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Stimulation of protein secretion in *Trichoderma reesei* by Tween surfactants is not correlated with changes in enzyme localization or membrane fatty acid composition

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1. SUMMARY

Tween surfactants (Tween 20, 40, 60 and 80) stimulated the excretion of protein by *Trichoderma reesei* QM9414 during growth on cellulose. Tween 60 gave maximal secretion of protein. The presence of Tween decreased the lag time of growth and weakly stimulated growth. Almost the same proportion of cell-wall-bound activity was found in cultures supplemented with Tween for β-glucosidase, acid phosphatase and β-*N*-acetyl-glucosaminidase. The addition of various Tweens (20, 40, 60 or 80) had only a small effect on the fatty acid composition of the mycelium of *T. reesei*. Among 5 mutant strains with different secretory capacity, all but one showed stimulation of protein secretion by Tween surfactants. There was no observable correlation between the stimulation of secretion by Tween and the degree of unsaturation of mycelial fatty acids. It is concluded that the stimulation of protein secretion by Tween surfactants does not involve release of surface bound enzymes or changes in membrane fatty acid unsaturation.

2. INTRODUCTION

Non-ionic surfactants of the Tween (polyoxyethylene sorbitan mono-fatty acid) type have been shown to stimulate the secretion of various microbial products, particularly enzymes [1–5], with maximal stimulation often observed being that by Tween 80—the only Tween bearing an unsaturated fatty acid residue (oleate). In bacteria, oleic acid becomes incorporated into membrane lipids; the corresponding increase in membrane fluidity is suggested to facilitate and enhance the rate of enzyme secretion [4,5]. Recently, however, Jacques et al. [6] provided evidence that an increase in membrane fatty acid unsaturation alone is insufficient for stimulation of exoprotein secretion by Tween 80.

The phenomenon of stimulation of protein secretion by Tweens in filamentous fungi has so far hardly been dealt with; despite the common phenomenon, explanations offered for bacterial systems can only cautiously be applied with fungi

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because of the pronounced differences in membrane properties (see [7,8]). Particularly, membrane fluidity does not seem to be related to fatty acid unsaturation [9].

The aim of the present paper was to investigate whether the stimulation of protein secretion by Tween in the cellulolytic fungus *Trichoderma reesei* [2] involves changes in membrane fatty acid composition or cell surface binding of extracellular enzymes.

3. MATERIALS AND METHODS

3.1. Organisms and conditions of cultivation

Trichoderma reesei QM9414 was used throughout these studies, and was grown in a medium containing cellulose (1%, w/v) as a carbon source [10] and citrate-phosphate buffer [11]. For certain experiments, the mutant strains M 5, M 6, MHC 15 and MHC 22 [12] were used, which were obtained from Dr. V. Farkas, Bratislava, Czechoslovakia. Conditions for cultivation and harvesting of extracellular protein have been documented [13].

3.2. Measurement of the specific rate of exoprotein secretion

Extracellular protein was quantified by precipitating protein from 10 ml culture fluid with 10% (w/v) trichloracetic acid (TCA) (final concentration) for 3 h at 4°C, centrifuging (10 000 × *g*, 4°C, 10 min), washing the precipitate with 50% (w/v) ethanol, dissolving it in 0.5 ml distilled water, and quantifying the amount of protein by means of the dye-binding procedure [14].

The validity of this procedure was routinely checked by means of labelling the secreted protein with a pulse of [^{14}C]leucine (10 μCi per ml medium) for 8 h, then spotting 1 μl of the culture filtrate onto filter paper squares, washing them 3 times with 1% (w/v) TCA for 30 min, and measuring the activity remaining on the filter in a liquid scintillation counter.

For calculation of specific rates of secretion, mg (or μCi) of protein formed within a certain time interval were calculated on an hourly basis and referred to g mycelial protein. Mycelial protein was extracted and quantified as described [15]. Dry weight determination on cellulose-containing media was considered to lead to false results.

3.3. Fatty acid extraction and quantification

Flasks of the fungus growing on cellulose were harvested by suction filtration at the time growth had reached 2 g mycelial protein per l, the solids washed with tap water followed by distilled water, and ground in a mortar under liquid nitrogen to a fine powder. The powder was subsequently extracted for total lipids [16] in the presence of 5 mg · l^{-1} of 2,6-ditertiary butyl-*p*-cresol as antioxidant. Esters of total fatty acids were prepared by the BF_3 method [17]. The samples, dissolved in *n*-hexane, were analysed on a Perkin-Elmer gas chromatograph equipped with a $\frac{1}{8}$-inch stainless steel column packed with 5% DEGS on chromosorb G, using an injection temperature of 300°C and a temperature gradient on the column of 150–200°C with 8°C · min^{-1}. Fatty acid esters were identified by comparing retention time with that of known standards. In selected cases, gas chromatography-mass spectrometry (GC-MS) was used to verify the identification. The total lipid content of the mycelia was quantified gravimetrically [16].

