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Abstract
A solid-reagent dispenser for use in the azocoll protease assay (and other insoluble substrate analyses).

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Azocoll (Calbiochem) has been used for many years as a substrate for the assay of collagenases and other proteases (Oakley et al., 1946 J. Pathol. Bacteriol. 58:232). The assay is sensitive, simple to perform, and the reaction has essentially no background absorbance. Despite these advantages, azocoll has not been extensively used, primarily because the reagent is insoluble, a property that requires the investigator to measure precise amounts of dry azocoll for each assay sample.

We have developed a solid-reagent dispenser to aliquot azocoll rapidly and accurately, and have used the machine to demonstrate multiple proteolytic activities in Neurospora crassa (Gaertner et al., 1979 in Limited Proteolysis in Microorganisms, DHEW Publ. No. 79-1591:197-202). The dispenser, consisting of three parts, is shown in Figs. 1 and 2. The first part is a hopper, machined from a block of aluminum and containing 12 wells from which azocoll is funneled into the second part, a machined teflon rod. This has 12 holes drilled into it, each positioned below a well and containing space for approximately 18 mg of azocoll. It may be rotated 180° so that in one position (holes pointed up) all 12 holes are simultaneously filled with azocoll from the hopper, while in the second position (holes pointed down) the aliquots of azocoll are released and drop directly into test tubes positioned below the holes. The third part consists of an ordinary test tube rack with a Plexiglas template to hold each row of test tubes vertically and in position directly below the rod.

![Figure 1. -- Solid reagent dispenser.](image1)

![Figure 2. -- Sectioned detail drawings of the solid reagent dispenser.](image2)

Figure 2 contains sectioned detailed drawings of the apparatus. The cross section of the turning knob (Section AA-1) shows the position of the positive lock pin, which allows the knob to be turned only 180° in either direction and thus aligns the holes in the rod with the hopper or with the test tubes. Section BB shows the hopper, the teflon rod, and the Plexiglas template that houses the test tubes. The azocoll is fed directly into the hole in the rod, the rod is turned 180°, and azocoll is deposited into the test tube. Section BB-2 gives the location of one of the positive lock pins which serves to align the template directly underneath the rod. Another lock pin is located on the left-hand side of the apparatus (not visible), and the corresponding stops in the template are shown in Figure 1C and in Section BB-3 of Figure 2.

The volume of the aliquot delivered is dependent solely upon the diameter of the rod and the diameter and depth of the hole drilled into it. Investigators would need to determine their own requirements and construct accordingly. The overall size of the apparatus is determined principally by the size of the test tube rack and the test tubes that are used. We have not given any dimensions, but our rack holds forty-eight 13 x 100 mm test tubes.

Because azocoll is stable (in the absence of proteolytic activity), we could increase the reaction time to 10 hours, thereby increasing the assay's sensitivity to a small amount of protease. Each reaction mixture contains 18 mg of azocoll, 0.05 ml of enzyme (either from a column fraction or a known concentration of a commercial protease), and 0.45 ml of buffer. We use either 0.1 M potassium phosphate (pH 7.0 or 8.5) or 0.1 M sodium citrate (pH 5.0), depending upon the protease being assayed. The azocoll is dispensed into 13 x 100 mm test tubes, the buffer and enzyme are added to initiate the reaction, and the tubes are shaken with sufficient vigor to keep the azocoll in suspension. The reaction is terminated by centrifuging the test tubes at 2000 x g for 10 min to pellet the unsolubilized azocoll. Each supernatant is aspirated into clean test tubes, and its absorbance is measured at 520 nm.
We compared the sensitivity of this procedure to two other common protease assays using hemoglobin (Kunitz 1947 J. Gen. Physiol. 30:291) and casein yellow (Anson 1938 J. Gen. Physiol. 22:79). The latter assays are short-term, so we modified our procedure to include a reaction termination step, adding 0.5 ml of 10% trichloroacetic acid at appropriate time intervals. We found that azocoll is approximately 100-fold more sensitive, over a 30 min interval, to hydrolysis by trypsin than are either casein yellow or hemoglobin (Figure 3). To demonstrate further the sensitivity of the azocoll assay, we assayed several commercial proteases over a ten h. period and readily detected one nanogram quantities of trypsin, subtilisin, and thermolysin (Sigma).

