A Micromethod for Determination of Proteolytic Enzymes in the pH Range of 2.8 to 4.8

Radial Enzyme Diffusion into Skim Milk-Containing Agarose Gel

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Radial diffusion of enzymes into a casein-containing gel is a fast and inexpensive technique for determination of proteolytic enzymes. A simple method for the preparation of substrate plates with a homogeneous distribution of caseinate and buffered at selected pH values between 2.8 and 4.8 has been described in this communication. By determination of pepsin in caseinate gels the accuracy was better than that described for photometric pepsin assays, and the sensitivity was 300 times greater.

A sensitive and highly reproducible method for determination of milk-clotting enzymes above pH 5.2 has been described by Lawrence and Sanderson (1). The method is based on radial diffusion of enzymes into a thin layer of agarose gel containing purified calcium caseinate. During investigations into the activity and the pH-profile of proteolytic enzymes, we found that the method of Lawrence and Sanderson could be modified to cover also the pH range of 2.8 to 4.8. Furthermore, the method could be simplified by using ordinary skim milk instead of purified calcium caseinate in the gel. In the present paper, porcine pepsin has been used for the demonstration of the technique as it is representative for a group of enzymes where time consuming photometric methods (2–4) can advantageously be substituted by the simple and inexpensive radial diffusion method.

MATERIALS AND METHODS

Pepsin. Porcine pepsin, twice crystallized, was obtained from Worthington Biochemical Corporation, Freehold, N.Y.

Agarose gel. One gram of agarose (Litex, Glostrup, Denmark) was dissolved in 100 ml of boiling water. The gel was kept above 55°C in a thermostated waterbath.

Skim milk. One gram of skim milk powder was reconstituted by gradually adding 30 ml of sodium acetate buffer, pH 5.5, ionic strength 0.10.
Buffers for equilibration of the pH in the substrate plates. Sodium acetate and sodium formate buffers in the pH range 2.8 to 4.8 were used. The ionic strength was kept at 0.10. To all buffers sodium azide was added to a concentration of 0.015M.

Preparation of the agarose–substrate plates. A glass plate, 10 × 10 × 0.1 cm, was coated with a thin film of agarose gel by use of a gel-moistened paperwipe. The plate was allowed to dry. The coating helped the substrate-containing gel to adhere to the plate. Agarose gel (13.5 ml) at 55°C was mixed with 1.5 ml of reconstituted skim milk and poured onto the leveled glass plate. After solidification the substrate plate was placed in a plexiholder, and the pH in the gel was adjusted to a selected value by incubation for 2 hr in 100 ml of the appropriate acetate or formate buffer. During the acidification, calcium caseinate was precipitated homogeneously in the gel. The substrate plates can be stored at 4°C in a moist chamber for several months.

Application and incubation of samples. Wells 2.5 mm in diameter were punched out in the substrate gel using a puncher connected to a water suction device (5). Five microliters of standards and samples were applied to the wells with a double constriction pipet. Incubation took place in a moist chamber at a selected temperature between 4 and 45°C.

Transparent circular zones appeared in the dense white gel where the calcium caseinate had been digested by the enzyme. The diameters of the transparent zones could be measured directly. Incubation times of 2 to 72 hr were used.

Staining of plates. The sensitivity and accuracy of the method were increased when the unhydrolyzed substrate was stained with a protein stain. This was also an easy way of stopping the reaction, and the stained plates can be stored indefinitely for documentation purposes. Staining was performed as follows: The wells were filled with water and the plate was covered with a 10 × 10 cm piece of wet filter paper. A 2 cm thick layer of soft cellulose tissue or eight layers of filter paper were placed on the plate, and a pressure of about 10 g/cm² was maintained for 10–15 min using a thick glass plate. By this procedure the gel was effectively squeezed, and the liquid phase of the gel containing enzyme and hydrolyzed substrate was eliminated. The filter paper was carefully removed, and the plate was dried under a stream of hot air. Staining was performed for 5 min in a solution of Coomassie Brilliant Blue R (Microme 1137, Edward Gurr Ltd., London), or Amido Black as described by Weeke (5).

