A Lethal Toxic Substance for Brewing Yeast in Wheat and Barley

Part II. Isolation and Some Properties of Toxic Principle.

By Tsutomu OKADA and Hajime YOSHIZUMI

*The Central Research Institute, Suntory Limited
43, 2-chōme Dojima-naka, Kitaku Osaka, Japan*

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A toxic substance contained in wheat and barley causing death of brewing yeast was purified and isolated as a single protein. The molecular weight of the toxic substance was estimated to be 9800 by Archibald method. The substance was found to be a simple protein by amino acid analysis. The isoelectric point was higher than pH 10. The UV absorption spectrum showed λ_{max} 278 m μ . By the measurement of the circular dichroic spectrum, the α -helix content was estimated to be 34.5%. The toxic effect was easily inactivated by trypsin, but not by chymotrypsin and carboxypeptidase.

It has been shown in the previous paper^{1,2)} that a lethal toxic substance for brewing yeast contained in wheat and barley was easily extracted with a diluted sulfuric acid solution, and the toxicity was neutralized with a divalent metallic ion such as Ca^{2+} , Zn^{2+} and Fe^{2+} . There may be involved a physiological and physicochemical significance on function of the toxicity, in the extractability of the toxic substance and in the effect of metallic ions on the toxicity. Present paper describes the isolation and purification of the toxic substance from wheat, and some physical and chemical properties of the toxic substance.

EXPERIMENTAL AND RESULTS

Isolation and purification of toxic substance

1. *Isolation of toxic fraction from wheat.* The isolation procedure is summarized in Table I.

The grain of wheat was ground with a roller mill to 100 mesh powder, and the powder was immersed in five volumes of 0.05 N sulfuric acid solution for 3 hr at 30°C. After centrifuging the extract at 8000 rpm for 10 min, the supernatant was neutralized with sodium hydroxide solution to pH 7.0, and resulting precipitate was removed by centrifugation. The supernatant was concentrated to about one tenth volume *in vacuo* at 40°C. To the concentrated solution, equal volume of ethanol was added and allowed to stand over night in ice box. The resulting precipitate was removed by filtration, and the filtrate was concentrated to about one-tenth volume *in vacuo*, and to the concentrate, trichloro acetic acid (TCA) was added to the final concentration of 0.1 M. The resulting denatured protein was removed by centrifugation, and remaining TCA was extracted with ether using Soxhlet's apparatus for 12 hr at 30°C. Then, pyridine was added to the residual solution to the final concentration of 80%, and resulting precipitate was collected by centrifugation.

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2) T. Okada, H. Yoshizumi and Y. Terashima, Agr. Biol. Chem., 34, 1084 (1970).

TABLE I. ISOLATION OF TOXIC FRACTION

Grain powder		Yield of toxicity
after immersion in five volumes of 0.05 N H ₂ SO ₄ , centrifuged and neutralized with NaOH.	(discarded)	
Supernatant	(Precipitate)	100%
concentrated and precipitated with ethyl-alcohol at final concentration 50%.		
Supernatant	(Precipitate)	90%
denatured with TCA at final concentration 0.1 M.		
Supernatant	(Precipitate)	75%
for removing TCA, extracted with ether.		
Residual solution	(Precipitate)	75%
precipitated with pyridine at final concentration of 80%.		
Precipitate	(Supernatant)	50%
dissolved in water, and adjusted to pH 10.0 with NaOH.		
Supernatant	(Precipitate)	50%
after neutralization with H ₂ SO ₄ at pH 7.0, precipitated with pyridine at final concentration of 80%.		
Precipitate	(Supernatant)	30%
dissolved in water.		
Toxic fraction		

The precipitate was dissolved in distilled water and pH was adjusted to 10.0 with a sodium hydroxide solution. The resulting isoelectric precipitate was removed by centrifugation. The supernatant was neutralized with a sulfuric acid solution to pH 7.0, and again precipitated with pyridine at final concentration of 80%. The resulting precipitate was collected by centrifugation and dried *in vacuo*. The toxicity per dry weight was about 100 fold higher than that of the grain powder. The yield of the toxicity was calculated to be about 30% of the grain powder.

2. *Purification by ion-exchange resin column chromatography.* The toxic substance isolated as above was dissolved in water and subjected to a column chromatography using Amberlite CG 50 (-COOH form). The column was

FIG. 1. Chromatography on Amberlite CG 50.

