# Modified Milk-Clotting Procedure for Determination of Papain Activity

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A technically simple and rapid method for estimating enzyme activity was desired for investigation of the chromatographic behavior of papain and chymopapain on ion exchange columns. A modified milk-clotting procedure, using cysteine as the activator, gives a linear relationship between inverse time of clotting and enzyme concentration over a fifteenfold range of enzyme concentration. Ammonium sulfate and sodium chloride inhibit enzyme activity and determination of activity in their presence necessitates use of correction factors. The method is rapid and precise (2%) and suitable for routine determinations of enzyme activity in both absence and presence of inhibitory salts.

In a study of the ion exchange fractionation and purification of the proteases, papain and chymopapain, of commercial papain (*4*), a technically simple and rapid method for estimating enzyme activities was needed.

For this purpose the milk-clotting procedure of Balls and Hoover (*1*) was modified by a reduction in volume and utilization of cysteine for activation. In addition, inhibitory effects of the salts, sodium chloride and ammonium sulfate, used in fractionation and precipitation experiments were studied. These effects were found to be independent of enzyme concentration, over the range studied, but dependent on salt concentration. Accordingly, correction factors for inhibition by the salts in question were calculated and used in routine analyses.

Thioglycolic acid may also be used as an activator. However, maximal activation by this activator is decidedly less than that given by cysteine and time of activation is critical. Accordingly, cysteine was utilized routinely.

### Reagents

Stock acetate buffer, pH 4.6.

Acetate buffer for use, a 1 to 10 dilution of the stock.

Milk powder suspension [buffer and milk prepared according to Balls and Hoover (*1*)], stored at 3° C. under toluene when not in use and not used for more than 4 days.

Neutral cysteine solution, 4%, 0.25*M*. C.P. cysteine hydrochloride dissolved in water, neutralized with the equivalent amount of solid sodium bicarbonate, and diluted to volume. This solution was not used for more than 8 hours. If oxidation became pronounced in less than 8 hours (precipitation of cysteine), the solution was discarded and made anew.

Enzyme solutions. A 1% aqueous suspension of commercial papain (inactive in absence of activators) was filtered and the clear filtrate was diluted to give the needed enzyme concentrations. The sample of commercial papain used contained both papain and chymopapain. However, as papain and chymopapain exhibit the same milk-clotting activity (*6*), the simultaneous presence of both enzymes does not affect standardization.

### Procedure

All determinations were carried out at 40° C. in a water bath. Into a 13 × 95 mm. test tube, 0.5 ml. of diluted buffer, 0.3 ml. of water, 0.2 ml. of neutral cysteine solution, and 0.2 ml. of enzyme solution were pipetted in succession. After 10 minutes had been allowed for activation and temperature equilibration, 2.0 ml. of milk suspension at 40° was rapidly added by being blown out from a 2-ml. wide-tipped pipet. Simultaneously, an electric stopclock was started. The tube was then shaken in the bath sufficiently to give a film of the test suspension on the upper sides of the tube and the film was allowed to drain. This operation was repeated until clotting was noted—i.e., separation of small milk curds in the falling film rather than the rapidly ensuing complete clotting. At this point the stopclock was stopped and the time in seconds noted. A bright lamp placed externally at right angles to the tube in the bath helps observation of the end point.

The pH of the test system, checked after clotting, must be in the range of 5.3 to 5.7. If the buffer and milk suspension do not have sufficient capacity to maintain this pH range on addition of the sample, the pH of the sample must be adjusted to this range.

The unit of enzyme activity utilized in this work was defined by $1000 \times 1/(t - c)$, where $t$ is equal to time of clotting in seconds when measured by the above system and $c$ is a constant determined for the system, as shown under results.

For testing the effect of various substances, up to 0.3 ml. of water was replaced by an equal volume of test solution, unless otherwise noted.

### Results

**Cysteine Required for Complete Activation.** Figure 1 presents a plot of the data obtained by determining clotting times as a function of cysteine concentration over the range $2.25 \times 10^{-5}M$ to $3.97 \times 10^{-2}M$ in the presence of a constant amount of enzyme. The data indicate a leveling off in activation at approximately $0.8 \times 10^{-2}M$. For the routine procedure about twice this cysteine concentration, $1.588 \times 10^{-2}M$, was used.

Figure 1 also shows the effect of varying concentration of thioglycolic acid on clotting times. Activation with thioglycolic acid begins to level off at approximately $0.6 \times 10^{-2}M$. However, maximum activation observed with thioglycolic acid is not quite two thirds of that observed with cysteine. Similar results have been commented on (*3*).

Experiments were also performed to determine the time required for maximum activation (Table I). With cysteine, time of activation was not critical from 3 to 25 minutes. Accordingly, the 10-minute activation period was chosen to permit rapid successive determinations. With thioglycolic acid there was a marked decrease in enzyme activity with increasing time of activation. For this reason zero activation times were used to obtain the thioglycolic acid data of Figure 1.