RESULTS

Eight different samples containing from 0.100 to 10 μg of pepsin were applied six times each to substrate plates which had been equilibrated in an acetate buffer of pH 4.00 and ionic strength 0.10. After incubation of 20 hr
at 37°C, the plates were stained. The zones of proteolysis appeared on the dark background as clear circular areas with well-defined borders (see Fig. 1).

The mathematical relationship between the amount of enzyme applied to the wells in the substrate plates \((x, \text{ ng})\) and the diameter of the hydrolysis zone \((y, \text{ mm})\) was investigated. The following combinations: \(x \text{ versus } y\), \(x \text{ versus } y^2\), \(\log x \text{ versus } y^2\), and \(\log x \text{ versus } y\) were tested. The best fit to a straight line was found when \(y\) was plotted against \(\log x\). The coefficient of correlation was 0.9971 and the standard error of the slope 1.29%.

A linear relationship between the diameter of the zone of hydrolysis and the logarithm of the amount of enzyme was observed in the whole pH range of 2.8–4.8, at temperatures from 20–55°C, and from 30 ng to 10 μg of enzyme. Ten micrograms was the upper limit investigated. Below 30 ng linearity was no longer observed, but 10 ng of pepsin still produced well-defined zones of hydrolysis.

The experimental error inside the same plate was determined by applying 0.250 μg of pepsin to each of 12 different wells. When the enzyme reaction was stopped, the mean diameter of the zones of hydrolysis was 8.80 mm. The standard deviation was ±0.085 mm, corresponding to 0.97%. Inside the same plate, the standard deviation on the diameter of the zones of hydrolysis produced by 10 ng of pepsin was 1.14% (eight determinations).

It was found that equilibration of the pH in the substrate plates by immersion in buffer could only be done in the pH range of 2.8–4.8 where calcium caseinate is insoluble. Experiments showed that at least 2 hr of incubation was necessary for equilibration of the gel with respect to pH and ionic strength.

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**Fig. 1.** Proteolytic activity of pepsin determined in skim milk-containing agarose gel. Samples, from the left to the right, were 2.50, 1.67, 0.250, and .167 μg pepsin. Experimental conditions: sodium acetate buffer, pH 4, ionic strength 0.10. Incubation time; 20 hr at 37°C.
**DISCUSSION**

Radial diffusion into substrate-containing agarose is a well known principle for determination of enzymes (1,6,7). For proteolytic enzymes, casein is an inexpensive and easily available substrate. It is well suited for the radial diffusion technique as partial hydrolysis caused by milk-clotting enzymes appears as opaque precipitates (1), while more extensive hydrolysis is seen as clear zones on a whitish background. For enzyme assays at pH values above 5, the substrate plates can be prepared by mixing skim milk, or purified casein, directly into the warm buffered agarose before the gel is cast (1). This cannot be done in the pH range of 4.8 to 2.8 because the glycoside bondings in the agarose begin to hydrolyze in hot solutions of pH below 4, and because calcium caseinate is insoluble in this pH range, and a homogeneous distribution of caseinate in the gel is therefore difficult to obtain. However, by the technique described here, where substrate plates containing skim milk are prepared at pH 5.5 and the caseinate is then precipitated in situ in the gel by immersion in an appropriate buffer at room temperature, these problems are overcome.

Pepsin determinations are frequently performed by absorption photometry where the digestion of denatured hemoglobin or a synthetic substrate is measured (2,3). The sensitivity is about 3 μg pepsin in a 1 ml specimen, and the experimental error has been reported to be ±8% (4).

Compared with the photometric methods, pepsin determination by the radial diffusion technique has several advantages: Simple and inexpensive
equipment is used, the sensitivity is 300 times greater, the experimental error is about ±1%, and only a few microliters of sample is necessary. Many samples can be assayed with a minimal investment of time, and on the same substrate plate enzyme amounts differing by a factor 10³ can be quantitated. Furthermore, the stained plates can be stored for re-investigation or documentation purposes. Simply by varying parameters like caseinate concentration, incubation time, and incubation temperature, a still wider range of pepsin concentrations than those described in this paper can be covered. We have found the radial diffusion method useful for following enzymatic activity in effluents from column chromatography, and for comparing the activity and pH profile of chemically modified enzymes with the native enzymes. Also, for investigation of proteolytic enzymes in tissue homogenates and in viscous biological fluids, the method might be advantageous.

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REFERENCES