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Phospholipid Requirement of Microsomal Chitinase from *Mucor mucedo*

Anne M. Humphreys and Graham W. Gooday

Department of Microbiology, University of Aberdeen, Aberdeen, UK

Abstract. Microsomal and supernatant chitinase activities have been prepared from mycelial cultures of *Mucor mucedo*. Studies of their responses to changing temperature and phospholipid composition indicate that the lipid environment is important in regulating membrane-bound chitinase activity, but that supernatant chitinase activity does not have a phospholipid requirement. Membrane-bound chitinase was solubilized by different types of non-denaturing detergents. Maximum solubilization was achieved with 1 m*M* Zwittergent-14 or 1.2% Triton X-100 (93% and 90% solubilization, respectively). This solubilized chitinase activity could not be activated by protease treatment, i.e., was nonzymogenic, as was the supernatant chitinase. The insoluble residual chitinase activity was, however, zymogenic after treatment with 1.2% Triton X-100, but fully active after treatment with 3% Triton X-100.

We have described the characterization of two main sources of chitinase activity, supernatant and microsomal, in *Mucor mucedo* [4]. Microsomal chitinase is a membrane-bound zymogen, as is chitin synthase [3]. We suggested that microsomal chitinase and chitin synthase may be regulated in consort to play a morphogenetic role in hyphal growth. Studies have shown that the phospholipid environment is important in regulating chitin synthase activity [2, 5, 9]. This paper presents results of an investigation of the phospholipid requirements of chitinase activities from *M. mucedo*, as determined by effects of temperature, different phospholipids and detergents on activity.

Materials and Methods

Organism and growth conditions. *Mucor mucedo* (L) Fresenius (Z46 *plus*) was grown and maintained on agar plates, as described by Humphreys and Gooday [4].

Preparation of mycelial extract. Mycelia were harvested at 18 h, and supernatant and microsomal fractions were obtained exactly as described by Humphreys and Gooday [4].

Chitinase assay. Chitinase activity was measured with [^{3}H] chitin substrate, as described by Humphreys and Gooday [4]. Protein concentration was determined using the method of Bradford [1].

Treatment with phospholipids. Pure phospholipids (Sigma Chemical Co., England) were dissolved in chloroform–methanol (2:1, vol/vol), dried in a stream of nitrogen, and freeze dried for 3 h. Enzyme preparation (40 μl) and assay buffer (50 μl) were added to the tubes containing the phospholipids and treated in an ultrasonic water bath (Hilbre Ultrasonics Ltd, England) for 2 min. The samples were incubated at 25°C for 30 min and then assayed for chitinase activity.

Membrane solubilization. The technique was based on that described by Navarrete and Serrano [6]. Microsomal fractions were prepared and resuspended in 2 ml 2[N-morpholino]-ethanesulphonic acid (MES) buffer containing 10 m*M* MES, 1 m*M* EDTA, 1 m*M* dithiothreitol, 20% glycerol, pH 5.6, so that the protein concentration was 2–3 mg/ml. Samples of 0.4 ml were left at room temperature for 5 min; then 0.1 ml of detergent solution (in water) or water was added, mixed well, and left for a further 10 min. The samples were centrifuged at 100,000 *g* for 1 h at 10°C in a Beckman SW 50.1 rotor. The supernatant was carefully removed and the pellet resuspended in 0.23 ml buffer. Both fractions were assayed for chitinase.

Treatment with trypsin. Prior to the chitinase assay, the enzyme preparation (40 μl) was preincubated with assay buffer containing 60 μg/ml trypsin at 30°C for 5 min; 7 μl of a solution of soybean trypsin inhibitor was added, and the tubes were cooled. The final concentration of inhibitor was twice that of trypsin present. Samples were then assayed for chitinase.

Results

Temperature dependence. Arrhenius plots for microsomal and supernatant chitinase showed biphasic and monophasic plots, respectively (Fig. 1). For microsomal chitinase the transition temperature was 12°C.