Column size; 2.0 × 30 cm. Fraction volume; each 20 ml. x—x; absorbance at 280 mμ. o—o; toxicity. Flow rate; 5 ml/10 min.

stepwisely eluted with water, 2 N formic acid and 2 N formic acid containing 20% ethanol.

FIG. 2. Chromatography on Dowex 1×2.

Column size; 2.0×30 cm. Fraction volume; each 20 ml. x—x; absorbance at 280 mμ. O—O; toxicity. Flow rate; 5 ml/min.

FIG. 3. Re-chromatography on Amberlite CG 50.

Column size; 2.0×40 cm. Fraction volume; each 20 ml. x—x; absorbance at 280 mμ. O—O; toxicity. Flow rate; 5 ml/10 min.

The result is shown in Fig. 1. The toxicity was found only in the fraction of peak III eluted by 2N formic acid containing 20% ethanol, and in this procedure, the toxicity increased approximately 20 fold per dry weight. The fraction was then subjected to a column chromatography using Dowex 1×2 resin. The column was equilibrated at pH 8.5 with 0.5M pyridine-collidine buffer and eluted with the

same buffer. As shown in Fig. 2, the toxicity was found only in the fraction of peak A. The toxic fraction was further subjected to a re-chromatography using Amberlite CG 50. As shown in Fig. 3, the toxicity was contained only in the fraction of peak γ eluted by 2N formic acid containing 20% ethanol. After removing formic acid and ethanol by evaporation *in vacuo* at 30°C, purified toxic substance

was obtained in a yield of 50%. Since the minimum concentration of the toxic substance to inhibit the yeast growth in wort was 4 ppm (1 unit), it was shown that the toxicity per dryweight increased to approximately 2000 fold from the starting material.

Some physical and chemical properties of purified toxic substance

1. *Sedimentation coefficient.* Homogeneity of the toxic substance was confirmed by the sedimentation analysis with a Hitachi ultracentrifuge apparatus. The toxic substance was dissolved in a sodium sulfate solution with ionic strength $\mu=0.10$, and analyzed at 21°C and 41,300 rpm. The sedimentation coefficient s_{20} was calculated to be 1.5 according to the method described by Schachman.³⁾

2. *Electrophoretic analysis.* For further determination of the purity, paper electrophoresis was performed in ice box with 0.1 M acetate buffer adjusted pH and ionic strength at 5.0 and 0.1, respectively. As shown in Fig. 4, the purity of the preparation was shown by the toxicity and color reaction. The disc-electrophoresis pattern also revealed the homo-

FIG. 4. Paper Electrophoresis Pattern.

Condition; toxicity 20 units. Solvent; acetate buffer (pH 5.0 and ionic strength 0.1). Temperature; 5°C, Amperage; 3 mA. Migration; 120 min, Standard; amino acids (Products of Wakô Chemicals Ltd.).

geneity of the preparation.

3. *Ultraviolet absorption spectrum.* As shown in Fig. 5, UV spectrum of 0.7% aqueous solution of the toxic substance shows $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ 278 m μ , and absorption of 1% solution at 280 m μ was 0.414.

FIG. 5. Ultraviolet Spectrum.

4. *Molecular weight*

a. *Permeability through collodion bag.* Permeability through the collodion bag was determined by detection of the protein reaction (color reaction and absorption at 280 m μ), and molecular weight of the toxic substance was estimated to be below 10,000.

b. *Permeability through Diaflo-membrane.* Permeability through Diaflo membranes⁴⁾ (UM 1 and XM 50) was determined by the toxicity and absorption at 280 m μ . As shown in Fig. 6, the toxic substance could permeate through the membrane XM 50, but could not permeate through the membrane UM 1. Therefore, the molecular weight was presented within the value from 10,000 to 50,000.

c. *Gelfiltration.* Andrews method⁵⁾ was applied using Sephadex G-75, and the molecular weight was estimated to be about 8500, as shown in Fig. 7.

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4) Data booklet 960 by Amicon corporation.

5) P. Andrews, *Biochem. J.*, **91**, 222, (1964).

FIG. 6. Permeability through *Diaflo* Membrane.

Diaflo model 50 ultrafiltration cell (capacity 65 ml) and membrane UM 1 and XM 50 were used at 3.0 kg/cm pressure. Fraction volume; each 20 ml, $\times$ — $\times$; absorbance at 280 mμ. $\circ$ — $\circ$; toxicity (in unit).

FIG. 7. Gel-filtration on Sephadex G-75.

