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Protoplasts from *Podospora anserina*: Isolation, Purification, and Transformation

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Abstract. Protoplasts from *Podospora anserina* mycelium were produced using the commercially available enzyme Novozym 234. Different parameters involved in protoplast isolation were analyzed in order to establish optimal conditions, and protoplast production was notably increased. For the purification of protoplasts, several techniques based on both centrifugation and filtration were assayed, with filtration yielding the best results. Regeneration of protoplasts was studied on different media and osmostic stabilizers, and about 80% regeneration was obtained. The good physiological condition of the protoplasts produced with this method is demonstrated by the lack of cell wall and high regeneration rate and transformation frequencies.

The isolation of protoplasts from fungi using lytic enzymes is now a well-established technique [19]. Initially, protoplasts were very useful for biochemical studies [11], but more recently interest has been focused on the use of protoplasts as genetic tools in experiments on fusion [2, 10] and transformation [3, 5, 30].

In *Podospora anserina*, senescence occurs when mycelia are maintained under continuous vegetative growth [23]. A mitochondrial plasmid, p1DNA, was identified as the causative agent for aging [27]. This plasmid is able to replicate autonomously [28, 30] and transform juvenile cells to senescence. For these reasons, p1DNA can serve for the construction of eukaryotic vectors for molecular cloning [9].

The development of transformation systems in *P. anserina* requires the previous isolation of protoplasts. Most experiments with protoplasts of this fungus used natural *Trichoderma* lytic extract to get them, but its production and regeneration were low: about 10^7 protoplasts/g and 1%–3% regeneration [29]. In some experiments, the commercial enzyme cytohelicase was employed with the isolation of 10^6 protoplasts/ml and a 5%–15% regeneration rate [4]. We have tried to increase the yield of the process as an essential step toward the development of the best transformation systems in *P. anserina*.

Materials and Methods

Organisms. The wild *s*(+) strain of *Podospora anserina* and SF8 of *Escherichia coli* containing the plasmid pSP24 used in this work were kindly provided by K. Esser (Lehrstuhl für Allgemeine Botanik, Ruhr-Universität, Bochum, FRG). For details about the *s*(+) strain, see Esser [8]. *Trichoderma harzyanum* CBS 354-33 strain (Centraalbureau voor Schimmelcultures, Baarn, The Netherlands) was used to produce the lytic enzyme against *P. anserina*.

Media. The following media were employed for *P. anserina*: CZM, Czapeck Dox medium (Oxoid); and MEM, malt extract medium containing malt extract 5 g, yeast extract 5 g, and dextrose 5 g/liter (J. F. Peberdy, personal communication). We also suggest RFM, rabbit food medium (5 g of rabbit food commercial pellets were boiled for 30 min in 1 liter of distilled water and filtered through cheesecloth); and SSM, soluble starch medium (soluble starch 5 g, yeast extract 4 g, K_2HPO_4 1 g, and $MgSO_4$ 0.5 g/liter); both media were at pH 6.0. In addition to these media, we employed CMM, cornmeal medium; MGM, microconidia germination medium; MPM, microconidia production medium [8]; and AMM, acetate minimal medium [22]. Stock cultures of *T. harzyanum* CBS 354-33 were maintained on AG medium [24].

Enzymes and chemicals. Novozym 234 was kindly provided by Novo Industri A/S (Denmark), chitin, chitinase, chromomycin A3, dextran, 1,4-dithiothreitol, Ficoll type 400, 2-mercaptoethanol, and polyethylene glycol (PEG) 4000 were purchased from Sigma (USA), cellulase CP from Sturge (UK), cellulase Onozuka R-10 from Yakult (Japan), cytohelicase from I.B.F. (France), Lymphoprep from Nyegaard (Norway), Percoll from Pharmacia (Sweden), and Tinopal 5BMS from Ciba-Geigy (Switzerland).