Address reprint requests to: G. W. Gooday, Department of Microbiology, University of Aberdeen, Aberdeen AB9 1AS, United Kingdom.

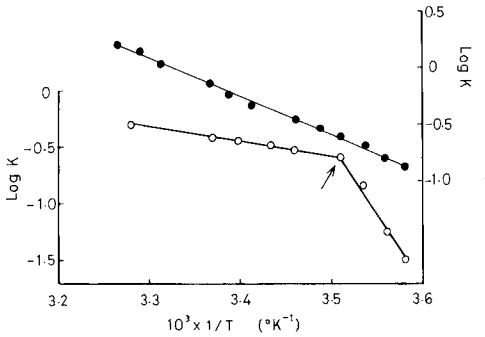

Fig. 1. Arrhenius plots of chitinase from *Mucor mucedo*. ○, microsomal chitinase; the arrow indicates the transition temperature, which is 12°C. ●, supernatant chitinase; K represents enzyme activity in nmol diacetylchitobiose min^{-1} (mg protein)$^{-1}$.

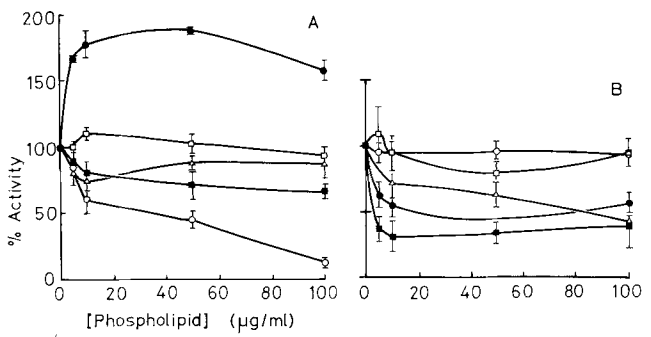

Fig. 2. Effect of phospholipids on chitinase activity from *Mucor mucedo*. (A) Microsomal chitinase; 100% activity represents 1.38 nmol diacetylchitobiose min^{-1} (mg protein)$^{-1}$. (B) Supernatant chitinase; 100% activity represents 3.19 nmol diacetylchitobiose min^{-1} (mg protein)$^{-1}$. Bars represent +/− standard error; △, dipalmityl phosphatidylethanolamine; ■, dipalmityl phosphatidylcholine; ●, dimyristoyl phosphatidylcholine; □, distearoyl phosphatidylcholine; ○, dioleoyl phosphatidylcholine.

The effect of phospholipids on chitinase activity. The five phospholipids had different effects on both microsomal and supernatant chitinase (Fig. 2). Only dimyristoyl phosphatidylcholine had a stimulatory effect on microsomal chitinase (Fig. 2A). Distearoyl phosphatidylcholine had no effect, dipalmityl phosphatidylcholine and dipalmityl phosphatidylethanolamine had a slight inhibitory effect, and dioleoyl phosphatidylcholine greatly inhibited the activity. Supernatant chitinase activity was inhibited by all five phospholipids to some extent (Fig. 2B).

The effect of detergents on microsomal chitinase. Zwittergent-14 was the most effective solubilizing agent, solubilizing 90% of the chitinase activity at 6 m*M*, with a twofold increase in activity (Table 1).

Table 1. Effect of detergents on chitinase activity and solubility

Detergent	Concentration	Total activity[b]	% Solubilization
None	0	0.72	19.6
Zwittergent-08[a]	6 m*M*	1.135	72.5
Zwittergent-10[a]	6 m*M*	0.815	64.9
Zwittergent-12[a]	6 m*M*	0.805	77.4
Zwittergent-14[a]	6 m*M*	1.496	90.0
Zwittergent-16[a]	6 m*M*	0.947	79.4
CHAPS	6 m*M*	0.34	64.4
Octyl glucoside	6 m*M*	0.52	47.8
Triton X-100	1.2%	3.56	81.6

[a] Chemical formula: $C_nH_{2n+1}-N(CH_3)_2-(CH_2)_3-SO_3^-$: where n = 8–16.

