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Simple Spectrophotometric Assay for Calcium-Activated Neutral Proteases (Calpains)[1]

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MOSS, D. E., Y. R. GUTIERREZ, R. G. PEREZ AND H. KOBAYASHI. *Simple spectrophotometric assay for calcium-activated neutral proteases (calpains).* PHARMACOL BIOCHEM BEHAV **39**(2) 495–497, 1991.—A simple spectrophotometric assay for calcium-activated neutral proteases (calpains) is a simple modification of other popular spectrophotometric assays using casein substrate and precipitation by trichloroacetic acid (TCA). The assay presented here, however, uses azocasein as substrate, and activity is measured by the amount of azo chromophore that remains in solution after TCA precipitation. Because the chromophore is specific to the substrate and the azo substrate absorbs strongly, this assay provides improved spectrophotometric performance in a practical and sensitive procedure.

Calcium-activated neutral proteases Assay method Calpain Thiol proteases

CALCIUM-ACTIVATED neutral proteases (EC 3.4.22.17) have been shown to be virtually ubiquitous in a wide variety of tissues. These enzymes have been known in brain tissue for decades (2). In view of the potential interest in calpain in behavioral pharmacology, a simple spectrophotometric method of assaying enzyme activity was developed. The method uses azocasein as a substrate and is, therefore, a simple modification of an older general assay for proteolytic activity (1).

Azoprotein substrates are made by coupling diazotized aryl amines with proteins in alkaline solution to yield chromophoric protein derivatives. Spectrophotometric characteristics of azoproteins are well understood (5). Proteolytic activity such as that produced by calpain results in the formation of colored protein fragments that are soluble in trichloroacetic acid (TCA). The intensity of the color in the supernatant from TCA precipitation is a function of the proteolytic activity of the enzyme solution.

The method described in this paper uses the simplicity of a spectrophotometric assay with azocasein substrate. This produced two main advantages. One is that the color detected spectrophotometrically is specific to the substrate added to the assay (i.e., low background and no confounding from TCA-soluble proteins in the tissue sample being assayed). This is important for assaying unpurified samples. Secondly, the assay produces high levels of color without improving detection by reacting the residual components from proteins with chromophores. Azocasein substrate is inexpensive and commercially available. The method is simple, reliable, and sensitive.

EXPERIMENT 1

The purpose of Experiment 1 was to develop a method using azocasein substrate that would be a useful and simple spectrophotometric assay of unpurified calpain in brain tissue. This would be useful in studying the effects of endogenous inhibitors, ions, or interactions with other factors present under near in vivo conditions.

METHOD

Solutions

Rat and rabbit brains were homogenized on ice as 20% w/v in an homogenizing buffer containing 20 mM Tris HCl [*tris*-(hydroxymethyl) aminomethane, HCl] (Calbiochem, La Jolla, CA), 1 mM EDTA (ethylenediaminetetraacetic acid, free acid) (Sigma Chemical, St. Louis, MO), 5 mM 2-mercaptoethanol (2-ME) (Sigma), 15 mM cysteine HCl (Sigma), and 0.25 M sucrose (Sigma), pH 7.4 (2,4). Assay buffer was 50 mM Tris HCl, pH 7.4, containing 15 mM 2-ME.

Azocasein substrate stock solutions were made by placing 40 mg/ml azocasein (sulfanilamine-azocasein, $E^{1\%}_{440} = 36$) (Sigma) into deionized water with gentle heating and stirring. When the material was in solution, 0.005 g/ml sodium bicarbonate was added followed by sufficient assay buffer, pH 7.4, to make a final substrate stock of 20 mg/ml, about pH 7.7.

The degree to which the aromatic diazonium compounds have been reacted with the protein tyrosyl, histidyl, or possibly lysine residues (5) may vary with different suppliers. Because of this variability, standardization can be accomplished by reporting the absorbance of a 1% solution at 440 nm ($E^{1\%}_{440}$).

Calpain has an absolute requirement of calcium for activity. Therefore, calcium chloride (30 mM) was used in a stock solution so that calcium could be added as required to the assay.