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Table 1. Comparison of enzyme systems for the production ($\times 10^5$ protoplasts/ml) of protoplasts from *Podospora anserina*

Enzyme system	Time (h)				
	1	2	3	4	5
Cytohelicase, 20 mg/ml	1.0 ± 0.1	2.5 ± 0.4	5.0 ± 0.5	7.5 ± 0.2	7.5 ± 0.8
Novozym 234, 20 mg/ml	340 ± 10	470 ± 40	520 ± 70	470 ± 10	470 ± 10
Novozym 234, 10 mg/ml + cellulase Onozuka, 10 mg/ml	250 ± 40	400 ± 10	650 ± 20	670 ± 50	620 ± 80
Novozym 234, 10 mg/ml + cellulase CP, 10 mg/ml	150 ± 70	260 ± 90	510 ± 10	650 ± 60	520 ± 10
Novozym 234, 10 mg/ml + chitinase, 2 mg/ml	450 ± 10	520 ± 10	650 ± 70	660 ± 40	680 ± 40
Trichoderma extract	0.5 ± 0.07	2 ± 0.1	2.5 ± 0.3	3 ± 0.9	3 ± 0.6
Trichoderma extract + Novozym 234, 10 mg/ml	50 ± 4	70 ± 4	120 ± 80	160 ± 20	200 ± 20

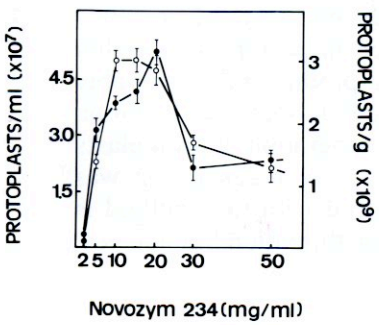

Fig. 1. Effect of Novozym 234 concentration on the liberation of *Podospora anserina* protoplasts. Protoplast number was determined after 3 (●) or 5 (○) h of treatment.

Culture conditions. Liquid cultures were obtained in 250-ml Erlenmeyer flasks containing 25 ml of medium placed on a rotatory shaker at 200 rpm. Microconida of *P. anserina* were harvested from grown solid agar plates of MPM and inoculated into liquid MGM. After three days of incubation, they were transferred to liquid AMM for six days and then the mycelial pellets were ground with a homogenizer and stored at 4°C, making up the stock of juvenile mycelium. For protoplast isolation, the mycelium was obtained by inoculating 1 ml of thick stock in liquid MEM (if not otherwise specified) and incubating it for 20–24 h. All the cultures were at 27°C.

Preparation of protoplasts. Mycelia from various shake culture flasks were recovered aseptically by filtration, washed three times with osmotic stabilizer, and resuspended in stabilized buffer solution containing the lytic mixture. Incubations were made at 27°C with gentle shaking, and protoplast number was periodically determined by using a Neubauer hemocytometer counting chamber. Pretreatment assays of mycelium with a thiol compound prior to digestion with lytic enzymes were done with 10 m*M* 1,4-dithiothreitol or 90 m*M* 2-mercaptoethanol in stabilized buffer for 1 h.

Purification and regeneration of protoplasts. Protoplasts were filtered through a Jena no. 2 (40- to 100-μm pore size), sintered glass filter to remove the hyphal fragments. Several systems were assayed to eliminate the lytic enzymes: direct centrifugation [1], discontinuous gradient centrifugation [12, 13, 16], flotation [7, 14], aqueous two-phase systems [15, 18], and filtration (V. Moreno, personal communication). The purified protoplasts were regenerated at different concentrations on solid AMM, CMM, or MEM. Sucrose, KCl, and $MgSO_4$ were assayed at different molarities as osmotic stabilizers.

Staining of mycelia and protoplasts. The absence of cell walls in protoplasts was checked by staining with the fluorescent brightener Tinopal 5BMS [11] and the ratio of nuclei per protoplast was determined by staining with chromomycin A3 [25]. The number of nuclei per septum was tested with Tinopal 5BMS and chromomycin A3 applied simultaneously [20, 21].

Transformation of protoplasts. pSP24 is a recombinant plasmid from pBR322 and p1DNA from *P. anserina* [26], and was isolated from SF8/pSP24 as described by Maniatis et al. [17]. Protoplasts were transformed with pSP24 by the method described by Tudzynski et al. [30], regarding the induction of premature colony aging.

All experiments were repeated a minimum of three times. The results represent the mean and the standard deviation.

