	211.0±20.8
YE	500	i.d.	2.57±0.28	194.8±27.8	141.6±19.7[b]
YCW	500	i.d.	2.01±0.29[b]	147.6±16.9[b]	118.6± 8.3[c]
ADF-YCW	250	i.d.	1.93±0.26[b]	138.7±25.9[b]	124.4±14.6[c]
PADF-YCW	250	i.d.	1.61±0.17[c]	76.1±28.9[c]	91.6±17.5[d]
PADF-YCW	100	i.d.	2.72±0.49	217.4±48.4	178.2±29.8
Control	—	i.p.	2.77±0.25	344.9±37.5	312.6±36.8
PADF-YCW	100	i.p.	0.88±0.09[d]	48.0± 5.1[d]	55.3±10.6[d]
PADF-YCW	50	i.p.	1.21±0.09[d]	119.2± 9.5[d]	109.6± 9.7[d]
PADF-YCW	25	i.p.	2.23±0.25	254.1±33.4	257.2±33.3

a) The sample was administered immediately after pylorus-ligation. All values represent the mean±S.E. (n=8—10) per 100 g body weight. Significantly different from the control group: *b*) $p<0.05$, *c*) $p<0.01$, *d*) $p<0.001$.

TABLE II. Gastric Secretion-Inhibitory Activities of Fractions Obtained during the Purification and SP-I in Pylorus-Ligated Rats (4 h)

Sample[a]	Dose (mg/kg)	Route	Gastric volume (ml)	Total acid output (μeq)	Total peptic activity (mg as tyrosine)
Control	—	i.p.	3.02±0.19	342.6±36.4	227.4±11.8
EtOH 0—50% ppt.	25	i.p.	1.11±0.15[d]	108.0±16.4[d]	107.9± 9.4[d]
EtOH 30—40% ppt.	25	i.p.	0.93±0.10[d]	80.3±10.5[d]	79.4±8.9[d]
Control	—	i.p.	3.09±0.46	355.4±55.4	260.1±35.6
DEAE peak I	10	i.p.	1.25±0.22[c]	125.3±25.6[c]	129.3±11.7[c]
Control	—	i.p.	3.47±0.49	416.6±61.6	289.8±33.0
SP-I	10	i.p.	1.31±0.28[c]	108.9±31.3[d]	107.2±17.4[d]
SP-I	5.0	i.p.	1.48±0.17[c]	147.9±21.3[c]	133.9±12.2[d]
SP-I	2.5	i.p.	1.65±0.15[c]	182.2±26.4[c]	148.6±12.3[c]
SP-I	1.0	i.p.	2.85±0.24	285.8±23.3	186.7±25.0[b]
Control	—	i.v.	3.12±0.37	337.1±47.6	243.4±24.3
SP-I	5.0	i.v	1.05±0.21[d]	85.8±17.7[d]	104.8±15.7[d]
SP-I	2.5	i.v.	0.97±0.17[d]	86.6±21.4[d]	95.7±10.2[d]
SP-I	1.0	i.v.	1.60±0.35[b]	179.1±47.5[b]	140.7±22.4[c]
Control	—	i.d.	2.94±0.14	335.7±30.6	180.3±17.8
SP-I	25	i.d.	2.09±0.31[b]	204.6±37.8[b]	85.8±20.2[c]
SP-I	10	i.d.	2.51±0.38	250.3±40.5	120.1±25.8

a) The sample was administered immediately after pylorus-ligation. All values represent the mean±S.E. (n=8—10) per 100 g body weight. Significantly different from the control group: *b*) $p<0.05$, *c*) $p<0.01$, *d*) $p<0.001$.