Column size; 2.5 × 50 cm, Buffer; 0.05 M Tris-HCl buffer (pH 7.5), Flow rate; 30 ml/hr.

d. *Molecular weight by Archibald method.*⁶⁾ Centrifuge was operated at 21,000 rpm and 26.0°C, and photographs were taken at intervals of 10 min. The molecular weight was estimated to be 9800.

5. *Circular dichroism.* Circular dichroic (CD) spectrum⁷⁾ of the toxic substance was measured over a wave length range from 200 mμ to 250 mμ. From the negative band at 222 mμ, the α -helix content was calculated to be 34.5% by comparing with L-poly glutamic acid.

6. *Amino acid constitution.* Amino acid con-

stitution of the toxic substance was analyzed by a Hitachi auto-amino acid analyzer and sugar content was determined by Anthrone method.⁸⁾ On the basis of the value of molecular weight 9800, 85 of amino acid residues and 9 of ammonia were estimated per one mole of the toxic substance, and no sugar component was found. Also, no free sulfhydryl-group was found by Ellman reagent.⁹⁾

7. Stability

a. *Effect of pH and temperature.* The effect of temperature on the toxicity was examined at various pH. In acidic solution (pH 4.0), the toxicity was stable and tolerant even at 100°C for 10 min. However, in alkaline solution (pH 10) the toxicity was unstable and easily inactivated by heating.

b. *Hydrolyzability.* As shown in Table II, the toxic substance was easily decomposed and inactivated by 1/10N sodium hydroxide solution, but not by ammonium hydroxide solution, at 30°C for 24 hr. Also, the toxic substance was half inactivated by 1/10N sulfuric acid solution, but not by organic acid such as formic and acetic acids.

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9) G. L. Ellman, *Arch. Biochem and Biophys.*, **82**, 70 (1959).

6) K. Kakiuchi, *Biophysics*, **7**, No. 1, 38 (1967).

7) G. Holzwarth, *J. Am. Chem. Soc.*, **87**, 218 (1965).

TABLE II. HYDROLYZABILITY

Acid hydrolysis at 30°C for 24 hr		Toxicity
H ₂ SO ₄	1/10 N	half inactivated
	1/20 N	no effect
Formic acid	2 N	no effect
Acetic acid	2 N	no effect
Alkaline hydrolysis at 30°C for 24 hr		Toxicity
NaOH	1/10 N	completely inactivated
	1/100 N	no effect
NH ₄ OH	2 N	no effect

8. *Digestibility.* Trypsin, chymotrypsin and α -carboxypeptidase used for this experiment were all the products of Sigma. Chem. Comp. It was shown that the toxic substance was easily decomposed by trypsin, but not by the other proteinases.

DISCUSSION

It was reported in the past by A. Balls, W. Hale and T. Harris,¹⁰⁾ that a toxic substance named "purothionin" which was extracted from wheat with petroleum ether, and contained 20% arginine inhibited the growth of *Saccharomyces cerevisiae* at a concentration of 5 ppm and inoculum size 10^5 cells/ml. There is, however, some doubt with the extractability of the toxic substance of wheat, and with the homogeneity of "purothionin." The toxic substance of wheat obtained in our experiment is ultracentrifugally and electrophoretically homogeneous as described above. The differences between our toxic substance and "purothionin" are also pointed out in the

effective concentration on the inhibition of the yeast growth (in our case 2 ppm at inoculum size 10^5 cells/ml) and in the content of arginine (6%). It may be suggested that "purothionin" may be a complex of the toxic substance and other proteins in wheat. The remaining of the toxicity in hydrolysis products by proteinase or acid is considered as very important information for chemical structure of active site.

Since the toxic substance is a basic protein of which isoelectric point is higher than pH 10, as described above, it may combine with the acidic groups such as carboxyl and phosphoric groups on the cell surface of yeast. If so, it must inhibit the yeast growth by binding with these acidic groups in yeast cell membrane. Neutralization of the toxicity by divalent metallic ions such as Ca^{2+} , Zn^{2+} and Fe^{2+} may be due to the competitive binding with the acidic groups on the cell surface. It is known that the yeast flocculency has a relation with the situation and distribution of the acidic groups on the cell surface. Accordingly, as a matter of course, there must exist close relationship between the sensitivity of yeast to the toxicity and the yeast flocculency. In order to clarify the mode of binding of the toxic substance to the cell surface, structural studies on the binding sites would further be necessary. With the role of the toxic substance in the seeds of wheat and barley *per se*, there may be a significance in the metabolic regulation of endosperm of dormant seeds.

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10) A. K. Ball, W. S. Hale and T. H. Harris, *Cereal Chem.*, **19**, 279 (1942).