Results and Discussion

Factors affecting protoplast isolation. To improve the production of protoplasts, we attempted first to choose the best enzymatic system, and the results of some tested lytic mixtures are shown in Table 1. *Trichoderma* extract, obtained as described [24], provides a low number of protoplasts, according to data from the literature [29], whereas the addition of Novozym 234 to the lytic system leads to a 50- to 200-fold increase in production. Cytohelicase behaves similarly, as expected [4]. This group of enzymes produces a weak liberation of protoplasts. Novozym 234, however, alone or combined with other enzymes, has been more efficient and is capable of producing under our conditions up to 5.2×10^7 protoplasts/ml. Combinations of Novozym 234 with cellulase CP, cellulase Onozuka R-10, or chitinase could only increase this value weakly. For

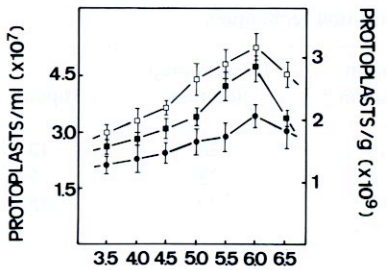

Fig. 2. Influence of pH on the isolation of *Podospora anserina* protoplasts. Protoplast number was determined after 1 (●), 2 (■), or 3 (□) h of treatment.

Table 2. Effect of different osmotic stabilizers on the formation of protoplasts from *Podospora anserina* ($\times 10^5$ protoplasts/ml)

Osmotic stabilizer	Protoplasts
Sucrose, 0.56 *M*	79 ± 6
$MgSO_4$, 0.7 *M*	520 ± 70
Mannitol 1.1 *M*	0.4 ± 0.04
NaCl, 0.7 *M*	2.9 ± 0.4
Sorbitol, 1.1 *M*	0.5 ± 0.02
KCl, 0.9 *M*	59 ± 6

all of these reasons, we selected Novozym 234 as the enzymatic system for further experiments. Figure 1 shows the effect of the Novozym 234 concentration on protoplast production at different times. Maximum yield was obtained at 20 mg/ml for 3 h or at 10 mg/ml for 5 h. We suggest using 20 mg/ml because, at short time intervals, protoplasts are less vacuolizated, less degraded, and more viable.

Potassium phosphate, 50 m*M*, was the most effective buffer among those tested, although the incompatibility of this buffer with $MgSO_4$ [6] forced us to sterilize them separately. Others, such as Tris–HCl, Tris-maleate–NaOH, citrate–phosphate, sodium phosphate, or ammonium phosphate, yielded lower numbers of protoplasts. Experiments using no buffer produced more protoplasts, but they were smaller and less viable. For potassium phosphate (50 m*M*) buffer, the best pH was 6.0 (Fig. 2). As osmotic stabilizers, we employed mineral salts and sugars (Table 2). It can be seen clearly that the highest yields were obtained with $MgSO_4$, and even protoplasts liberated with it were the biggest. As observed in Fig. 3, the optimal concentration of $MgSO_4$ is 0.7 *M*.

Pretreatment of the mycelium with dithiothreitol had no effect on protoplast yield, and even mercaptoethanol sometimes reduced its production. Preplasmolysis with osmotically stabilized buffer was also ineffective. Another factor that affects the liberation of protoplasts is the mycelium concentration in the lytic system (Fig. 4), but the protoplast number obtained per gram of mycelium showed very few variations. A concentration of 50–100 mg mycelium/ml was used in further experiments.

Podospora anserina becomes senescent with the age of mycelium, and accumulates a pigment that can affect the liberation of protoplasts. Thus, the experiments were done with juvenile myce-

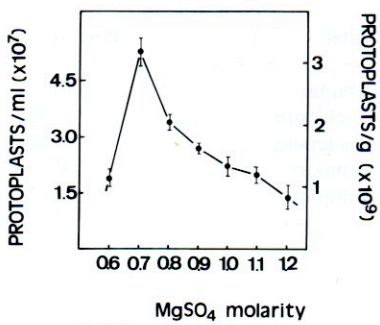

Fig. 3. Isolation of *Podospora anserina* protoplasts at different $MgSO_4$ molarities.