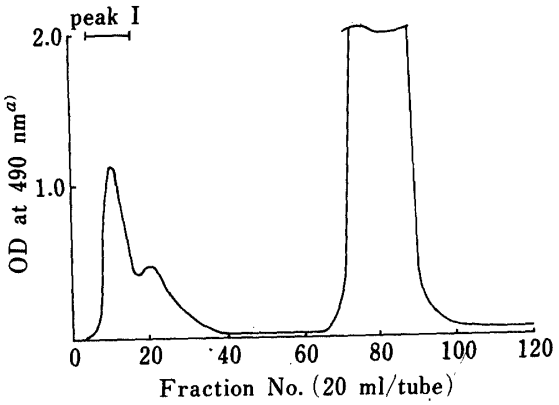

Fig. 2. Chromatography of Ethanol 30—40% ppt on a DEAE-Sephadex A-50 Column

Ethanol 30—40% ppt. (500 mg/20 ml) was applied to the column (80 ml volume), which was equilibrated with 10 mM Tris-HCl buffer (pH 8.0). The column was eluted with buffer followed by a linear gradient formed from 800 ml each of buffer and 0.5 M NaCl in the buffer at fraction No. 60.

a) A 0.4 ml aliquot was taken for the phenol–H_2SO_4 reaction.

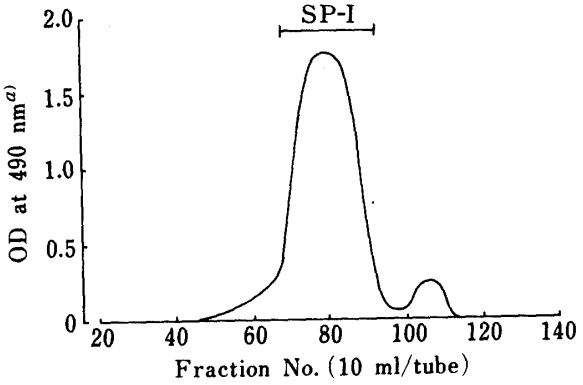

Fig. 3. Chromatography of DEAE Peak I on a Sepharose 6B Column

DEAE peak I (100 mg/10 ml) was applied to the column (1400 ml. volume), which was eluted with 10 mM phosphate buffer (pH 7.0).

a) A 0.4 ml aliquot was taken for the phenol–H_2SO_4 reaction.

