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Inhibition of Brewer's Yeasts by Wheat Purothionins

C. HERNANDEZ-LUCAS, R. FERNANDEZ DE CALEYA, AND PILAR CARBONERO

Departamento de Bioquímica, E. T. S. de Ingenieros Agrònomos, Universidad Politècnica, Madrid-3, Spain

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Purothionins are basic polypeptides with antimicrobial properties that are present in the endosperm of wheat and other *Gramineae*. Susceptibility to crude and electrophoretically purified purothionins among brewing starters has been investigated. Seven yeast strains of *Saccharomyces uvarum* (syn. *carlsbergensis*), four strains of *Saccharomyces cerevisiae*, and four wild strains (*Saccharomyces* spp.) have been tested in three culture media. All the strains were susceptible to the crude preparation in a yeast extract-glucose medium. Determinations of minimal inhibitory and biocidal concentrations yielded double end points in about half of the assays. The highest sensitivity to purothionins was obtained in malt extract medium. Sensitivity to electrophoretically purified purothionins was of the same order or smaller than to the crude preparation. Possible explanations for this unexpected result are presented.

It has been repeatedly cited (12, 17) that, as early as 1895, Jago observed that added wheat flour would inhibit beer-yeast fermentations and that other workers attempting to separate the substance possessing this activity showed that it had toxic amine characteristics.

Recently, Nose and Ichikawa (11) found that a water-soluble substance from wheat flour increased the leakage of ultraviolet absorbing substances from yeast cells, and Okada et al. (12) reported on a substance toxic to brewing yeast, extracted from wheat and barley endosperm with a 0.05 N sulfuric acid solution.

Purothionins are protein moieties of proteolipids extracted with petroleum ether from the endosperm of wheat and other *Gramineae*. They are highly basic polypeptides (one-fourth of their amino acid residues are lysine and arginine) and have a high proportion of disulfide bridges (about 10 per 100 residues). Crystallized purothionin from *Triticum aestivum* L. is really a mixture of related polypeptides that appear as a closely spaced doublet on starch-gel electrophoresis-designated purothionins α and β (1, 2, 4, 7, 8, 10, 15, 16).

Purothionins possess antimicrobial properties against human pathogenic gram-positive bacteria and against yeasts (17). Data from this laboratory showed that they also inhibit gram-positive and gram-negative phytopathogenic bacteria (6). The effect of purothionin on fermentation of wheat mashes by one unidentified strain of distiller's yeast was studied by Balls and Harris (3).

The purpose of this paper is to study the effect of crude and of electrophoretically purified α and β purothionins from *T. aestivum* L. against several top and bottom strains of brewer's yeasts.

MATERIALS AND METHODS

Yeasts. Yeasts used in this study are listed in Table 1, and include strains of *Saccharomyces uvarum* (syn. *carlsbergensis*) and *Saccharomyces cerevisiae*. Four wild strains (*Saccharomyces* spp.) were also studied. All the strains were kindly supplied by J. Conde (Cervezas Cruzcampo, Sevilla, Spain).

Purothionins. Crude purothionin (CP) was obtained from *T. aestivum* L. (varieties Negrillo and Candeal) as previously described (8). CP was directly dissolved in sterile water (5 mg/ml) with the aid of short pulses in an ultrasonic bath (Varian AG). A small insoluble residue was eliminated by centrifugation at 20,000 $\times$ *g* for 15 min.

Purified purothionin (PP) was a mixture of α and β purothionins obtained by preparative electrophoresis. This was carried out on 10% polyacrylamide columns (1.5 by 10 cm) with 0.1 M acetic acid buffer, pH 2.9, at 20 V/cm. An electro-elution adaptor similar to that described by Popescu et al. (14) was used to collect the purothionin peak, which was monitored by ultraviolet absorption at 280 nm (Isco UA-40). A 50-mg amount of CP in 0.5 ml of 3 M urea was applied in each run. The purothionin solution was directly freeze-dried.

CP in solution was quantified by biuret reaction, using bovine serum albumin (Sigma) as the standard. The proportion of α and β purothionins in CP was estimated by densitometry of electrophoretic bands (starch gel, aluminum lactate buffer, 3 M urea, pH 3.2) using PP as standard. Purothionins were stained with 0.5% nigrosine in acetic acid:water (1:1), and densitometry was performed with a 620-nm filter (Chromoscan, Joyce & Loebl). The estimated α and β purothionin content in CP was 42% of total protein.