[b] Total activity: nmol diacetylchitobiose produced min^{-1} by 0.4-ml enzyme preparation.

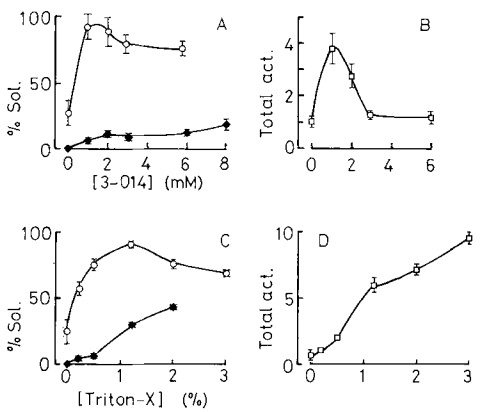

Fig. 3. Effect of Zwittergent-14 and Triton X-100 on microsomal chitinase activity of *Mucor mucedo*. Zwittergent-14 (3-014): (A) ○, % chitinase activity solubilized; ◆, % protein solubilized. (B) □, total chitinase activity (nmol min^{-1}). Triton X-100: (C) ○, % chitinase activity solubilized; ◆, % protein solubilized: (D) □, total chitinase activity (nmol min^{-1}). Bars represent +/− standard error.

Triton X-100 solubilized 82% of the chitinase activity and increased activity by fivefold; octyl glucoside and (3-[3]cholamidopropyl)-dimethylammonio]-1-pronane sulfonate (CHAPS) solubilized a portion of the chitinase activity, but inactivated the enzyme.

Zwittergent-14 achieved maximum chitinase solubilized (93%) at a concentration of 1 m*M* (Fig. 3A), with a fourfold increase in chitinase activity (Fig. 3B). Further increase in concentration resulted in a small decline of enzyme solubility, but a dramatic decrease in total enzyme activity. A small proportion of the total protein was solubilized (Fig. 3A). Triton X-100 at 1.2% achieved 90% solubilization of chitinase activity and 31% solubilization of the total protein (Fig. 3C). Beyond the optimum

Table 2. Zymogenic nature of solubilized and insoluble chitinase

Treatment	% Triton X-100	Fraction[a]	Activity[b]	% Change[c]	Total Activity[b]	% Solubilization
None	0	S	0		0.38	0
		P	0.38			
	1.2	S	4.10		4.62	88.7
		P	0.52			
	3.0	S	4.46		6.24	71.5
		P	1.78			
Trypsin[d]	0	S	0	0	0.69	0
		P	0.69	180		
	1.2	S	2.58	37	4.26	60.6
		P	1.68	320		
	3.0	S	2.70	31	3.74	72.2
		P	1.04	42		

[a] S. Supernatant from 100,000 *g* centrifugation after treatment with Triton X-100 or water; P, pellet from 100,000 *g* centrifugation as above.
[b] Activity is nmol diacetylchitobiose produced min^{-1} by 0.4-ml enzyme preparation.
[c] Percentage change in activity after trypsin treatment.
[d] Trypsin treatment was for 5 min with 60 μg trypsin ml^{-1} at 30°C.

concentration for maximum solubility, total activity did not decrease (Fig. 3D) as seen for Zwittergent-14, but continued to increase.

The zymogenic nature of solubilized and insoluble chitinase differed (Table 2). Chitinase activity in the control pellet was activated 1.8-fold by trypsin. At both 1.2% and 3% Triton X-100, the solubilized chitinase was fully activated and was degraded by trypsin, but the zymogenic nature of insoluble chitinase varied. At 1.2% Triton X-100, the insoluble chitinase was zymogenic and was activated 3.2-fold by trypsin; at 3% Triton X-100, the insoluble chitinase was fully active, and the activity decreased when treated with trypsin.