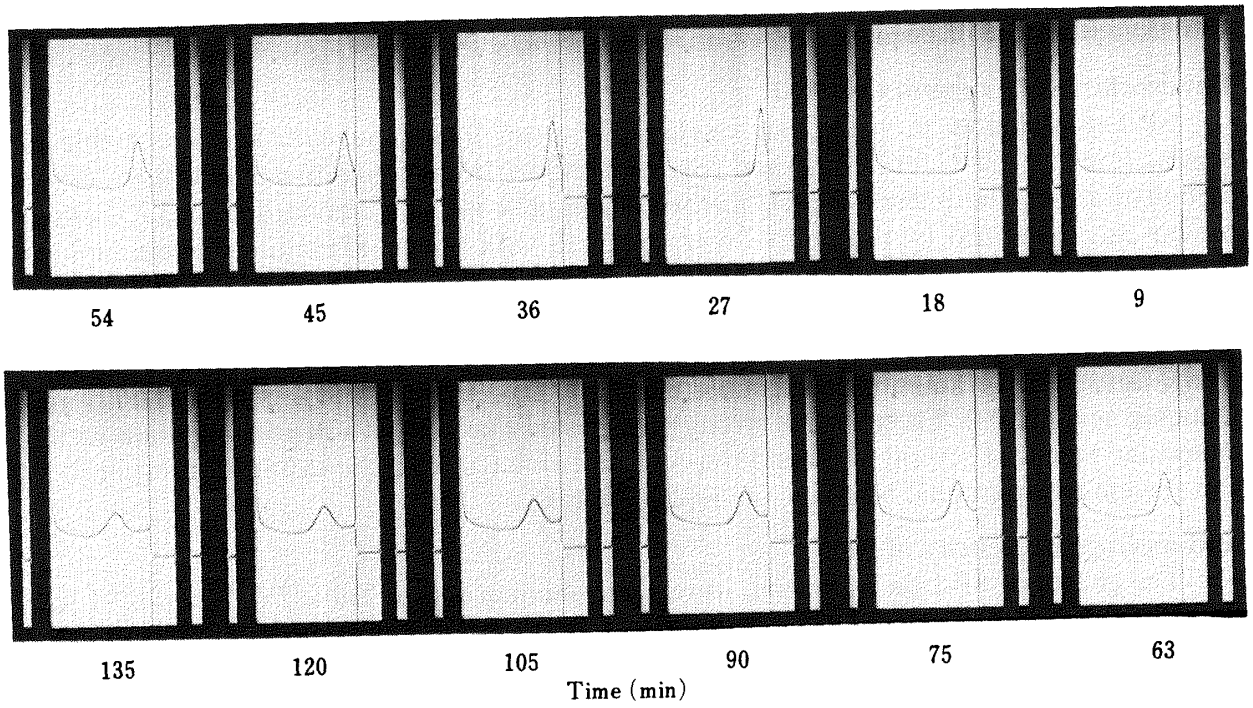

Fig. 4. Ultracentrifugal Pattern of SP-I

The sample concentration was 1.0% in 0.2 M phosphate buffer (pH 7.3).
The photographs were taken at 9 min intervals (up to 63 min) after reaching 53000 rpm at a bar angle of 65°, then at 75 min, and there after at 15 min intervals.

stronger than when it was intraduodenally administered, in terms of the minimum effective dose. When PADF-YCW was fractionated with ethanol, the inhibitory activity was recovered in the ethanol 0—50% ppt. then the ethanol 30—40% ppt. as shown in Table II. The active fraction, ethanol 30—40% ppt. was further purified by DEAE-Sephadex A-50 column chromatography, followed by Sepharose 6B column chromatography, as shown in Figs. 2 and 3. The active principle collected as peak I from a DEAE-Sephadex A-50 was symmetrically

TABLE III. Physicochemical Properties of SP-I

Carbohydrate content	94.8% (w/w)
Carbohydrate composition	Man (mol%: 99.89), GlcN (0.06), GalN (0.05)
Protein content	3.2% (w/w)
Amino acid composition	Asp (mol%: 7.31), Thr (5.30), Ser (14.09), Glu (11.43), Pro (17.00), Gly (12.72), Ala (9.89), Val (4.28), Met (0.32), Ile (2.65), Leu (4.35), Tyr (0.89), Phe (1.96), Lys (3.82), His (2.04), Arg (1.97)
Sedimentation constant	Apparent s_{20w}: 10.7
Extinction coefficient	$E_{1\%}^{1cm}$ at 270 nm (s) in water: 0.385

but broadly eluted as SP-I at a position of K_{av}: 0.35 from a Sepharose 6B column. Thus purified SP-I was found to be homogeneous in an ultracentrifugal analysis, showing symmetrical sedimentation at a constant rate of 10.7 S (Fig. 4 and Table III).

Intraperitoneally or intravenously administered SP-I showed dose-dependent gastric secretion-inhibitory activity in pylorus-ligated rats, as shown in Table II. It had a significant effect in terms of three parameters of gastric secretion at a dose of 2.5 mg/kg i.p. or 1.0 mg/kg i.v. It was also found that intraduodenally administered SP-I significantly inhibited the gastric secretion at a dose of 25 mg/kg.

Physicochemical Properties of SP-I

SP-I was soluble in water, but insoluble in most organic solvent, and was stable to heating (even at 120 °C for 20 min) and pepsin-digestion. It was found to contain 94.8% (w/w) carbohydrate by the phenol–H_2SO_4 method, using glucose as a standard, and 3.2% (w/w) protein as determined by the Folin–Lowry method, with bovine albumin as a standard, as shown in Table III.

Almost all of the carbohydrate in SP-I was mannan (mol%: 99.89), with very small amounts of glucosamine (0.06) and galactosamine (0.05). The protein moiety contained comparatively large amounts of proline (mol%: 17.00), serine (14.09), glycine (12.72) and glutamic acid (11.43). These results strongly suggested that SP-I is a peptide-mannan with a molecular weight of several hundred thousand, as estimated from the sedimentation constant and the chromatographic behavior on Sepharose 6B.