TABLE 1. *Inhibition of yeasts by crude purothionin (CP): broth dilution test in YG broth*[a]

Yeasts	MIC[b]	MBC[b]
Bottom fermentation strains		
Saccharomyces uvarum CC-2	128	256
S. uvarum CC-11	32	32
S. uvarum CC-14	64	128
S. uvarum CC-15	128	256
S. uvarum CC-21	64/16[c]	64/16[c]
S. uvarum CC-22	128	128
S. uvarum CC-23	128/8[c]	128
Top fermentation strains		
Saccharomyces cerevisiae CC-51	256/32[c]	256/32[c]
S. cerevisiae CC-86	256/32[c]	256
S. cerevisiae CC-90	256/16[c]	256
S. cerevisiae CC-98	128/8[c]	256
Wild strains		
Saccharomyces spp. CC-100	64	64
Saccharomyces spp. CC-101	256/8[c]	512
Saccharomyces spp. CC-102	256	256
Saccharomyces spp. CC-103	256/4[c]	256/4[c]

[a] Inoculum 10^5 yeasts/ml; incubation at 25 C with shaking.

[b] Minimal inhibitory concentration (MIC) and minimal biocidal concentration (MBC) expressed as micrograms of protein per milliliter.

[c] Double end point.

Media. The sensitivity tests were performed in the following liquid media: (i) YG medium, containing 0.3% yeast extract (Oxoid) and 0.5% glucose, pH adjusted to 5.5 prior to sterilization; (ii) G-YNB medium, containing 0.67% yeast nitrogen base (Difco) and 0.5% glucose, pH 5.5; and (iii) ME medium, containing 1.5% malt extract (Difco), pH 5.0. ME medium was autoclaved for 20 min, and then filtered and finally treated at 115 C for another 20 min.

Plate counts were performed on Wickerham agar medium (0.3% yeast extract [Oxoid], 0.3% malt extract [Oxoid], 0.5% peptone, 1% glucose, 2% agar) (18).

Maintenance of yeast cultures was in Wickerham agar slants.

Sensitivity tests. Yeast sensitivity to CP and PP was tested in liquid media. An inoculum of 10^5 cells/ml was used in all cases except in some ME medium tests, where a lower inoculum (10^3 cells/ml) was also used. Purothionin concentrations ranged from 512 to 1 μg/ml, following a twofold dilution series. Incubation was at 25 or 30 C with shaking until good growth on controls without purothionin was observed (24 to 48 h).

The minimal inhibitory concentration (MIC) was taken as the lowest concentration completely inhibiting growth as judged visually. The minimal biocidal concentration (MBC) was determined through subculture of a loopful of inhibited broth into fresh medium which was further incubated. The MBC was defined as the lowest concentration yielding no growth after subculture.

RESULTS

All strains tested were inhibited by CP in YG medium (Table 1). MBC values in all cases were no higher than twice the MIC values. It is remarkable that, in over half of the above assays, one or more tubes showing good growth were followed in the series by one tube with no apparent growth, although it contained a lower concentration of purothionin. Thus, there were two end points in the same assay. Not all assays of a given strain showed the lower end point. Isolates from tubes containing subinhibitory concentrations of CP did not present the double end point.

Three of the strains (*S. uvarum* CC-21 and CC-23; *S. cerevisiae* CC-51) were chosen for further investigation. These were tested against CP in G-YNB and in ME media. Although no inhibition was detected in the first medium even at the maximum concentration used (512 μg/ml), a high sensitivity was observed in the second (Table 2).

The same three strains tested in YG medium against PP showed identical MIC values (256 μg/ml) and MBC/MIC = 1.

The activity of CP and PP in ME broth was compared by plate counting (Table 3).

DISCUSSION

According to Okada et al. (12), Lecourt in 1928 found that only bottom fermentation yeasts were inhibited by wheat flour. Our results show that wheat purothionin inhibits both top and bottom yeasts, although the more sensitive strains are to be found among the latter group.