Discussion

The biphasic Arrhenius plot obtained for microsomal chitinase is characteristic of membrane-bound enzymes. The break in the plot (transition temperature) can be attributed to phase changes in the bound lipid and is the midpoint between a fluid and a crystalline bilayer (but see suggestion by Silvius et al. [8] that breaks in Arrhenius plots can arise from factors other than changes in enzyme conformation).

Altering the phospholipid environment of microsomal chitinase had pronounced effects on enzyme activity. Only dimyristoyl phosphatidylcholine stimulated enzyme activity, presumably as it forms a favorable environment, creating bilayers that reflect the phospholipid environment of the enzyme in vivo. Greater differences in enzyme activity are observed when the composition of the acyl chain is altered, compared with the polar head group (Fig. 2A). Chitin synthase activity from *Coprinus cinereus* showed a specificity towards acyl chain length [5], but a membrane-bound ATPase from human erythrocyte ghosts showed specificity toward the phospholipid head group [7].

At the concentrations used in these experiments, phospholipids are imiscible with water and therefore probably form bilayers or monolayers, with only the free ends of the chains interacting with the enzyme. Also the sonicating step may create liposomes, causing the enzyme to become trapped, either within the vesicle or bound to the phospholipid.

Since Triton X-100 is a mild detergent, this suggests that the chitinase may be less strongly bound to the membrane than chitin synthase, which is not solubilized by Triton X-100. As a large increase in Triton X-100 concentration resulted in an increase in total enzyme activity (Fig. 3D), there may be a conformational change to the enzyme caused by the detergent. The zymogenicity of insoluble chitinase varied (Table 2), but solubilized chitinase activity was fully activated at all Triton X-100 concentrations used. In this respect, solubilized chitinase is like the supernatant chitinase [4]. We suggest that at low Triton X-100 concentrations the membrane-bound proteolytic activity responsible for chitinase activation [4] may activate solubilized chitinase only, but at high Triton X-100 concentrations the membrane-bound proteolytic ac-

tivity may be solubilized and would activate both the soluble and insoluble forms of chitinase.

ACKNOWLEDGMENTS

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Literature Cited

1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry **72**:248–254.
2. **Duran, A., Cabib, E.** 1978. Solubilization and partial purification of yeast chitin synthase: confirmation of the zymogenic nature of the enzyme. Journal of Biological Chemistry **253**:4419–4425.
3. **Gooday, G. W., Trinci, A. P. J.** 1980. Wall structure and biosynthesis in fungi, pp. 207–251. In: Gooday, G. W., Lloyd, D., Trinci, A. P. J., (eds.), The eukaryotic microbial cell: 30th Symposium of the Society of General Microbiology. Cambridge: Cambridge University Press.
4. **Humphreys, A. M., Gooday, G. W.** 1984. Properties of chitinase activities from *Mucor mucedo:* evidence for a membrane-bound zymogenic form. Journal of General Microbiology **130**:1359–1366.
5. **Montgomery, G. W. G.** 1983. Studies on the purification of chitin synthase from *Coprinus cinereus*. M.Sc. thesis, Aberdeen University, Aberdeen.
6. **Navarrete, R., Serrano, R.** 1983. Solubilization of yeast plasma membranes and mitochondria by different types of non-denaturing detergents. Biochimica et Biophysica Acta **728**:403–408.
7. **Roelofsen, B., Van Deenen, L. L. M.** 1973. Lipid requirement of membrane-bound ATPase: studies on human erythrocyte ghosts. European Journal of Biochemistry **40**:245–257.
8. **Silvius, J. R., Read, B. D., McElhaney, R. N.** 1978. Membrane enzymes: artifacts in Arrhenius plots due to temperature dependence of substrate-binding affinity. Science **199**:902–904.
9. **Vermeulen, C. A., Wessels, G. H.** 1983. Evidence for a phospholipid requirement of chitin synthase in *Schizophyllum commune*. Current Microbiology **8**:67–71.