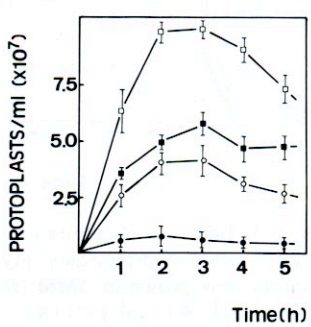

Fig. 4. Effect of mycelium concentration on the liberation of *Podospora anserina* protoplasts: 10 mg/ml (●), 25 mg/ml (○), 50 mg/ml (■), and 100 mg/ml (□).

lium, and the influence of the growth medium on the production of protoplasts was also studied (Fig. 5). The best results were obtained with MEM, which provides 3×10^9 protoplasts/g mycelium. Other media, such as CMM or RFM, produced smaller protoplasts and a very low regeneration rate, according to Tudzynski and Esser [29]. Mycelial growth in other media provided fewer protoplasts, but they were in good condition.

Table 3. Recovery percentages of protoplasts from *Podospora anserina* by different centrifugation techniques

Centrifugation technique	Direct $MgSO_4$	Direct KCl	Direct flotation	Ficoll flotation	Percoll flotation	Lymphoprep flotation	Biphase
Supernatant	65	60	65	30	10	28	10
Recovered	16	15	—	21	5	23	5
Lost	19	25	35	49	85	49	85

All centrifugations were carried out at 1500 rpm for 15 min.

Table 4. Recovery percentages of protoplasts from *Podospora anserina* by different filtration systems

Filter	Pore size (μm)	Recovery percentages		
		Filtered solution	Without membrane resuspension	With membrane resuspension
Jena no. 4	10–16	—	—	—
Nuclepore	0.45	—	10	36
Nuclepore	0.22	—	40	70
Millipore	0.45	—	15	50
Millipore	0.22	—	60	88

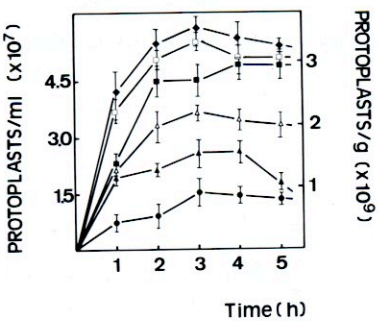

Fig. 5. Influence of culture medium on the production of *Podospora anserina* protoplasts. Mycelia for the isolation of protoplasts were grown in AMM (■), CMM (△), MEM (□), MGM (●), RFM (♦), and SSM (▲).

In conclusion, the best system was Novozym 234 at 20 mg/ml for 3 h in 50 m*M* potassium phosphate buffer (pH 6.0), and osmotically stabilized with 0.7 *M* $MgSO_4$. The final concentration of the 24-h-MEM-grown mycelium was 50–100 mg/ml. Under these conditions, we have increased the production of protoplasts up to 150–1500 times compared with that reported in the literature [4, 28, 29].

Factors affecting protoplast purification. The density of the protoplasts obtained in $MgSO_4$ was variable, and their behavior was more complicated than that of protoplasts obtained in other osmotic stabilizers, as previously described [7]. We confirmed this fact when the direct centrifugation method sedimented the protoplasts, at different speeds, only slightly. Similar results were obtained with protoplasts isolated with 0.43 *M* KCl (Table 3), so we tried to recover the protoplasts by flotation, from 0.7 *M* $MgSO_4$ to a less dense layer of 0.43 *M* KCl, but they did not migrate to the interphase; this fact agrees with microscopic examinations where vacuoles were not detected in protoplasts isolated under optimal conditions. The addition of commercial polymers that increase the density without varying the osmotic strength (Ficoll, 10%; Percoll, 10%; or Lymphoprep) to the $MgSO_4$ layer, makes it possible to recover some protoplasts from the interphase, but only 5%–25% (Table 3). Also we assayed the aqueous two-phase system, based on the migration of protoplasts to the partition zone of two phases of PEG–dextran or PEG–$MgSO_4$ [15, 18]. Now again, only 5% of protoplasts were recovered efficiently.

