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Purification and Partial Characterization of the Gastric Ulcer Inhibitory Substance from Culture Filtrate of *Bacillus subtilis* H[1)]

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The inhibitory substance against gastric ulceration in pylorus-ligated rats was purified through extraction with 5% acetic acid–50% ethanol, fractionation with ethanol, isoelectric precipitation, DEAE-cellulose column chromatography, and gel filtration on Sephadex G-100 column from isoelectric precipitate if the 72 hr culture filtrate of *Bacillus subtilis* H IAM 1521, and it was named gastric ulcer inhibitory substance (GUIS). This substance reduced ulceration in pylorus-ligated rats by 84.2% at the dose of 5.0 mg/kg, it also significantly repressed aspirin-induced gastric lesions under pylorus ligation at the same dose, but its activity was weak in stress-induced ulceration. It markedly decreased gastric juice volume, acidity, and peptic activity in pylorus-ligated rats when administered intraperitoneally at 5.0 mg/kg. This substance was a glycoprotein which showed homogeneous patterns in various kinds of electrophoresis, and its isoelectric point was pH 4.5.

Keywords——*Bacillus subtilis*; gastric ulcer inhibitory substance; glycoprotein; Shay ulcer; aspirin-induced gastric lesion; stress-induced gastric lesion

Recently, many studies have been made to find effective substances for therapeutic use among microbial products. We have already purified an effective substance for peptic ulcers, especially in ulcers resulting mainly from gastric digestion, from *Streptomyces bottropensis*.[3,4)] We also obtained a gastric acid inhibitory peptide-lipid from the culture filtrate of *Bacillus subtilis* H IAM 1521,[5)] but this was found to have no anti-ulcerogenic activity according to gastric bleeding time after perfusion of artificial gastric juice.[6)]

In the present work, we found a component in isoelectric precipitate from the culture filtrate of *B.subtilis* H IAM 1521, which inhibits ulceration caused by pylorus ligation, and it is not the peptide-lipid[5)] mentioned above. Purification of this active component and investigation of its properties are reported here.

Experimental

Organism——*Bacillus subtilis* H IAM 1521 was used for isolation of the substance with anti-ulcerogenic activity.

Animals——Male Wistar rats were used as experimental animals.

Medium and Culture Condition——Cultivation was carried out in 1 l shaking culture flasks containing 500 ml of semisynthetic medium (NH_4Cl 2.0 g, Na_2HPO_4 6.0 g, KH_2PO_4 3.0 g, NaCl 3.0 g, $MgCl_2$ 0.04 g, Na_2SO_4 0.11 g, glucose 10.0 g, Casamino acids 2.0 g, and tryptophan 0.02 g in H_2O 1 l, pH 7.0) sterilized by autoclaving. Each flask was inoculated with a loopful of *B. subtilis* H IAM 1521 cells, placed on a reciprocating shaker (95 rpm) shaken at 37° for 72 hr, and then stood at 37° for 24 hr.

Purification——The culture filtrate was separated by centrifugation of the medium and its supernatant

1) A part of this work was presented at the 95th Annual Meeting of the Pharmaceutical Society of Japan, Nishinomiya, April 1975.
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was collected as the culture filtrate. A crude active fraction was obtained from the culture filtrate as an isoelectric precipitate at pH 3.0, treated with 5% AcOH–50% EtOH, and fractionated with chilled EtOH. Further purification was performed with DEAE-cellulose, hydroxylapatite gel, and Sephadex G-100 columns. Hydroxylapatite gel was prepared according to the method of Tiselius, *et al.*[7]

Experimental Gastric Ulcers in Rats——1) Gastric Ulceration in Pylorus-ligated Rats: The rats (150—200 g) were deprived of food but allowed free access to water for 24 hr before the experiment. Surgical procedures were carried out according to the method described by Shay, *et al.*[8] After 18 hr, the animals were sacrificed and the stomach was removed. The stomach was opened along the greater curvature and the gastric ulcer that developed in the forestomach was macroscopically examined. The degree of gastric ulceration was estimated by the method of Narumi, *et al.*[9] and given an ulcer index from 0 to 5 according to its severity. A sample of gastric ulcer inhibitory substance was given intraperitoneally immediately after the ligation. This method was used to purify the effective component.

2) Aspirin-induced Gastric Lesions: The rats (150—200 g) were fasted for 24 hr before the experiment. According to the method of Okabe, *et al.*,[10] the rats orally received 100 mg/kg of aspirin suspended in 1% carboxymethylcellulose solution just after the pylorus ligation. After 5 hr, the rats were sacrificed and the stomach was removed, treated with 1% formalin, and examined for lesions in the glandular portion. The ulcer index was calculated as the sum of the length of each lesion. A sample of gastric ulcer inhibitory substance was administered intraperitoneally immediately after the pylorus ligation.

