A recipe for the crystallization of enzymes

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Abstract
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Aspartate transcarbamylase (ATCase) activity has been determined by a calorimetric assay for carbamyl aspartate (Davis and Woodward 1962 Genetics 47: 1075). The calorimetric assay can be adapted to ornithine transcarbamylase activity by assaying for citrulline. These same enzymes can be assayed by titration of hydrogen ion released in a pH-stat. The following equation describes the reaction in question:

\[
\text{NH}_2\text{CO}_2\text{PO}_3^- + \text{CO}_2\text{H}_2\text{CHNH}_2\text{COO}^- \rightarrow \text{CO}_2\text{H}_2\text{CHNHCOCH}_2\text{COO}^- + \text{HPO}_3^2^- + \text{H}^+. 
\]

Reagents: 0.005 M sulfamic acid (primary standard solution); 0.01 M NaOH, standardized against sulfamic acid; 0.05 M L-aspartate, standard solution; 0.0204 g dissolved in 25 ml cold water and kept in ice bath; H\text{}_2\text{O}, boiled and cooled to exclude CO\text{}_2; protein solution adjusted to pH 8.5 with NaOH.

Apparatus: Sargent pH-stat equipped as follows: 2.5 ml barrel and plunger; 10 ml reaction container, with stopper permitting entrance of electrodes, thermocouple, thermometer, NaOH delivery tube, nitrogen delivery tube, and reaction delivery syringe.

Procedure: The pH-stat is calibrated against standard pH 8.0 buffer at 30°C. The barrel is filled with 0.01 M NaOH and the system is flushed with nitrogen. Water, enzyme, and aspartate are introduced into the reaction vessel and the mixture is titrated to pH 8.5. The reaction is begun with the addition of carbamyl phosphate. At the end of the assay an aliquot of 0.005 M sulfamic acid is titrated under the conditions of the assay to standardize the NaOH. From this titration the number of micromoles of H\text{+} released during the assay can be calculated. Also, carbamyl phosphate can be assayed by letting the reaction go to completion, i.e., by permitting all the carbamyl phosphate to convert to carbamyl amino acid, and determining the number of micromoles of H\text{+} released.

Starting with 2.00 ml 0.05 M aspartate, 0.180 units of enzyme, H\text{2}O, 0.01 M NaOH, 0.50 ml 0.00425 M carbamyl phosphate in a total final volume of 8.5 ml, it was determined that the NaOH was 0.00947 M, and that the initial velocity of the reaction was 0.1843 micromoles H\text{+} per minute. Subtracting carbamyl phosphate hydrolysis, 0.0057 micromoles H\text{+} per minute, leaves an initial velocity of 0.1786 micromoles H\text{+} per minute. Aliquots of this reaction mix were assayed calorimetrically, and the initial velocity was shown to be 0.180 micromoles carbamyl aspartate per minute. This close agreement speaks for the validity of the pH-stat assay method at this pH and under these conditions.

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Jakoby, W. B. A recipe for the crystallization of enzymes. The crystallization of a protein is often a final step resulting in further purification and, hopefully, serving as one index of purity. Although confidence in equating such an esthetically appealing and ordered structure as a crystal with purity is often misplaced, more critical methods are available for that decision. More often the problem has been that of inducing crystallization.

During the past year we have enjoyed uniform success in attempting the crystallization of twelve enzymes by a technique of extracting and protein with ammonium sulfate solutions of decreasing concentration. No claim for originality is made since the method is derived from the early work of Warburg. Crystallization of an enzyme by this method was described by Pontremoli et al. (1961 Proc. Natl. Acad. Sci. U. S. 47: 1942) and several laboratories have used it successfully.

The routine which we have adopted for crystallization involves the complete precipitation of 10-20 mg protein with ammonium sulfate and the successive extraction of the resultant precipitate with 1 ml volumes of solutions of decreasing ammonium sulfate concentration; all such solutions are 0.05 M with respect to potassium phosphate at pH 7.0 although other buffers have been used also. All operations are conducted at or near 0°C. The precipitate is evenly suspended in the salt solution by triturating with a glass rod and then centrifuged for 10 minutes at 10,000 x g. The supernatant fluid is poured off and allowed to stand at room temperature while the residue is resuspended in a solution of lower salt concentration and the process is repeated. The first few salt concentrations are somewhat higher than that expected from the solubility of the protein. Thus, for a protein known to precipitate at an ammonium sulfate concentration of 30% to 40% of saturation, extraction is performed sequentially with solutions which have a concentration of 30%, 25%, 20%, 15%, and 10% of saturation. Crystallization often begins within an hour, occasionally within minutes, of the last centrifugation from the cooled vessel to one at room temperature. The crystals obtained in this manner are usually about 1 micrometer in their largest axis and are difficult to distinguish without the aid of a dark-field condenser and an oil immersion lens. With dark-field optics, the highly refractive outline of the crystals easily allows differentiation from possible amorphous material. Crystal shape is characteristic of the individual system. Electron microscopic studies of three proteins by Labaw, using the technique of shadowed carbon replicas, (1964 J. Ultrastruct. Res. 10: 66) has confirmed the crystallinity of the products. After one or two recrystallizations, carried out in the same manner, a
Klingmuller, W. and H. G. Truper. Determination of hexokinose and other enzymes which possibly phosphorylate fructose in Neurospora crassa.

by three different enzymes: hexokinase (ATP: D-hexose 6-phosphotransferase, E.C., No. 2.7.1.1), fructokinase (ATP: D-fructose 6-phosphotransferase, E.C., No. 2.7.1.4) and ketohexokinase (ATP: D-fructose 1-phosphotransferase, E.C., No. 2.7.1.3).