There are two common problems in these centrifugation systems: (a) The high loss rate of protoplasts because of breakage during centrifugation; we tried in each case at different times and speeds and no variation was distinguishable. (b) These data are the result of only one centrifugation, and we had to repeat this process two or three more times in order to eliminate completely the lytic system; so the final yield of the complete process was insignifi-

Table 5. Regeneration percentages of protoplasts from *Podospora anserina* in different combinations of solid media and osmotic stabilizers

Medium	Osmotic stabilizer		
	KCl (0.42 *M*)	Sucrose (20%)	$MgSO_4$ (0.7 *M*)
AMM	7.7 ± 0.3	16.0 ± 0.7	6.3 ± 0.3
CMM	61.8 ± 0.2	81.4 ± 0.9	31.2 ± 0.5
MEM	33.6 ± 0.6	62.3 ± 0.3	17.5 ± 0.4

cant. For these reasons, we assayed other purification systems based on filtration.

Several types of filters were tested (Table 4). Jena filter no. 4 was totally ineffective because its pore size (10–16 μm) is similar to the diameter of the protoplasts (10 μm) and so they were broken or lost. The use of filters with smaller pore size, i.e., Nuclepore and Millipore, was more effective. The best results were obtained in all cases with the employment of the Millipore filter because its rough structure prevents the blockage of the pores by the protoplasts. A pore size of 0.22 μm was optimal. The resuspension of filters in the medium to liberate trapped protoplasts increased the final yields to 88% of recovered protoplasts.

Factors affecting protoplast regeneration. Several combinations of solid media and osmotic stabilizers were assayed (Table 5), and the highest percentages were obtained with CMM. The nature of the osmotic stabilizer was conclusive: in all assayed media, 20% sucrose provided the best results. An optimal regeneration percentage of 81.4% ± 0.9% was obtained when we used CMM stabilized with sucrose. This value is higher than percentages reported in the literature, which varied between 1% and 15% [4, 29]. Purification of protoplasts considerably affected regeneration rates; under optimal conditions, unpurified protoplasts showed only a regeneration of 0.1%.

Physiological condition of protoplasts. The lack of a detectable cell wall was revealed by absence of fluorescence in a suspension of purified protoplasts treated with Tinopal 5BMS. The ratio of nuclei per protoplast was tested with chromomycin A3. The mean ratio was 1.47 ± 0.86. The mode was one nucleus per protoplast, in accordance with the fact that in the mycelium of *P. anserina* there is only one nucleus per septum. However, a significant number of protoplasts (41%) had more than one nucleus, probably due to spontaneous protoplast fusion also detected in microscopic observations. The good state of the protoplasts obtained with the method proposed here was also established because they were capable of reversing in colonies morphologically identical to those of the parental strain.

When we assayed genetic transformation of these protoplasts with the hybrid plasmid pSP24, we obtained a frequency of 1.68×10^{-2} transformants per viable protoplast. This rate is higher than that of 2.8×10^{-3} described previously [30], pointing out the good physiological condition of the protoplasts.

The protoplast system developed in the present study provides a large number of viable protoplasts: 3×10^9 protoplast/g mycelium; 88% of them are recovered after purification, with an 81% regeneration rate. Protoplasts produced in this way can serve as a useful tool for studies about somatic fusion and genetic transformation of *Podospora anserina*.

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Literature Cited

1. Anné J (1977) Somatic hybridization between *Penicillium* species after induced fusion of their protoplasts. Agricultura 25:1–117
2. Anné J, Peberdy JF (1975) Conditions for induced fusion of fungal protoplasts in polyethylene glycol solutions. Arch Microbiol 105:201–205
3. Ballance DJ, Buxton FP, Turner G (1983) Transformation of *Aspergillus nidulans* by the orotidine-5′-phosphate decarboxylase gene of *Neurospora crassa*. Biochem Biophys Res Commun 112:284–289
4. Belcour L (1975) Cytoplasmic mutations isolated from protoplasts of *Podospora anserina*. Genet Res 25:155–161
5. Case ME, Schweizer M, Kushner SR, Giles NH (1979) Efficient transformation of *Neurospora crassa* by utilizing hybrid plasmid DNA. Proc Natl Acad Sci USA 76:5259–5263
6. De Vries OMH, Wessels JGH (1972) Release of protoplasts from *Schizophyllum commune* by a lytic enzyme preparation from *Trichoderma viride*. J Gen Microbiol 73:13–22
7. De Vries OMH, Wessels JGH (1975) Chemical analysis of cell wall regeneration and reversion of protoplasts from *Schizophyllum commune*. Arch Microbiol 102:209–218
8. Esser K (1974) *Podospora anserina*. In: King RC (ed) Handbook of genetics, vol 1. New York: Plenum, pp 531–551
9. Esser K, Kuck U, Stahl U, Tudzynski P (1983) Cloning vectors of mitochondrial origin for eukaryotes: a new concept in genetic engineering. Curr Genet 7:239–243
10. Ferenczy L, Kevei F, Zsolt J (1974) Fusion of fungal protoplasts. Nature 248:793–794