3) Stress-induced Gastric Lesions: Stress-induced gastric lesions were produced according to the method of Takagi and Okabe.[11] The rats (200—250 g) were placed in a stress cage and immersed to the xiphoid process in a water bath (23°) for 20 hr. The animals were sacrificed and the stomach was removed. After the stomach was treated with 1% formalin, gastric lesions in the glandular portion were examined. The ulcer index was estimated as the sum of the length of each lesion. A sample of gastric ulcer inhibitory substance was administered intraperitoneally 15 min before the stress exposure.

Gastric Secretion in Rats——Inhibitory activity on gastric secretion was examined by the method described in the preceding report.[3]

Cellulose Acetate Membrane Electrophoresis——According to the method of Kohn,[12] electrophoresis was carried out in Veronal buffer (pH 8.6, $\mu=0.05$) for 30 min and the membrane was stained with Ponceau 3R.

SDS-polyacrylamide Gel Electrophoresis——Electrophoresis was performed using 7.5% acrylamide gel by the method described by Fairbanks, *et al.*[13] and the gel was stained with 0.05% Coomassie Brilliant Blue R250.

Isoelectric Focusing——According to the method of Matsuo and Hori,[14] a column was prepared at the concentration of 0.5% of carrier ampholite ranging from pH 4 to 6. After electrophoresis for 24 hr at 4°, pH and absorbance at 280 nm were measured.

Analysis of Chemical Composition——Protein and hexose contents were determined respectively by the method of Lowry, *et al.*,[15] using bovine serum albumin as a standard and according to phenol–H_2SO_4 method[16] using glucose as a standard. After hydrolysis with 4 N HCl in a sealed vial at 110° for 8 hr, glucosamines were determined by the method modified by Blix.[17] Sialic acids were examined by the direct Ehrlich method[18] after hydrolysis with 0.1 N H_2SO_4 in a sealed vial at 80° for 1 hr, using N-acetylneuraminic acid as a standard. Amino acids, after hydrolysis with 6 N HCl at 100° for 24 hr, were analyzed using the Hitachi amino acid analyzer.

Results

Purification

Purification procedures are shown in Chart 1. The culture filtrate was adjusted to pH 3.0 with 1 N HCl and the precipitate formed was collected by centrifugation. To the precipi-

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culture filtrate (1 l)
↓ adjusted to pH 3.0 with 1N HCl, centrifuged
ppt
↓ extracted with 5% AcOH–50% EtOH
extract ⋮ gastric acid inhibitory substance
residue
↓ dissolved in H_2O (pH 7.0), fractionated with chilled EtOH
50—75% EtOH ppt
↓ dissolved in H_2O (pH 7.0), adjusted to pH 4.0 with 1N HCl
ppt
↓ fractionated on DEAE-cellulose column
0.05N NaOH eluate
↓ fractionated on hydroxylapatite column
active fraction
↓ fractionated on Sephadex G-100 column
gastric ulcer inhibitory substance (GUIS) (4.9 mg)

Chart 1. Purification Procedure of Gastric Ulcer Inhibitory Substance produced by *Bacillus subtilis* H

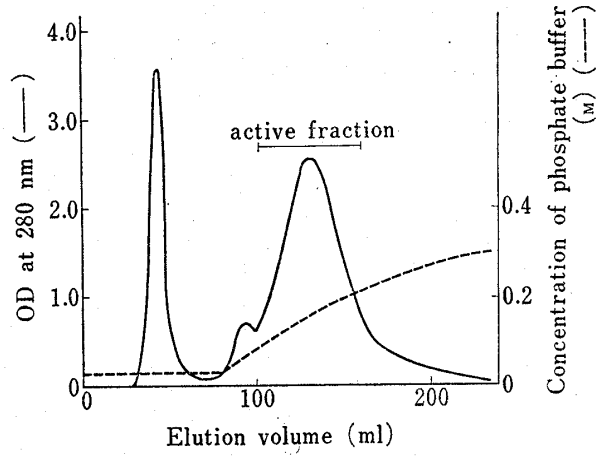

Fig. 1. Chromatogram of 0.05N NaOH Eluate on Hydroxylapatite Column

0.05N NaOH eluate was applied on a column (2.0×13.0 cm) of hydroxylapatite equilibrated with 0.02M phosphate buffer (pH 7.0). Flow rate, 10 ml/hr.

tate was added 5% AcOH–50% EtOH and the supernatant was discarded. At this step, the residual fraction clearly inhibited gastric ulceration in pylorus-ligated rats at the dose of 20 mg/kg (*i.p.*).

