SUMMARY

1. The possibility has been shown of obtaining 5,6-ureylene-3-methylindan-1-one and 6,7-ureylene-3,5-dimethylindan-1-one in one stage by condensing the corresponding benzimidazolin-2-ones with γ-butyrolactone or crotonic acid in the presence of an excess of anhydrous aluminum chloride.

2. The plant growth inhibiting activity of the indanones synthesized has been studied.

3. Derivatives of the indanones with hydroxylamine, semicarbazide, and 2,4-dinitrophenylhydrazine have been obtained and their fungicidal activities have been studied.

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ISOLATION OF A LIPASE INHIBITOR FROM THE FUNGUS *Rhizopus microsporus*

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A factor capable of causing inhibition of the activity of extracellular lipase has been detected in the mycelium of the fungus Rhizopus microsporus. By fractionating a homogenate of the mycelium followed by chromatographic purification on a column of DEAE-cellulose and on Sephadex G-75 this inhibitor has been isolated in the electrophoretically homogeneous state. It is a substance of protein nature with M ~ 24,000, consisting of two subunits. The inhibitor acts on the isoenzymes of the lipase to different extents.

The fungus Rhizopus microsporus is known as a producing agent of active lipases. On studying various components, we detected a protein factor capable of exerting an inhibiting action on extracellular lipase.

Figure 1A presents the results of gel filtration of the supernatant liquid from a homogenate on a column of Sephadex G-75. When the influence of the individual fractions on the activity of the extracellular lipase the preparation of which has been described previously [1] was studied, it was found that elution fractions 42-46 contained a protein causing inhibition (Fig. 1A). These fractions were combined, dialyzed against distilled water, and freeze-dried. The resulting preparation was subjected to ion-exchange chromatography on a column (1× 15 cm) of DEAE-cellulose in 0.01 M phosphate-citrate buffer, pH 7.4. The proteins were eluted with a linear gradient of NaCl from 0 to 1 M at the rate of 12 ml/h. Fractions with a volume of 3 ml were collected (Fig. 1B). Of the five protein peaks obtained, only the proteins of fractions 51-56 possessed inhibitory capability.

A repeat of gel filtration on Sephadex G-75, after dialysis and lyophilization, permitted us to obtain the lipase inhibitor in an electrophoretically homogeneous state (Fig. 1B). The homogeneity of the inhibitor was shown by rechromatography (ion-exchange chromatography on DEAE-cellulose – the protein eluted as one peak

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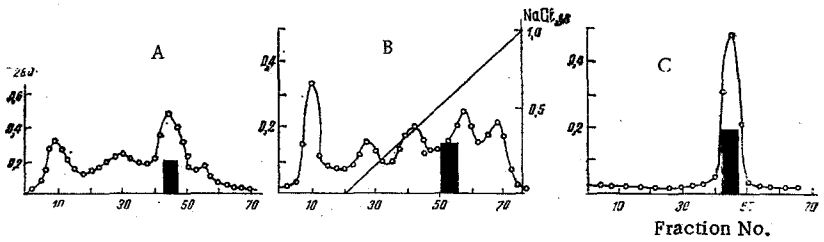

Fig. 1. Purification of the lipase inhibitor from the fungus Rhizopus microsporus: A) gel filtration of the supernatant liquid from a homogenate of the mycelium on Sephadex G-75 (here and below the blackened zone represents the inhibitory fraction); B) ion-exchange chromatography of fractions 42-46 after Sephadex G-75 on DEAE-cellulose; C) repeated gel filtration of the inhibitory fraction on Sephadex G-75.

at 0.55 M NaCl; by gel filtration on Sephadexes G-75 and G-100 – the protein eluted as a single symmetrical peak) and by disc electrophoresis in 7.5% polyacrylamide gel (in Tris-glycine buffer, pH 8.9, only one band was detected, with R_f 0.78). The homogeneous protein obtained also eluted as a single band on disc electrophoresis on polyacrylamide gel in a buffer containing 1% of sodium dodecyl sulfate, but with a different R_f value, 0.81, which shows the existence of a subunit structure.