A method for measurement of kinases is that described by Sherman (1962 Analyt. Biochem. 5: 548) which takes advantage of the adsorption characteristics of phosphorylated and unphosphorylated sugars on ion exchange paper. Since this method does not permit differentiation between the three different kinases mentioned, it was discontinued after some preliminary investigation. A useful technique permitting such differentiation, at least to a certain extent, is the application of combined optical enzyme tests (B. D. Sanwal and H. G. Schlegel, personal communications). The use of these enzyme techniques for the above measurements of kinases in Neurospora crassa is described in this communication.

Reparation of crude extracts: Mycelia were grown in Fries-minimal solution with 1% filter-sterilized fructose (3 x 10^4 conidio of wild type 74-OR23-1A per 100 ml solution in 200 ml Erlenmeyer flask on a shaking machine at 25°C). Mycelia were harvested at 3-6 days, washed twice with distilled water, pressed slightly to remove excess water, and taken up in 0.05 M triethanolamine HCl/NaOH buffer of pH 7.6 (ca. 40 mg dry weight/ml buffer).

Good enzyme activities were obtained by grinding the mycelia with quartz sand in an ice bath, but minute pieces of sand and mortar debris, produced during the grinding process, remained in the crude extract after low speed centrifugation and interfered with subsequent optical measurements. High speed centrifugation is not feasible, since kinases have been reported in soluble and particle-bound forms (Medina and Sols 1956 Biochim. Biophys. Acta 19: 378). This complication was avoided, and good consistent results were obtained by disrupting mycelia by means of a Hughes press (Hughes 1951 Brit. J. Exp. Pathol. 32: 97) at -4°C. In this case low speed centrifugation (0°C, 4,000 x g, 30 min.) of the broken mycelial mass gave a clear, slightly opalescent supernatant, containing 10-20 mg protein/ml, depending on the buffer volume used.

Protein was measured by the biuret method, comparing the readings with a calibration curve obtained for bovine serum albumin. Since several shortcuts of the biuret method are in use, it should be mentioned that the protein of the samples to be measured has to be precipitated with 3.0 M trichloroacetic acid. Triethanolamine HCl/NaOH buffer, recommended for kinases and applied here, produces a strong blue color itself with the reagent and interferes with the measurements. If such care is taken, protein contents from 5-25 mg/ml can be measured with reasonable precision.

Enzyme measurements: Phosphorylation of fructose to fructose-6-phosphate was measured according to the Baehringer instructions for hexokinase (Gottschalk 1964 Arch. Mikrobiol. 49: 96) in the following coupled enzyme test:

\[
\text{fructose} + \text{ATP} \xrightarrow{\text{kinase}} \text{fructose-6-phosphate} + \text{ADP}.
\]

\[
\text{fructose-6-phosphate} \xrightarrow{\text{phosphoglucoisomerase}} \text{glucose-6-phosphate}.
\]

\[
\text{glucose-6-phosphate} + \text{NADP} \xrightarrow{\text{glucose-6-phosphate dehydrogenase}} 6\text{-phosphogluconate} + \text{NADPH}.
\]

The Eppendorf photometer connected to an automatic recorder was found useful for tracing the increase in adsorption of NADPH at 366 mp against time. By use of a cell holder with temperature control adjustment, connected to an ultrathermostat, it could be demonstrated that the reaction rate at 25°C is higher than that at 20°C, but equals that at 30°C. Crude extracts may be stored at 4°C for several days without loss of activity. Also storage of mycelia at -20°C does not influence the activity of crude extracts.

Three and six day old wild type mycelia exhibited activities of 114.0 ± 2.6 mmoles/min/g protein and 144.0 ± 8.8 mmoles/min/g protein, respectively. Two sorbose-resistant mutants (sor^R^Al and sor^R^BS^7^), mapping in separate linkage groups, gave similar results. Thus their resistance cannot be explained by an alteration of their hexokinase and/or fructokinase activities.

Reaction rates in crude extracts with fructose and glucose as substrates were equal. At first sight this seems to indicate that the hexokinase produced during growth of mycelia in fructose media has equal affinity for both hexoses, which is in contrast to other reports on hexokinase activity in glucose-grown mycelia (Medina and Nicholas 1957 Biochem. J. 66: 573; Sols et al. 1960 Biochem. Biophys. Res. Commun. 2: 126). It should be kept in mind, however, that in our case besides hexokinase a specific fructokinase could be at work as demonstrated in other organisms. The latter enzyme, together with a hexokinase of high glucose affinity and low fructose affinity, could well effect results as indicated. Only purification (and separation) of the respective enzyme(s) would permit a decision in favor of one or the other possibility. This task was not in the scope of our present research.