This fraction was fractionated with chilled EtOH and 50—75% EtOH precipitate was effective in pylorus-ligated rats. After isoelectric purification at pH 4.0, the precipitate was applied on DEAE-cellulose column (3.3×15.0 cm) equilibrated with 0.05M phosphate buffer (pH 7.0). As the active component was eluted with 0.05N NaOH, further purification was done by chromatography on hydroxylapatite gel. The column (2.0×13.0 cm) was equilibrated with 0.02M phosphate buffer (pH 7.0) and developed with a linear gradient concentration of phosphate buffer (pH 7.0). As shown in Fig. 1, active fraction was eluted with higher concentration of phosphate buffer. Further, this fraction was eluted on Sephadex G-100 column (2.0×80.0 cm) with 0.05M phosphate buffer (pH 7.0) and the high molecular weight fraction obtained from this procedure was effective on gastric ulceration in pylorus-ligated rats.

This fraction, dialyzed against distilled water and lyophilized, was named gastric ulcer inhibitory substance (GUIS). The yield of this fraction was 4.9 mg from 1 liter of the culture filtrate.

Inhibitory Effect on Gastric Ulceration

Effect of GUIS on gastric ulceration in pylorus-ligated rats, aspirin-induced gastric lesions, and stress-induced gastric lesions is shown in Table I. GUIS at 5.0 mg/kg, when administered intraperitoneally in pylorus-ligated rats, significantly prevented gastric ulceration and the inhibition percentage of ulcer index was 84.2%. While perforation was found in four out of nine animals in the control group, no rats in GUIS-treated group had a perforation and no disorder was recognized in five rats. Further, GUIS at 5.0 mg/kg (*i.p.*) produced a significant reduction of ulcer index in aspirin-induced gastric lesions and its inhibition percentage was 72.4%. The ulcer index in stress-induced gastric lesions was not significantly lessened by the intraperitoneal administration of GUIS at 5.0 mg/kg.

Table I. Anti-ulcerogenic Activity of GUIS on Several Experimental Ulcers in Rats

	Treatment	Dose (mg/kg, *i.p.*)	No. of rats	Ulcer index (mean ± s.e.)	Inhibition (%)
Shay ulcer	Control	—	9	3.8±0.3	—
	GUIS	5.0	8	0.6±0.2[a)]	84.2
Aspirin ulcer	Control	—	8	35.2±5.1	—
	GUIS	5.0	8	9.7±2.2[a)]	72.4
Stress ulcer	Control	—	8	26.9±4.0	—
	GUIS	5.0	8	21.3±2.7	20.8

a) Significantly different from control group, $p<0.01$.

Table II. Effect of GUIS on Gastric Secretion in Pylorus-ligated Rats (4 hr)

Treatment	Dose (mg/kg, *i.p.*)	No. of rats	Gastric volume (ml/100 g b.w.)	Gastric acidity (mEq/liter)	Total acid output (μEq/100 g b.w.)	Total peptic activity (mg as tyrosine/ 100 g b.w.)
Control	—	6	2.08±0.53	105.0±8.7	220.6±57.0	132.4±32.0
GUIS	5.0	6	0.39±0.07[a)]	59.2±4.2[b)]	23.9± 6.3[b)]	37.7± 5.6[b)]

All values represent mean ± s.e.
Significantly different from control group, *a*) $p<0.05$, *b*) $p<0.01$.

Inhibitory Effect on Gastric Secretion

Effect of GUIS on gastric secretion in 4 hr pylorus-ligated rats is summarized in Table II. The gastric juice volume, total acid output, and total peptic activity were remarkably reduced by the administration of GUIS at 5.0 mg/kg, and total acidity was also significantly reduced.

Electrophoresis

As shown in Fig. 2a, GUIS acted as a single compound in cellulose acetate membrane electrophoresis. In SDS-polyacrylamide gel electrophoresis, GUIS gave a single band as shown in Fig. 2b and its molecular weight was estimated as about 9300.

The electrophoretic pattern of GUIS in isoelectric focusing is shown in Fig. 3. GUIS exhibited a single peak at pH 4.5.

