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Abstract

The *Age-1* gene family consists of 16 functionally redundant, closely linked loci on linkage group I. (Munkres 1985 Superoxide Dismutases, Vol. III, CRC Press, Boca Raton, FL). Dominant mutations at those loci reduce conidial lifespan (loc. cit.) and cause multiple deficiencies of at least 12 antioxidant enzymes; the endocellular superoxide dismutases (SOD), and exocellular cell wall bound SOD isozyme, catalase, peroxidases and reductases (Munkres et al. 1984 Mech. Age. Dev. 24:83-100; Munkres 1990 Free Radical Bio. Med., in press). Colonies of the mutants secrete superoxide radicals and H2O2, apparently as a consequence of their antioxidant enzyme deficiencies. The purpose of this note is to describe histochemical methods that detect the secretions.

Histochemical detection of superoxide radicals and hydrogen peroxide by *Age-1* mutants of Neurospora

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Yellow, water-soluble nitroblue tetrazolium (NBT) is reduced by superoxide radicals to blue, water- insoluble formazan (Halliwell and Gutteridge 1985 Free Radicals in Biology and Medicine, Clarendon Press, Oxford) Although other substances may also reduce NBT, specificity of the reaction can be demonstrated by the inhibitory effect of added SOD.

Superoxide radicals dismutate to H_2O_2 either spontaneously or by SOD catalysis. The secretion of H_2O_2 is detected by a colorimetric reaction with a dye that is catalyzed by an excess of peroxidase. Cultural procedures were described (Munkres and Furtek 1984 Meth. Enzymol. 105:263-270). The density of viable conidia plated should be adjusted to obtain not more than 50-100 colonies per plate. The growth and reaction of crowded colonies is retarded. In the case of wild type, it is usually preferable to test 3-4 day old colonies before conidiophores are formed. *Age-1* mutant colonies do not form conidiophores. Mutant and wild type colonies as old as 10 days can be used, but wild type conidiophores must be removed with a forcible stream of water because they obscure the reactions.

The staining solution for superoxide contains 5 mM 3-(N-morpholino) propane sulfonate-NaOH buffer, pH 7.6, and 2.5 mM of NBT. The solution is shielded from light because the dye slowly photo-oxidizes. The colonies are flooded with 5-10 ml of the solution and incubated at 35°C for 15-60 min. The solution is discarded and plates are incubated for 0.5 to 3 h at 35°C in the dark until the desired differential intensity of stain of mutant and wild type colonies is obtained. Overnight incubation increases the overall intensity, but does not increase the differential. Commercial bovine red blood cell SOD (30-300 units/ml) in the staining solution inhibits the differential reaction. Mutant colonies are intensely blue to purple, but wild type is colorless or faintly blue. Microscopically, large blue formazan granules occur on the surface of mutant colonies, whereas the faint stain of wild type is diffuse within hyphae. (In wild type, the reducing substance in unknown.)

To test hydrogen peroxide secretion, a solution of 100 mM potassium phosphate buffer, pH 6.9, 2.5 mM diaminobenzidine tetrachloride, and 5 purpurogallin units/ml of horseradish peroxidase (Type VI, Sigma Chem. Co., St. Louis, MO) is freshly prepared and shielded from light to

prevent spontaneous photooxidation. Plates are flooded with sufficient solution to submerge the colonies and incubated for an hour at 35°C in the dark. After decanting the solution, plates are inverted and incubated at 35°C in the dark for 24-48 h. Mutant colonies exhibit brown halos of various diameters and intensities and are uniformly brown, but wild type colonies do not stain.

Populations of colonies of our wild type (in Oak Ridge background) reproducibly exhibit about 15% spontaneous *Age-1* mutants (Munkres and Furtek 1984 Mech. Age. Dev. 25:47-62). About 15% of the colonies are also positive in the histochemical tests. Subsequent tests by present methods (*ibid*) indicated that the positively-stained colonies are indeed *Age-1* mutants. Thus, the secretion phenotype is a general feature of the mutants. We do not know whether the high spontaneous frequency of the mutants is a feature of other wild types, but one should be aware of the phenomenon in wild type control tests.

The superoxide test has been successfully applied to colonies derived from random ascospores in crosses of mutant to wild type or the linkage testers multicent and alcoy in analysis of segregation and recombination. - - - This research was supported by funds from the Laboratory of Molecular Biology. Contribution no. 3108 from the Department of Genetics.