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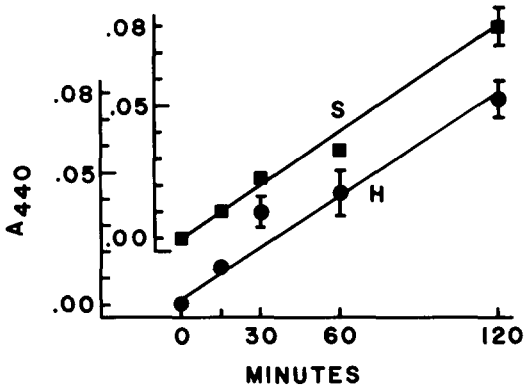

FIG. 1. Linearity of reaction with time. Line H is rat brain homogenate assayed at 25°, 2 mg/ml azocasein, 3 mM calcium. Note that more than standard amounts of enzyme were included to produce higher levels of activity for accurate measurement at shorter time intervals. Line S (offset Y axis) is rat brain supernatant assayed at 37°, 2 mg/ml azocasein, 3 mM calcium. Because of autoactivation and autodestruction, the reactions may not be linear under other experimental conditions. Error bars, if extending outside the size of the symbols, indicate standard error of the mean of the triplicate. Once the spectrophotometer is zeroed on the control (no calcium during incubation) at the beginning of the reaction, the controls at other time intervals (not shown in the figure) averaged 0.0028 (±0.0013 SEM) from that baseline.

Assay Procedure

Reactions prepared in triplicate included 1.0 ml of 50 mM Tris assay buffer containing 15 mM 2-ME. If cations other than calcium, inhibitors, or other test compounds were to be included in the assay, those solutions were made in assay buffer, and this volume was adjusted to keep the total reaction volume at 2.0 ml. Secondly, 0.2 ml of azocasein or various combinations of azocasein and buffer totaling 0.2 ml were added as required by each experiment. To the above solution, 0.2 ml of calcium chloride was added. The reaction was started by the addition of 0.6 ml of brain homogenate. The reaction volume was usually a total of 2.0 ml.

The reactions were typically run for 30 min, 1 h, or 2 h with continuous gentle inversions in a water bath at 25°. The reactions were linear for at least 2 h (Fig. 1). At the end of the reaction, the tubes were removed from the water bath and placed on ice. If calcium and/or azocasein concentrations were different in various reaction tubes because they were variables in the experiments, the reaction solutions were all brought to the same calcium and azocasein concentrations by the addition of a constant volume to each tube. Immediately following, 0.1 ml of 15% TCA was added to each tube. The tubes were placed in a freezer (−17°) for 5 minutes and then moved to a refrigerator (4°) for 15 minutes to facilitate TCA precipitation, and centrifuged for 10 min at about 5000 × g. The supernatant was pipetted off, and 0.9 ml of 0.5 M NaOH was added to maximize the absorbance of the azo chromophore at a high pH. The resulting solutions of NaOH with reaction supernatants were read at 440 nm relative to controls incubated without calcium. The color produced developed very quickly after the addition of NaOH and was stable at least overnight. There was no color change without calcium.

All reaction tubes must contain the same calcium/azocasein concentrations before the addition of TCA. The presence of calcium clearly affects the degree to which TCA will precipitate azocasein from the solutions.

Insofar as calpain is regulated by a divalent cation (calcium) and the regulation/interference/activation by other cations may be of experimental interest, we ran some exploratory experiments with magnesium included in the reaction. This caused a special problem in that the presence of magnesium caused a fine precipitate to form when 0.5 M NaOH was added at the end of the experiment. This interfered with spectrophotometric readings. This problem was readily avoided by the addition of 0.1 ml of a stock solution of 200 mM EDTA made in 0.5 M NaOH to the supernatant before the addition of the remaining 0.8 ml NaOH.

Determination of K_m and V_{max} With Regard to Azocasein and the Requirement for Calcium

Brain calpains, as most tissues, include a mixture of both calpain I (high sensitivity to calcium) and calpain II (low sensitivity to calcium) and the endogenous inhibitor calpastatin (2,3). In these experiments designed to develop an assay method, no attempt was made to separate calpain I from calpain II and, furthermore, to separate these enzymes from calpastatin.