Analysis of Chemical Composition

Chemical composition of GUIS is presented in Table III. A major constituent of GUIS was protein and hexose, and hexosamines were minor constituents. Amino acids

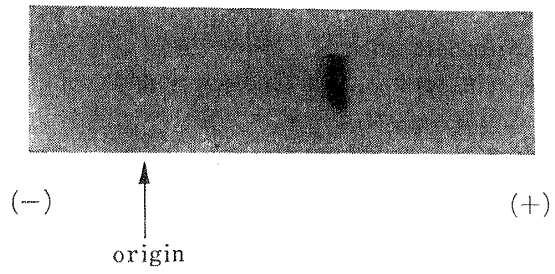

Fig. 2a. Cellulose Acetate Membrane Electrophoresis of GUIS

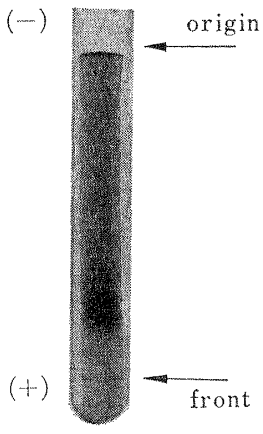

Fig. 2b. SDS-polyacrylamide Gel Electrophoresis of GUIS

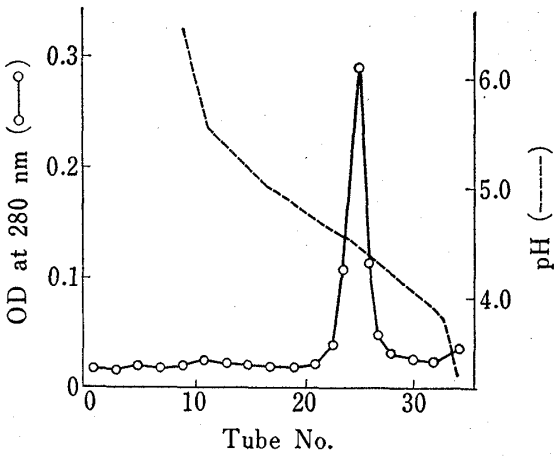

Fig. 3. Isoelectric Focusing of GUIS on Ampholine Column

Table III. Chemical Composition of GUIS

(A)

Component	Content (%)
Protein	90.4
Hexose	5.3
Hexosamine	1.1

(B)

Amino acid	mol/mg	Amino acid	mol/mg
Asp	0.53	Met	0.10
Thr	0.27	Leu	0.41
Ser	0.22	Ile	0.32
Glu	0.56	Tyr	0.18
Pro	0.20	Phe	0.23
Gly	0.36	His	0.11
Ala	0.40	Lys	0.31
Cys	0.04	Arg	0.16
Val	0.32		

which composed protein were 17 kinds, in which mainly aspartic acid and glutamic acid were detected in a large quantity. Tryptophan was not analyzed.

Discussion

The gastric acid inhibitory substance from the culture filtrate of *B.subtilis* H IAM 1521 was found to be soluble in 5% AcOH–50% EtOH[5] during purification and we first excluded this substance from the crude extract. After this procedure, the residual fraction was found to reduce gastric ulcers in pylorus-ligated rats at the dose of 20 mg/kg. Therefore, we confirmed that *B.subtilis* H IAM 1521 produces an inhibitory component against ulceration in pylorus-ligated rats, other than the gastric acid inhibitory substance. Gastric ulcer inhibitory substance (GUIS) was purified from this residual fraction, and it markedly inhibited ulceration at the dose of 5.0 mg/kg. Generally, ulceration in pylorus-ligated rats is said to be caused by digestion of accumulated gastric juice,[8] and aspirin-induced ulcers under pylorus ligation are considered to depend on gastric acidity.[10] GUIS decreases gastric juice and pepsin secretion in rats at the dose of 5.0 mg/kg (*i.p.*), and also lowered the gastric acidity. Therefore, the mode of action of GUIS against these two kinds of ulcers is considered to be based on decrease of gastric juice, pepsin secretion, and gastric acidity. However, inhibitory activity of GUIS was weak in stress-induced ulceration. This indicates that GUIS does not serve strongly on vagi, splanchnici nerves, and hypophysis-suprarenal gland system, not less than three of which are considered to mediate stimuli of stress causing stress-induced ulceration through the central nervous system.[19]

GUIS was found to be a glycoprotein containing a small amount of sugar and mainly composed of protein, according to the result of analysis of the components. This substance gave a homogeneous pattern in cellulose acetate membrane electrophoresis or isoelectric focusing, and its isoelectric point was pH 4.5. Though it appeared to be macromolecule in gel filtration over Sephadex G-100 column, it was a micromolecule in SDS-polyacrylamide gel electrophoresis, and its molecular weight in the presence of SDS was 9300. These facts indicate that GUIS exists as a polymer of subfragments.

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