11. Hamlyn PF, Bradshaw RE, Mellon FM, Santiago CM, Wilson JM, Peberdy JF (1981) Efficient protoplast isolation from fungi using commercial enzymes. Enzyme Microb Technol 3:321–325
12. Harms ChT, Potrykus I (1978) Fractionation of plant protoplast types by iso-osmotic density gradient centrifugation. Theor Appl Genet 53:57–63
13. Hughes BG, White FG, Smith MA (1978) Purification of plant protoplasts by discontinuous gradient centrifugation. Biochem Physiol Pflanz 172:223–231
14. Isaac S, Ryder NS, Peberdy JF (1978) Distribution and activation of chitin synthase in protoplast fractions released during the lytic digestion of *Aspergillus nidulans* hyphae. J Gen Microbiol 105:45–50
15. Kanai R, Edwards GE (1973) Purification of enzymatically isolated mesophyll protoplasts from C3, C4 and crassulacean acid metabolism plants using an aqueous dextran–polyethylene glycol two-phase system. Plant Physiol 52:484–490
16. Larkin PJ (1976) Purification and viability determinations of plant protoplasts. Planta 128:213–216
17. Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor NY: Cold Spring Harbor Laboratory
18. Mattiasson B (1983) Applications of aqueous two-phase systems in biotechnology. Trends Biotechnol 1:16–20
19. Peberdy JF (1979) Fungal protoplasts: isolation, reversion, and fusion. Annu Rev Microbiol 33:21–39
20. Peberdy JF, Buckley CE (1973) Adsorption of fluorescent brighteners by regenerating protoplasts of *Aspergillus nidulans*. J Gen Microbiol 74:281–288
21. Preece TF (1971) Fluorescent techniques in micology. In: Booth C (ed) Methods in microbiology, vol 4. New York: Academic Press, pp 509–516
22. Prillinger H, Esser K (1977) The phenoloxidases of the ascomycete *Podospora anserina*. XIII. Action and interaction of genes controlling the formation of laccase. Mol Gen Genet 156:333–345
23. Rizet G (1953) Sur la longévité des souches de *Podospora anserina*. CR Seances Acad Sci 237:838–840
24. Sánchez A, Pérez G, Vicente E (1982) Efecto de diversas fuentes de carbono sobre actividades glucanásicas producidas por *Trichoderma harzianum* CBS 354-33 implicadas en la liberación de protoplastos. Coll Bot 13:643–656
25. Slater ML (1976) Rapid nuclear staining methods for *Saccharomyces cerevisiae*. J Bacteriol 126:1339–1341
26. Stahl U, Kuck U, Tudzynski P, Esser K (1980) Characterization and cloning of plasmid like DNA of the ascomycete *Podospora anserina*. Mol Gen Genet 178:639–646
27. Stahl U, Lemke PA, Tudzynski P, Kuck U, Esser K (1978) Evidence for plasmid like DNA in a filamentous fungus, the ascomycete *Podospora anserina*. Mol Gen Genet 162:341–343
28. Stahl U, Tudzynski P, Kuck U, Esser K (1982) Replication and expression of a bacterial–mitochondrial hybrid plasmid in the fungus *Podospora anserina*. Proc Natl Acad Sci USA 79:3641–3645
29. Tudzynski P, Esser K (1979) Chromosomal and extrachromosomal control of senescence in the ascomycete *Podospora anserina*. Mol Gen Genet 173:71–84
30. Tudzynski P, Stahl U, Esser K (1980) Transformation to senescence with plasmid like DNA in the ascomycete *Podospora anserina*. Curr Genet 2:181–184