Anti-ulcerogenic Activity of SP-I

The effects of SP-I on ulceration in rats are summarized in Table IV. When SP-I was intraperitoneally administered at a dose of 10 × 2 or 25 × 2 mg/kg immediately and 8 h after pylorus-ligation, it significantly prevented gastric ulceration; the extents of inhibition in terms of the ulcer index were 27% and 81%, respectively. At a dose of 25 mg/kg, SP-I significantly prevented aspirin-induced ulceation in pylorus-ligated rats 56% inhibition. This peptide-mannan (10 or 25 mg/kg, i.p.) also greatly reduced (by 75—82%) phenylbutazone-induced ulceration in rats.

Effect of SP-I on Mucopolysaccharide Contents in Gastric Mucosa

The contents of hexosamine, uronic acid and sialic acids which we examined were greatly decreased at 7 h after aspirin administration in pylorus-ligated rats, as shown in Table V. However, the concomitant administration of SP-I (10, 25 mg/kg, i.p.) prevented the decreases of hexosamine and sialic acid caused by aspirin, but not that of uronic acid.

Effect of SP-I on Rectal Temperature in Rats

SP-I (2.5 or 5 mg/kg, i.p. or i.v.), which could significantly inhibit gastric secretion, had

TABLE IV. Anti-ulcerogenic Activity of SP-I in Rats

Ulcer	Sample	Dose (mg/kg, i.p.)	Ulcer index (mean ± S.E., n=8—9)	Inhibition (%)
Pylorus-ligated (16 h)[a]	Control	—	4.3 ± 0.3	—
	SP-I	5 × 2	3.6 ± 0.6	16
	SP-I	10 × 2	3.1 ± 0.4[d]	27
	SP-I	25 × 2	0.8 ± 0.3[f]	81
	Control	—	3.5 ± 0.3	—
	Atropine	5 × 2	1.6 ± 0.4[e]	54
Aspirin (pylorus-ligated, 16 h)[b]	Control	—	21.9 ± 2.9	—
	SP-I	10	13.4 + 2.8	39
	SP-I	25	9.7 ± 1.0[e]	56
	Control	—	30.6 ± 7.8	—
	Metiamide	50	11.1 ± 2.5[d]	64
Phenylbutazone (5 h)[c]	Control	—	25.4 ± 6.2	—
	SP-I	10	6.3 ± 1.5[d]	75
	SP-I	25	4.6 ± 1.4[e]	82
	Control	—	13.6 ± 3.5	—
	Cimetidine	100	3.6 ± 0.5[d]	74

a) The sample was administered twice immediately and 8 h after pylorus-ligation. *b*) The sample was administered immediately after pylorus-ligation. *c*) The sample was administered 30 min after administration of phenylbutazone. Significantly different from the control group: *d*) $p<0.05$, *e*) $p<0.01$, *f*) $p<0.001$.

TABLE V. Effect of SP-I on Mucopolysaccharides Contents in the Gastric Glandular Region in Aspirin-Induced Ulcerative Rats

Treatment	Sample	Dose (mg/kg, i.p.)	Hexosamine (μg GlcN HCl)	Sialic acid (μg NeuNAc)	Uronic acid (μg GlcUA)
Intact	Control	—	231.0 ± 12.8	19.5 ± 1.9	24.9 ± 1.4
Aspirin (pylorus-ligated, 7 h)[a]	Control	—	165.4 ± 13.2	14.6 ± 1.5	20.7 ± 2.5
	SP-I	10	211.2 ± 13.7[b]	16.8 ± 0.4	21.2 ± 1.9
	SP-I	25	223.5 ± 15.1[b]	22.4 ± 1.9[c]	22.2 ± 1.5

a) The sample was administered immediately after pylorus-ligation. All values represent the mean ± S.E. (n=8) per 100 mg wet tissue. Significantly different from the aspirin control group: *b*) $p<0.05$, *c*) $p<0.01$.