3.4. Isolation of plasma membranes

This was done according to [18].

3.5. Determination of the extent of cell-wall-bound extracellular enzyme activities

This was done as described before [15]. Enzyme activities were determined as described before [15,19].

4. RESULTS

4.1. Effect of Tween surfactants on growth, protein secretion and localization of exoenzymes

Fig. 1a shows that one main effect of the presence or absence of Tween 80 was the duration of the lag time preceding growth. Thus, culture grown in the presence of 0.2% (w/v) Tween 80 exhibited a lag of 18 h, whereas those in the absence of Tween 80 exhibited a longer one (27 h). The

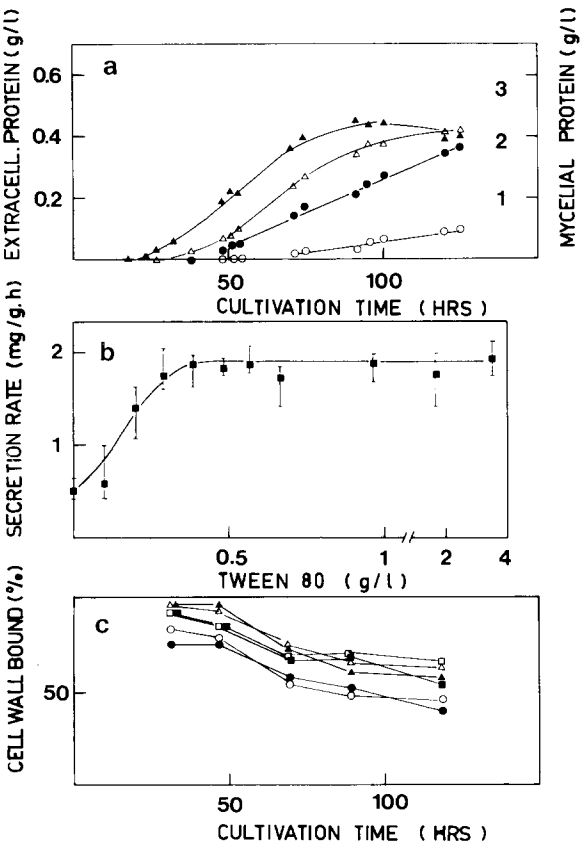

Fig. 1. Growth and total protein secretion of *T. reesei* QM9414 on cellulose medium in the presence (filled symbols) or absence (open symbols) of Tween 80 (0.2%, w.v). △,▲, mycelial protein (given as total g per 1 culture); ○,●, extracellular protein. (b) Effect of concentration of Tween 80 on the rate of protein secretion by *T. reesei* QM 9414. Bar markers indicate the standard deviation ($N = 4$). (c) Effect of Tween 80 (0.2%, w/v) on the cell-wall binding of (△,▲) β-glucosidase; (○,●) acid phosphatase; (□,■) *N*-acetyl-β-glucosaminidase. Filled symbols indicate the presence, and open symbols the absence of Tween 80.

subsequent specific growth rate was slightly lower (0.039 vs. 0.043 $g \cdot g^{-1} \cdot h^{-1}$), but the specific protein secretion rate was reduced by 76% in the absence of Tween 80. Thus the effect of Tween surfactants is not simply a result of its influence on growth of the organism. As can be deduced from Fig. 1a, the time interval used for calculation of the specific exoprotein secretion rate can significantly influence the value obtained; hence, for all cases, only the linear part of the secretion curve was used for these calculations and related to 2 g of mycelial protein (the secretion rate was linear at this point of growth).

Stimulation by Tween 80 was maximal at 0.6 $g \cdot l^{-1}$ (Fig. 1b), but it is noteworthy that higher concentrations were not inhibitory.

The observed increase in specific secretion rate was also not due to decreased binding of extracellular enzymes to the cell wall (Fig. 1c): the percentage of β-glucosidase, acid phosphatase and β-acetyl-*N*-glucosaminidase bound to the cell wall was only slightly influenced as compared to the influence of Tween 80 on the secretion rate. We have recently reported that Tween 80 solubilized the plasma membrane-bound β-glucosidase from *T. reesei* [18]. When mycelia, grown in either the absence or the presence of Tween were used to prepare intact plasma membranes, but the activity of β-glucosidase was almost the same (C. Umile and C.P. Kubicek, unpublished results). Hence the presence of Tween has little influence on exoprotein localization in vivo.