Homogenates of rabbit and rat brain were prepared as described above. The first experiments were conducted to study the behavior of the enzymes against azocasein substrate in concentrations from 0.2 to 2.0 mg/ml. All assays were conducted at 3.33 mM calcium, which is sufficient for complete activation of both calpain I and calpain II (2,4).

RESULTS

The assays were absolutely dependent upon calcium, as expected, and maximum activation was obtained at 3.33 mM. All enzyme activity was found in the supernatant.

Assays of the unpurified supernatant followed Michaelis kinetics as shown by strict linearity in double reciprocal plots. Rat brain showed a K_m of 0.75 mg/ml azocasein and a V_{max} of 0.064 OD_{440}/2 h ($r = +.996$). Rabbit brain showed a K_m of 0.27 mg/ml azocasein and a V_{max} of 0.036 OD_{440}/2 h ($r = +.999$).

EXPERIMENT 2

The purpose of Experiment 2 was to determine if the activity observed in Experiment 1 could be attributed to a specific enzyme that could be identified as calpain. In this experiment, enzyme activity was purified by molecular sieve gel chromatography followed by ion exchange chromatography.

METHOD

Frozen rabbit brain was thawed and homogenized 1:2 w/v in the Tris/sucrose buffer described in Experiment 1. The homogenate was centrifuged at 48,000 × g for 1 h at 0°. The supernatant was added to a G200 column (Pharmacia K50/60) and eluted with a buffer composed of 20 mM Tris, 1 mM EDTA, and 5 mM 2-ME. Fractions 24 through 31 showed activity (Fig. 2), and they were added to a DEAE column (Pharmacia K15), washed, and eluted with 400 ml of NaCl in a linear gradient.

RESULTS

The results of Experiment 2 are shown in Figs. 2 and 3. These results show that enzyme activity appears to follow specific protein(s). The proteins showing activity are consistent with similar methods of chromatographic separation of CANP from rat brain conducted by Murachi et al. (4).

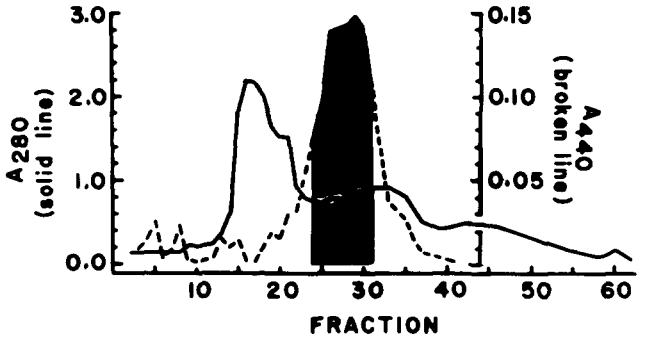

FIG. 2. Elution of proteins from the G200 column. Calpain activity (shaded area) from fractions 24–31 were pooled and added to the DEAE column.

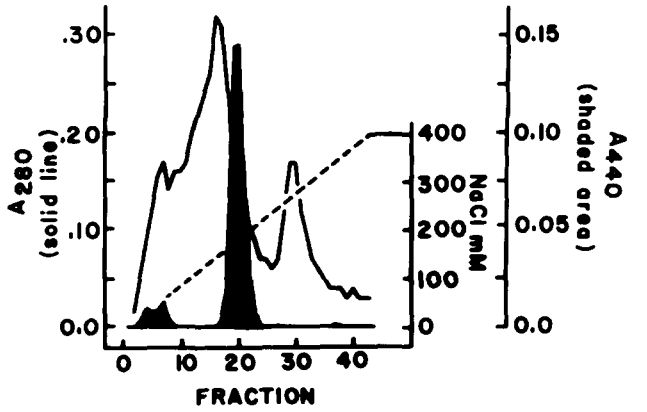

FIG. 3. Elution of proteins from the DEAE column by a linear gradient of NaCl up to 400 mM. Calpain activity in the fractions is shown by shaded area.

CONCLUSIONS

The simple spectrophotometric method reported here has some advantages over existing methods. The chromophore is specific to the substrate, the method provides a sensitive measure of enzyme activity, the procedure requires only common spectrophotometric equipment and a commercially available substrate, and there are very few steps required to complete the procedure.

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