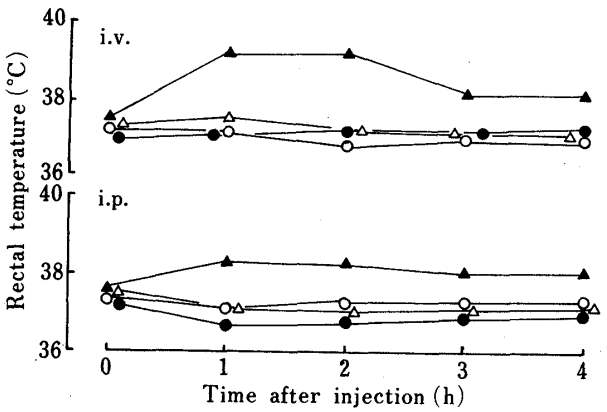

Fig. 5. Effect of SP-I on Rectal Temperature in Rats

Each point represents the mean of 3 rats.
●, control; ○, SP-I 5 mg/kg; △, SP-I 2.5 mg/kg; ▲, lipopolysaccharide 2.5 mg/kg.

no effect on the rectal temperature in rats, in contrast to lipopolysaccharide, as shown in Fig. 5.

Inflammatory Activity of SP-I in Rats

SP-I (dose of equivalent to 10 mg/kg body weight) caused only $6.5 \pm 0.6\%$ ($n=3$), $4.9 \pm 0.3\%$ and $4.2 \pm 0.2\%$ swelling at 3,4 and 5 h after the innoculation, respectively. On the other hand, carrageenin (0.1 ml of 1.5% solution per paw) caused $52.9 \pm 1.8\%$ ($n=3$), $57.9 \pm 2.0\%$ and $57.3 \pm 2.3\%$ swelling of the paws at the corresponding times.

Discussion

In our series of studies on useful pharmacologically active compounds in microbial products, a peptide-mannan, SP-I, has been isolated as a gastric secretion-inhibitory principle from autolysate of brewer's yeast.

In general, mannan and heteropolysaccharide containing large amounts of mannose exist on the surface layer of the cell wall, mainly as constitutive components. To date, only two groups of substances among yeast products have been demonstrated to have an inhibitory effect on gastric secretion. They are the fatty acids from brewer's yeast[3] and peptide-mannan A from baker's yeast.[14] Although both peptide-mannans, SP-I and A, were homogenous in centrifugal analysis and had the same gastric secretion inhibitory activity, there were apparent differences in their properties. Peptide-mannan A preparation (250 mg/kg, i.p.) obtained from the YCW of brewer's yeast by the same extraction procedure as used for peptide-mannan A from baker's yeast[14] did not show any gastric secretion-inhibitory activity in pylorus-ligated rats in the present study (data not shown). Peptide-mannan A was absorbed on DEAE-Sephadex A (Cl^--type), while SP-I was not. Peptide-mannan A consisted of 60% mannose and 25% peptide,[14] and SP-I of 95% mannan and only 3% peptide. As regards the amino acid compositions of peptide-mannans A and SP-I, there were differences in the contents of threonine and proline. Therefore, it is evident that SP-I differs chemically from peptide-mannan A in the peptide moiety.

The causes of peptic ulceration have been classified into two categories: aggressive factors and mucosal defensive factors. It is considered that the imbalance of these two factors (the relative dominance of defensive factors) initiates ulceration. SP-I was found to have a significant inhibitory effect on pylorus-ligation-, aspirin-, and phenylbutazone-induced ulcerations, together with pylorus-ligation-induced gastric juice secretion-inhibitory activity. Pylorus-ligation-induced ulceration is considered to be caused by the self-digestion of the gastric mucous membrane due to an increase of gastric juice secretion. Aspirin- and phenylbutazone-induced ulcerations are also caused by a deficiency of defensive factors due to the increase of gastric juice secretion. Therefore, it seems that the potent gastric secretion-inhibitory action of SP-I is one of the mechanisms of its antiulcerogenic effect.

