

Matile, Ph. Porosity of the cell wall as related

to the secretion of proteolytic enzymes in *N. crassa*.

Hyphae of *N. crassa* secrete two acid proteases into culture media which contain protein as the only nitrogen source (Matile 1965 Z. Zellforsch. 65: 884). These enzymes are localized intracellularly in small sacs, protease particles, which upon the supply of a proteinaceous nitrogen source are transferred to the outside of the plasmalemma (Matile et al. 1965 Z. Zellforsch. 68: 205). This process leads to the occurrence of free extracellular proteases which subsequently cross the cell wall and appear in the culture medium. Thus, the size of the secreted enzyme molecules must be of the same order of magnitude as the dimensions of the pores of the cell wall. In contrast, another secretion product, aminopeptidase, has never been observed outside the cells. This enzyme is localized somewhere between the plasmalemma and the cell wall (Motile 1964 Naturwissenschaften 51: 489). Travithick and Metzberg (1966 J. Bacteriol. 92: 1010) have described a process of molecular sieving in *Neurospora* cell walls with respect to secreted invertase; this finding suggests the existence of a similar mechanism resulting in the retention of secreted ominopeptidase molecules. In order to prove the validity of the above hypothesis, the approximate molecular size of both secreted proteases and aminopeptidase has been determined, using the gel filtration method of Whiteaker (1963 Anal. Chem. 35: 1950).

For isolation of cell walls, 50 g of wet mycelium (strain chol-1 (34486), FGSC #485) were mixed with an equal volume of glass beads (0.45 mm) and a small volume of 1% NaCl. The cells were broken by the action of a vibration mixer (1 hour). The resulting suspension, containing less than 1% of intact cells, was diluted with 1% NaCl and the glass beads were allowed to settle. Centrifugation for 15 min at 100 x g yielded a white pellet which contained the cell walls. This pellet was subjected to washings in 1% NaCl until the supernatant was perfectly transparent. After 20 washings, only clean fragments of cell walls could be observed in the phase contrast microscope.

Gel filtration with Sephadex-150 was carried out in a 15 x 300 mm column. Columns of isolated cell walls are completely impassable for the solvent: in order to obtain a homogeneous working column (11 x 65 mm), a suspension consisting of isolated cell walls and glass beads (0.18 mm) was added in small portions under continuous gentle stirring. IN NaCl in 0.1M Tris-HCl buffer pH 7.2 was used for the elution of both Sephadex and cell wall columns. Blue dextrane (Pharmacia Uppsala) was used for the determination of void volumes. Bovine serum albumin (Colbiochem. A-grade), cytochrome c and tyrosine (Fluka) served as reference substances. The proteolytic enzymes subjected to gel filtration were contained in a culture filtrate (acid proteases, pH optima 4.2 and 6.4) or in a high speed supernatant of a cell-free extract (aminopeptidase).

Both acid proteases were eluted from Sephadex-150 column at a  $V_e/V_0$  value of 2.9, the aminopeptidase at  $V_e/V_0 = 2.0$ . The corresponding molecular weights are ca. 22,000 and 85,000. Most probably these values don't represent true molecular weight since it has not been established that the secreted proteolytic enzymes are pure proteins. It may be assumed that the ominopeptidase is a glycoprotein like invertase (Metzberg 1963 Arch. Biochem. Biophys. 100: 503) and other extracellular enzymes of *Neurospora* and yeast (Lampen 1965 Symp Soc. Gen. Microbiol. 15: 115). However, the  $V_e/V_0$  values point to a considerably different size of protease and ominopeptidase molecules. This finding supports the hypothesis that the smaller molecules of acid proteases can, while the larger ones of ominopeptidase cannot, penetrate the pores of the *Neurospora* cell wall.

However, it appears from a comparison of the molecular sizes of invertase (which is partially released into the medium) and aminopeptidase (which is not released) that the situation is more complex. Invertase molecules should be smaller than

**aminopeptidase** molecules if molecular sieving alone is responsible for their different **behaviour** with respect to the wall. Gel filtration with **Sephadex**, however, indicates that the **invertase molecule** (monomer) is larger than the **aminopeptidase**. Therefore, in addition to molecular sieving, binding between cell wall constituents and **aminopeptidase** molecules must be considered.

The elution pattern from a cell wall column of acid **proteases** clearly **demonstrates** that these enzymes are **able to penetrate** the pores of the cell wall. The bulk of enzyme activity **occurs** in a **broad peak at  $V_e/V_o = ca. 3.5$** ; a small fraction of activity is eluted together with the blue **dextrane**. Since from columns of **Sephadex** the same enzymes are eluted in a uniform peak, it must be **concluded** that for possible reasons of the geometry of the cell wall fragments a fraction of enzyme **molecules** never enters the pores during the movement **through** the column. The **broad peak** of the bulk of **protease** activity points to the fact that **isolated** cell walls don't allow an ideal distribution of the molecules between the inner (pores) and the outer volume. Therefore, only **qualitative** results can be obtained from **molecular sieving** experiments with isolated cell walls.

**Invertase** filtered through **isolated cell walls** appears in an almost uniform peak together with the excluded blue **dextrane**. The small fraction of **enzyme** activity which is eluted after the **dextrane** indicates a limited penetration of the cell walls which is **possibly** responsible for the **appearance** of the enzyme in the culture medium. Similarly, the **elution** pattern of **aminopeptidase** regularly **shows** a tail of activity which does not **move** with the front. The bulk of **aminopeptidase** seems to **be** excluded and is therefore eluted together with the blue **dextrane**.

Since the **aminopeptidase** is **not** secreted into the culture medium, its **partial** retention in a cell wall column **must** be due to reversible binding rather than to penetration of the cell wall. The sizes of pores in the **Neurospora** cell wall determined by **Trevithick et al. (1966 J. Bacteriol, 92: 1016)** would permit the passage of even larger molecules than those of **aminopeptidase** and **isozymes of invertase**. The fact that the latter enzymes are released into the medium according to the principles of molecular sieving, whereas the former, smaller molecule **never** occurs outside the cell, **cannot** be explained by molecular sieving alone.

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