4.2. Stimulation of exoprotein secretion by various Tween surfactants and corresponding mycelial fatty acid composition

When different Tween-surfactants were examined with respect to their influence on protein secretion, all of them were found to stimulate to different extents but Tween 60 was most active. Corresponding analyses of mycelial fatty acid composition revealed more or less the same pattern (Table 1); only with Tween 80, the ratio of unsaturated to saturated fatty acids was slightly increased. Thus the protein secretion rate did not correlate with the fatty acid unsaturation ratio in *T. reesei*. Interestingly, the total lipid content increased with increasing secretion rates during these experiments.

In view of these findings it is noteworthy that when mycelia were grown in Tween-containing media, and then replaced on Tween-deficient medium, they still exhibited an increased rate of secretion (unpublished data). The effect of Tween-surfactants thus is not only the result of a physical contact between the cell surface and the surfactant.

Table 1

Influence of Tween surfactants on the secretion of protein and membrane fatty acid composition in *T. reesei* QM9414

Fatty acid	Fatty acid composition (% of total)				
	No Tween	Tween 20 [a]	Tween 40	Tween 60	Tween 80
Tetradecanoic (14:0)	1.4	1.4	1.2	1.0	1.0
Hexadecanoic (16:0)	20.8	20.4	20.3	23.2	13.0
Hexadecanoic (16:1)	TR [b]	TR	TR	TR	TR
Octadecanoic (18:0)	1.8	1.7	1.8	1.5	2.9
Octadecenoic (18:1)	7.3	9.0	8.9	8.8	8.4
Octadienoic (18:2)	68.7	67.3	65.3	63.6	71.2
Octatrienoic (18:3)	1.2	1.3	1.5	1.8	2.7
Specific protein secretion rate ($mg \cdot h^{-1} \cdot g^{-1}$) [c]	0.5	1.2	1.7	2.4	2.1
US-ratio [d]	3.1	3.1	2.8	2.6	3.9
Total mycelial lipid (mg/mg protein) [e]	0.08	0.12	0.15	0.27	0.24

[a] Added in concentrations of 0.2% (w/v).
[b] TR, only traces present.
[c] Defined as mg of protein produced per h and g of mycelial protein and calculated by referring to the presence of 2 g of mycelial protein.
[d] Ratio of unsaturated to saturated fatty acids.
[e] Determined from mycelia harvested when they had reached a density of 2 g mycelial protein per l of culture.

All Tweens showed their individual optimal effect at around 0.6 $g \cdot l^{-1}$.

4.3. Effect of Tween on protein secretion and fatty acid composition of T. reesei *mutant strains*

In order to establish the generality of the results shown above, certain mutant strains of varying protein secretion rate were examined in the presence and absence of Tween 60 (Table 2). In all these strains, Tween 60 was at least 20% more stimulatory than Tween 80, with the exception of *T. reesei* M 5, which did not respond to any Tween investigated. The corresponding fatty acid composition and unsaturation ratios showed some strain-dependent variation, but again no correlation was seen between the fatty acid pattern and the secretion rate.

Table 2

Secretion of protein and membrane fatty acid composition of mutants of *T. reesei* cultivated in the absence or presence [a] of Tween 60

Definitions and abbreviations as in Table 1

Fatty acid	Strains							
	M5		M9		MHC 15		MHC 22	
	+	−	+	−	+	−	+	−
C 14	TR	TR	TR	TR	TR	TR	TR	TR
C 16	13.2	18.1	19.6	23.2	26.0	52.0	27.0	38.1
C 16:1	TR	TR	TR	TR	TR	TR	TR	TR
C 18	1.2	1.5	TR	3.0	3.8	4.0	7.0	4.1
C 18:1	1.4	1.5	8.7	4.6	5.0	6.3	3.5	3.2
C 18:2	34.1	37.6	23.7	68.3	33.0	38.3	43.2	53.7
C 18:3	TR	TR	TR	TR	TR	TR	TR	TR
Specific protein secretion rate (mg/h·g)	0.7	0.7	2.3	0.9	0.8	0.4	1.2	0.4
US-ratio	2.5	2.0	1.7	2.8	1.3	0.8	1.4	1.4

[a] 0 or 0.2% (w/v), designated by − and +, respectively.

5. DISCUSSION

The addition of Tween surfactants to fermentation media is a commonly employed method for increasing the release of certain cellular products into the environment [1–5], although the mechanism appears more complicated than previously held. Recent results by Jacques and coworkers [6] have revealed that in *Streptococcus salivarius* stimulation of exoprotein synthesis by Tween 80 required not only an incorporation of oleic acid into the membrane, but also an additional physical contact of the membrane with Tween. Al-

though the authors demonstrated this effect very clearly, the requirement for membrane fatty acid unsaturation was less evident: in their experiments, Tween 60 also stimulated protein secretion 10-fold over the control, but fatty acid unsaturation was actually lowered. It is important to note in this context that these and most other authors measured protein secretion by means of determination of secreted enzyme activities (see [1–6]). Since Tween surfactants have been reported to release surface-bound enzymes [20,21] as well as to protect extracellular enzymes from denaturation by agitation [22], this could produce false results. Also, in most cases units per ml are quoted, although the specific secretion *rate* (units per ml *and* biomass unit) should be the truely influenced parameter.