Recently, more attention has been paid to the gastric mucous membrane barrier concept for a better understanding of the developmental mechanism of acute pathological changes in the mucous membrane of the stomach.[15] The destruction of mucopolysaccharides in the gastric mucosa, rather than the increase of the gastric juice secretion, was found to be a main cause of peptic ulceration.[16] Thus, the effect of SP-I on the contents of mucopolysaccharide components of the gastric mucosa were studied. The present findings indicated that the decreases of hexosamine and sialic acid caused by aspirin were prevented by the concomitant administration of SP-I with aspirin in pylorus-ligated rats. This result led us to assume that SP-I protects against ulceration by preventing the disappearance of hexosamine and sialic acid, and maintains the mucopolysaccharide content in the gastric mucosa at a normal level.

It is known that some microbial products such as the exothermic factor, pyrexine,[17] and

lipopolysaccharide,[18] have gastric secretion-inhibitory action due to their toxicity as endotoxins. However, SP-I did not show any exothermic or inflammatory effects in rats.

Thus, the antiulcerogenic activity of SP-I is considered to be based on both the inhibition of gastric juice secretion and the intensification of the defensive factors. However, the mechanism of SP-I inhibition of the gastric secretion and nature of the defensive factors still remain open questions at the present time. Further investigations on the effects of SP-I on the various functions of gastric juice secretion, and the biosynthesis and degradation of mucopolysaccharides in the gastric mucosa, should be done in order to evaluate further the preventive effect of SP-I on gastric ulceration.

References and Notes

1) T. Mimura, N. Muto, J. Tanaka, H. Oshita, N. Onishi and S. Aonuma, *Chem. Pharm. Bull.*, **26**, 998 (1978).
2) T. Mimura, N. Muto, H. Tsujibo, N. Onishi and S. Aonuma, *Chem. Pharm. Bull.*, **25**, 2770 (1977).
3) T. Mimura, H. Tsujibo, N. Muto, S. Otsuka and S. Aonuma, *Chem. Pharm. Bull.*, **28**, 1077 (1980).
4) H. Shay, S. A. Komarov, S. S. Fels, D. Meranze, M. Gruentein and M. Siplet, *Gastroenterology*, **5**, 43 (1945).
5) T. Mimura, N. Muto, E. Deguchi, K. Oyabu, S. Kondo and S. Aonuma, *Yakugaku Zasshi*, **96**, 621 (1976).
6) S. Narumi, Y. Hirata, K. Gomaibashi and M. Kanno, *J. Takeda Res. Lab.*, **29**, 85 (1970).
7) S. Okabe, K. Takeuchi, K. Nakamura and K. Takagi, *Jpn. J. Pharmacol.*, **24**, 363 (1974).
8) Y. Suzuki, M. Hayashi, M. Ito and I. Yamagami, *Jpn. J. Pharmacol.*, **26**, 471 (1976).
9) J. Hiroi, J. Seki, M. Ohtsuka, S. Katsuki and F. Honda, *Jpn. J. Pharmacol.*, **31**, 144 (1981); B. Gunner, *Acta Chem. Scand.*, **2**, 467 (1948).
10) L. Warren, *J. Biol. Chem.*, **234**, 1971 (1959).
11) T. Bitter and H. M. Muir, *Anal. Biochem.*, **4**, 330 (1962).
12) T. Mimura, M. Iwai, K. Maeda, I. Kohda, K. Tsujikawa and S. Aonuma, *J. Pharmacobio-Dyn.*, **7**, 718 (1984).
13) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
14) Y. Ito, Japan. Patent 49-69808 (1974) [*Chem. Abstr.*, **81**, 140865*h* (1974)].
15) F. Hollander, *Arch. Int. Med.*, **93**, 107 (1954).
16) K. Kira, *Jpn. J. Gastroenterol.*, **70**, 1182 (1973).
17) J. H. Wyllie, J. M. Limbosch and L. M. Nyhus, *Nature* (London), **215**, 879 (1967).
18) P. E. Baume, A. Nicholls and C. H. Baxter, *Nature* (London), **215**, 59 (1967).