Thus, a protein fraction possessing an inhibitory action with respect to extracellular lipase has been isolated from a homogenate of the mycelium of the fungus Rhizopus microsporus.

The molecular mass of the homogeneous protein inhibitor was determined by gel filtration on a calibrated column of Sephadex G-100 (M ~ 24,000 according to [2]).

It was interesting to establish whether the protein factor obtained, possessed an inhibitory action with respect to the different forms of the lipase of the fungus Rhizopus microsporus. All five known forms of the lipase differ by their structural composition [2] and also by their catalytic and physicochemical properties [3]. With respect to specific activity, they form the following sequence A + 2B > A + B > 3A + 2B > 2A + B > 4A + B (where A and B are subunits of the lipases) [2]. The protein factor that we isolated affected the activities of the individual forms of the enzyme differently. The degree of inhibition was studied at an equimolar concentration of the protein factor at pH 7.5.

The subunit composition and some properties of the various forms of the lipases of the fungus Rhizopus microsporus are given below:

Form of the lipase	Subunits	$M \cdot 10^3$	pH_{rel}	Amount subunits, %	Rel. activity of the forms	Degree of inhibition, %
1	A+B	28	7,8	50	1,0	8
2	2A+B	39	7,2	33	0,7	30
3	A+2B	43	8,2	66	1,5	37
4	4A+B	65	6,0	20	0,5	10
5	3A+2B	67	7,4	40	0,9	23

Thus, the protein factor not only inhibits the extracellular lipase but acts on the separated forms of the enzyme. All five isoenzymes are capable of interacting in different degrees with the protein inhibitor produced by the same fungus. This may have great value for regulating the lipase activity of the producing agent and will enable us to obtain new information on the nature of the multiplicity of forms of lipases and their interrelationship.

EXPERIMENTAL

The conditions of cultivation and the composition of the nutrient medium for the growth of the fungus Rhizopus microsporus and the biosynthesis of the lipases have been described previously [1-4]. After the completion of growth, the mycelium of the fungus was separated from the culture liquid by vacuum filtration on a Büchner funnel. The mycelium was carefully washed with distilled water and homogenized. For this purpose, 20 g of the crude biomass was ground with quartz sand in a mortar in an ice bath until the hyphae of the mycelium had been completely disrupted, the completeness of disruption being followed with the aid of a MBI-3 microscope. The homogenate obtained was treated with 0.1 M phosphate-citrate buffer, pH 7.4, containing 0.1

M $CaCl_2$ and the mixture was left for 1 h. After centrifugation at 600× g for 15 min, the supernatant liquid was separated off and was used for the purification of the protein inhibitor.

Lipase activity was determined titrimetrically [1]. The substrate was a 40% emulsion of olive oil stabilized in a 2% solution of polyvinyl alcohol. The conditions of stabilizing the substrate have been described previously [1, 4]. The reaction medium contained 2.5 ml of substrate, 6.5 ml of 0.1 M phosphate-citrate buffer, pH 7.5, and 1 ml of enzyme solution. The lipolysis reaction was stopped by the addition of 30 ml of 96% ethanol.

Protein was determined by Lowry's method or by spectrophotometry – from the change in the absorption of light at 280 and 260 nm on a SF-16 spectrophotometer.

Electrophoretic studies were carried out according to Davis [5] on a "Reanal" instrument in 7% polyacrylamide gel at pH 8.9. Proteins were visualized in a 0.1% solution of Amido Black 10B in 7% acetic acid.

The subunit structure of the isolated protein was investigated by disc electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate and β-mercaptoethanol by the Weber–Osborn method [6] and also according to Laemmli [7].

SUMMARY

1. A protein factor exhibiting an inhibitory action on extracellular lipase has been isolated from the intracellular fraction of the mycelium of the fungus Rhizopus microsporus in the electrophoretically homogeneous state. The molecular weight of the protein is ~24,000.

2. Different forms of the lipase have different sensitivities to the protein inhibitor and form the decreasing sequence 3–2–5–4–1.

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