TABLE 2. *Inhibition of brewer's yeasts by crude purothionins (CP): broth dilution test in ME medium*[a]

Yeast strains	Inoculum (cells/ml)	MIC[b]	MBC[b]
Saccharomyces uvarum CC-21	10^3	<4	<4
	10^5	<4	<4
Saccharomyces uvarum CC-23	10^3	2	2
	10^5	<4	<4
Saccharomyces cerevisiae CC-51	10^3	8	8
	10^5	32	32

[a] Incubation at 30 C with shaking.

[b] Minimal inhibitory concentration (MIC) and minimal biocidal concentration (MBC) expressed as micrograms of protein per milliliter.

TABLE 3. *Inhibition of brewer's yeasts by crude (CP) and electrophoretically purified α and β purothionins (PP): broth dilution test in ME medium*[a]

Yeast strains	Purothionin	Inoculum cells/ml	Plate counts per ml[b]						
			0[c]	32	16	8	4	2	1
Saccharomyces uvarum CC-21	CP	13×10^2	45×10^5	—[d]	—	—	—		
	PP	17×10^2	68×10^5	—	—	—	—	38×10^5	
Saccharomyces uvarum CC-23	CP	16×10^2	7×10^5	—	—	—	—	—	520
	PP	20×10^2	33×10^5	—	—	—	38×10^5		
Saccharomyces cerevisiae CC-51	CP	14×10^2	18×10^5	—	—	—	27×10^4		
	PP	17×10^2	21×10^5	—	—	9×10^2	84×10^4	16×10^5	

[a] Incubation at 30 C with shaking for 24 h.
[b] Plate counts in Wickerham agar.
[c] Purothionin added (micrograms of protein per milliliter).
[d] —, No colonies detected in agar plates with 0.1 ml of assay medium.

The double end point observed is difficult to explain. However, this same type of response has been reported by Kavanagh (9) for streptothricin against *Staphylococcus aureus* H. in liquid media and is well documented for the alkyldithiocarbamate fungicides. Also, bimodal inhibition curves have been obtained in vitro with certain enzymes under appropriate conditions (13).

Woolley and Krampitz (19) briefly mentioned that although yeast was inhibited by purothionin in a medium of salts and glucose, it was not inhibited in a richer medium. Accordingly, we have found great differences in sensitivity in the three media tested. Reversion of bacteriostatic activity of purothionin by phosphatides has been reported (19), and divalent cations have been shown to revert the toxic effect of flour extracts on yeast (11, 12). These and other such factors may be implicated in the above-mentioned differences in sensitivity.

An unexpected result is that yeast sensitivity to PP is of the same order or smaller than its sensitivity to CP. Denaturation during preparative electrophoresis, yeast inhibition by other components of the crude preparation (CP), or synergistic activity between some nontoxic substance in the crude extract and purothionin could account for the above results.

Inter- or intramolecular disulfide exchanges at acid pH could alter the cycle-like loops of purothionin molecules. The cyclic structure seems to be an important feature in determining activity in polypeptide antibiotics; for example, the straight-chain analogue of gramicidin S is less than 10% as effective (5). However, no evidence of polymerization was found by analytical electrophoresis, and no anomalous results were obtained when the same PP and CP preparations were tested against *Xanthomonas phaseoli* (ATCC 9563), PP being more than twice as active as CP.

The possibility that components of CP other than purothionins were active against yeast cannot be discarded, although we have previously demonstrated that purothionins are the only components of CP active against bacteria (6).

Another explanation could be that the stability of purothionins is enhanced in CP, since we have observed that a precipitate is formed after 24 h in YG medium PP blanks, especially at high concentrations, which is much less noticeable in CP blanks.

The inhibitory effect of purothionin on the fermentation of wheat mashes was investigated by Balls and Harris (3). They found that its activity could be destroyed by previous proteolysis with trypsin, chymotrypsin, papain, and chymopapain or by heating (partial destruction at 100 C for 1 h). However, we have found that purothionin is resistant to the intense proteolysis that takes place during the germination of wheat and barley and to the heat treatment during malt kilning (unpublished data).

A considerable genetic variability of purothionin content has been reported (8). Incomplete inactivation in high purothionin varieties could lead to undesirable levels of active substance in fermentation mixtures. Therefore, a more detailed study of the fate of purothionins during malting and wort production is justified.

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