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Abstract

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

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.

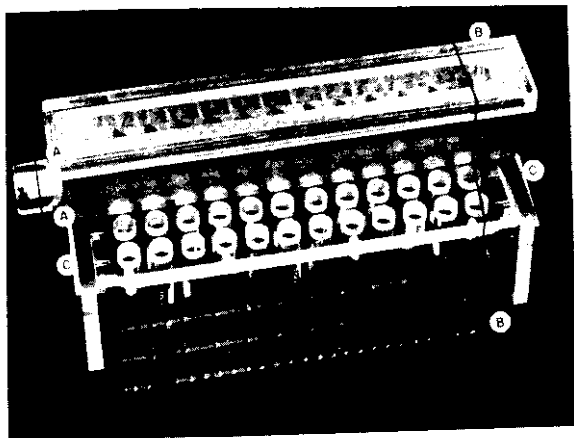


Figure 1. -- Solid reagent dispenser.

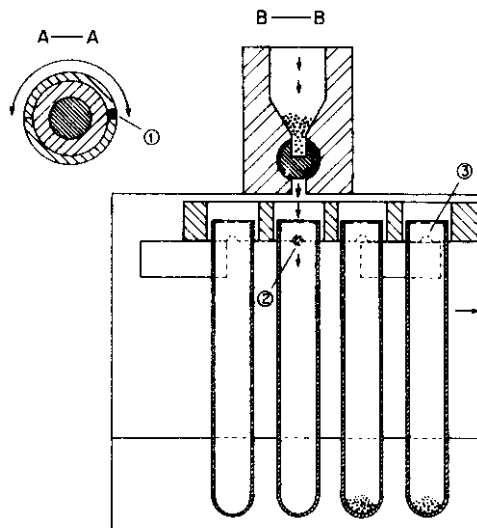


Figure 2. -- Sectioned detail drawings of the solid reagent dispenser.

Figure 2 contains sectioned detailed drawings of the apparatus. The cross section of the turning knob (Section AA-I) 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.

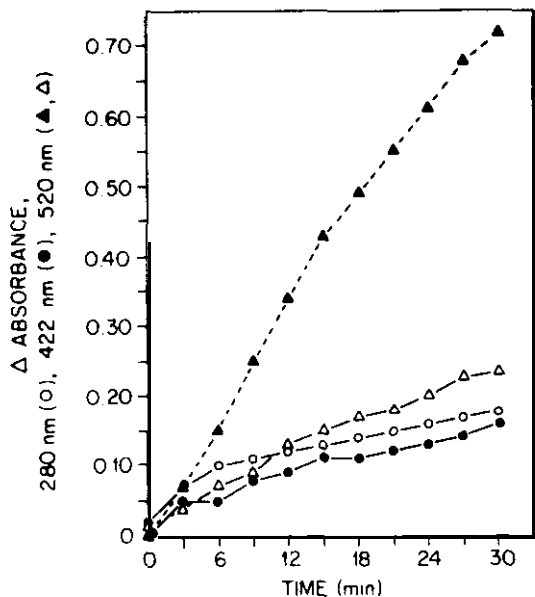


Figure 3. -- Comparison of three short-term protease assays. Hemoglobin, casein yellow, and azocoll are compared as substrates for trypsin over a short time interval. The hemoglobin (○) and casein (●) reaction mixtures each contained 1% substrate and 0.1% (1.0 mg/ml) trypsin. The azocoll reaction mixtures 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.

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 $\pm 4\%$). 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 PCM 76-80227 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.