The solid reagent dispenser greatly reduces the time needed to measure azocoll for each reaction mixture and makes feasible the use of azocoll for investigations requiring large numbers of protease assays. This method of measuring azocoll is both rapid and accurate (machine error is ±4%). The dispenser could readily be used to measure other insoluble substrates. Moreover, by using teflon rods with suitably sized holes drilled in them, one could dispense different amounts of solid substrates. (This research was sponsored jointly by NSF Grant PCM76-80227 and the Office of Health and Environmental Research, U. S. Dept. Energy Contract W-7405-eng-26 with Union Carbide. University of Tennessee Oak Ridge Graduate School of Biomedical Sciences and Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830)

Figure 3. -- Comparison of three short-term protease assays. Hemoglobin, casein yellow and azocoll are compared as substrates for trypsin over a short time interval. The hemoglobin (▲) and casein yellow (•) reaction mixtures each contained 1% substrate and 0.1% (1.0 mg/ml) trypsin. The azocoll reaction mixtures contained 18 mg of substrate and either 0.01 mg trypsin/ml (▲) or 0.033 mg trypsin/ml (●). Reactions were terminated by addition of 10% trichloroacetic acid.

The study of precursor ribosomal RNA (pre-rRNA) maturation in ribosome biosynthesis mutants of Neurospora crassa is facilitates by the isolation of RNA from purified nuclei. Problems have been encountered in attempts to purify nuclei with Ludox gradients. Specifically, Ludox precipitates at low temperatures when exposed to Triton X-100, which is an essential component of the buffer used in the nuclei isolation steps. Therefore, a new gradient medium Percoll (Pharmacia Fine Chemicals, Piscataway, N.J.) was tested for its applicability. The use of Percoll rather than Ludox eliminated problems with precipitation. In addition it was possible to determine the buoyant density of the nuclei accurately, since the colloidal silica particles are coated with polyvinylpyrroliodone to which the nuclear membrane is impermeable.

Flasks of liquid Vogel's minimal medium inoculated with wild type conidia (2x10^7 ml^-1) were incubated for 8 h. at 25°C. Crude nuclear pellets were prepared from these mid-logarithmic phase cultures using a modified version of the procedure described by Hautala et al. (1977) J. Bacteriol. 130:704). As in the original method, a French pressure cell was used for efficient cell breakage. Modifications included centrifuge of the supernatant liquid from the post-Omni mixer homogenized cell suspension at 2,300 g (rav 8.26 cm) rather than 5,300 g for each centrifugation. For subsequent steps, changes in buffer B were necessary to maintain the correct osmolarity for the Percoll gradient step. To generate a medium having an osmolarity of 320 mOs/kg H2O, it is necessary to mix Percoll with 2.5 M sucrose in a 9:1 ratio. Lower starting densities of Percoll can be obtained by adding the appropriate amount of 0.25 sucrose. Since, in the Hautala method, the crude nuclear pellet is suspended in buffer B which contains 1 M sucrose (i.e., 50 mM Tris-HCl, pH 7.5; 5 mM MgCl2; 10 mM CaCl2; 1 M sucrose; and 1% (v/v) Triton X-100), it was necessary to reduce the sucrose concentration in the experiments reported here from 1.0 to 0.25 M while keeping the other ingredients the same.

Thus, the crude nuclear pellets that were obtained were suspended in 8-10 ml of the modified buffer B and homogenized in 40-ml Potter-Elvehjem tissue grinders. The suspensions were then mixed with the appropriate amount of Percoll (isotonic in 2.5 M sucrose) in Beckman 1.6 x 7.62 cm 10.4 ml polycarbonate bottle assemblies.

Talbot, K. and P. J. Russell. Nuclear density determination and the purification of wild type Neurospora crassa nuclei using Percoll gradients.