To our knowledge the present investigation is so far the first examining the general effect of Tween-surfactants on the protein secretion rate in a filamentous fungus. From these we conclude that the surfactant exerts its effect not via surface release, nor changes in growth rate or the overall fatty acid composition. It is possible that alterations in fatty acid unsaturation occur only in small compartments, such as secretory vesicles [23], but in view of the maximal stimulation by Tween 60, which contains a saturated fatty acid residue (octadecanoic acid) this appears less likely. Furthermore, alterations in membrane fluidity in filamentous fungi do not seem to require changes in fatty acid unsaturation [9,24]. We are therefore forced to conclude that surfactant stimulation of protein secretion does not involve alterations in membrane fatty acid composition.

We have recently observed [25] that protein secretion by *T. reesei* QM9414 can be stimulated by phospholipid precursors, e.g., choline. In analogy, the present results might be summarized as stimulation of protein secretion by fatty acids. Ghosh and coworkers have stressed that protein secretion in *T. reesei* is limited by the amount of endoplasmic reticulum [26]. It is tempting to speculate whether low producer strains of *Trichoderma* are limited in their secretory capacity at the level of membrane biogenesis, which can be relieved by supplementation of lipid precursors. Our findings of increased lipid contents of mycelia supplemented with Tween 60 and Tween 80 would be in accordance with this view.

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REFERENCES

[1] Reese, E.T. and Maguire, A. (1969) Appl. Microbiol. 17, 242–245.
[2] Reese, E.T. and Maguire, A. (1971) Dev. Ind. Microbiol. 12, 212–224.
[3] Bhumibhamon, O. (1982) J. Ferment. Technol. 60, 167–169.
[4] Wittenberger, C.L., Beaman, A.J. and Lee, L.N. (1978) J. Bacteriol. 133, 231–239.
[5] Umesaki, Y., Kawai, Y. and Mutai, M. (1977) Appl. Environ. Microbiol. 34, 115–119.
[6] Jacques, N.A., Jacques, V.L., Wolf, A.C. and Wittenberger, C.L. (1985) J. Gen. Microbiol. 131, 67–72.
[7] Salton, M.R.J. and Owen, P. (1976) Annu. Rev. Microbiol. 30, 451–482.
[8] Rose, A.H. (1977) in The Filamentous Fungi, Vol. 2 (Smith, J.E. and Berry, D.E., Eds.) pp. 308–328. Edward Arnold, London.
[9] Chavant, L., Wolf, C., Fonvieille, J.L. and Dargent, R. (1981) Biochem. Biophys. Res. Commun. 101, 912–920.
[10] Mandels, M. and Andreotti, R.E. (1978) Proc. Biochem. 13, 6–13.
[11] Labudova, I. and Farkas, V. (1983) Biochim. Biophys. Acta 744, 135–140.
[12] Farkas, V., Labudova, I., Bauer, S. and Ferenzy, L. (1981) Folia Microbiol. 26, 129–132.
[13] Kammel, W.P. and Kubicek, C.P. (1985) J. Appl. Biochem. 7, 138–144.
[14] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[15] Kubicek, C.P. (1981) Eur. J. Appl. Microbiol. Biotechnol. 13, 226–231.
[16] Bligh, E.G. and Dyer, N.S. (1959) Can. J. Biochem. Biophys. 37, 911–917.
[17] Dittmer, J.C. and Wells, M.A. (1969) Meth. Enzymol. 14, 482–529.
[18] Umile, C. and Kubicek, C.P. (1986) FEMS Microbiol. Lett. 34, 291–295.
[19] Kubicek, C.P. (1982) Arch. Microbiol. 132, 349–354.
[20] Lisker, N., Katan, J., Chet, I. and Henis, Y. (1975) Can. J. Microbiol. 21, 521–526.
[21] Castanon, M. and Wilke, C.R. (1980) Biotech. Bioeng. 22, 1037–1053.

[22] Reese, E.T. (1980) J. Appl. Biochem. 2, 36–39.
[23] Calderbank, J., Keenan, M.H.J. and Rose, A.H. (1985) J. Gen. Microbiol. 131, 57–65.
[24] Aaronson, L.R. and Martin, C.E. (1983) Biochim. Biophys. Acta 735, 252–258.
[25] Schreiber, G., Kolar, H., Foisner, R. and Kubicek, C.P. (1985) Arch. Microbiol. 144, 41–47.
[26] Ghosh, A., Ghosh, B.K., Trimino-Vasquez, H., Eveleigh, D.E. and Montenecourt, B.S. (1984) Arch. Microbiol. 140, 126–133.