**Table I. Clotting Time as a Function of Time of Activation**

| Cysteine[a] | | Thioglycolic Acid[b] | |
|---|---|---|---|
| Activation time, min. | Clotting time, sec. | Activation time, min. | Clotting time, sec. |
| 1 | 72, 70 | 0 | 267 |
| 3 | 68, 68 | 3 | 277 |
| 5 | 68, 68 | 5 | 317 |
| 7 | 67, 67 | 7 | 371 |
| 10 | 68, 70 | 10 | 425 |
| 20 | 68, 69 | 15 | 520 |
| 25 | 68, 69 | 25 | 840 |

[a] Regular test system, 0.2 ml. of 1% enzyme solution.
[b] Regular test system, 0.2 ml. of 5× diluted 1% enzyme solution, $8.72 \times 10^{-2}$ millimole of sodium thioglycolate per tube.

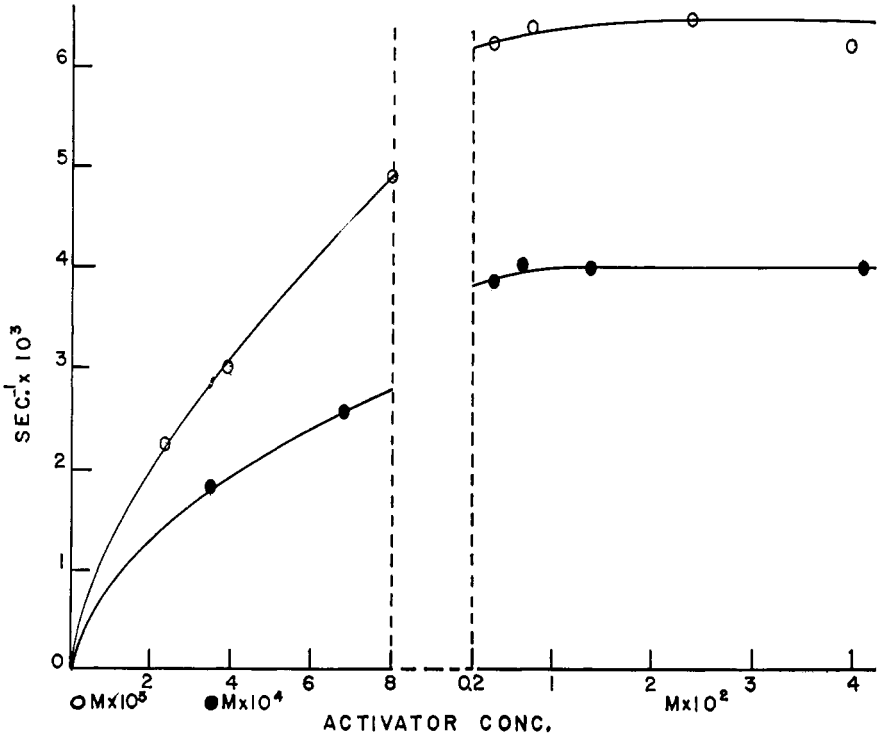



Figure 1. Reciprocal of clotting time as function of activator concentration

Regular test system employed in both cases, 1% enzyme solution being diluted 5 times

○ Cysteine run, sodium chloride concentration kept constant at $4 \times 10^{-2}$ M

● Thioglycolic acid run, solution neutralized with sodium bicarbonate prior to use

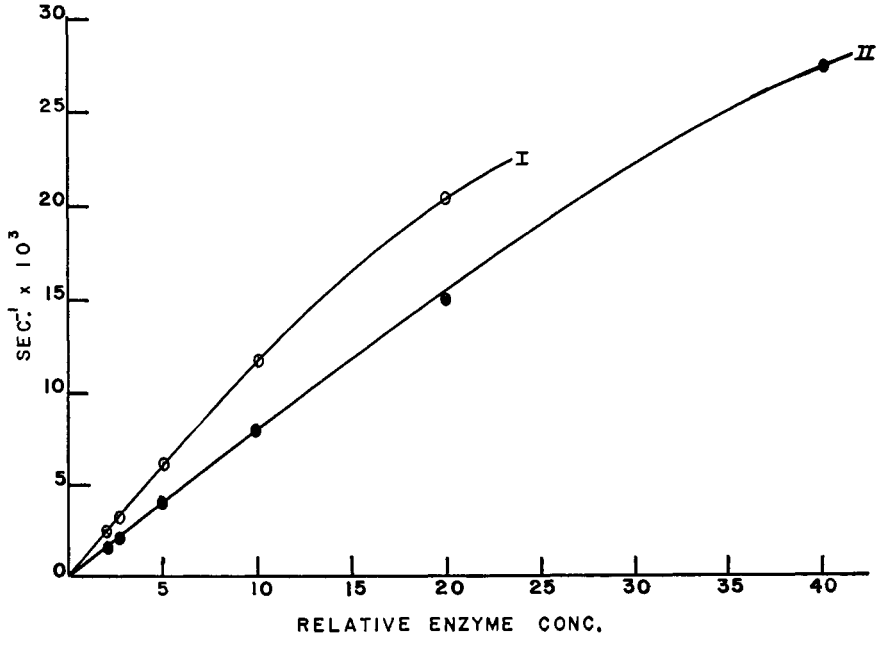



Figure 2. Reciprocal of clotting time as function of enzyme concentration

I. Regular test system
II. Regular test system, but 3 ml. of milk suspension employed

Relative enzyme concentration of 40 represents 0.4 ml. of 1% enzyme (partially purified chymopapain) solution

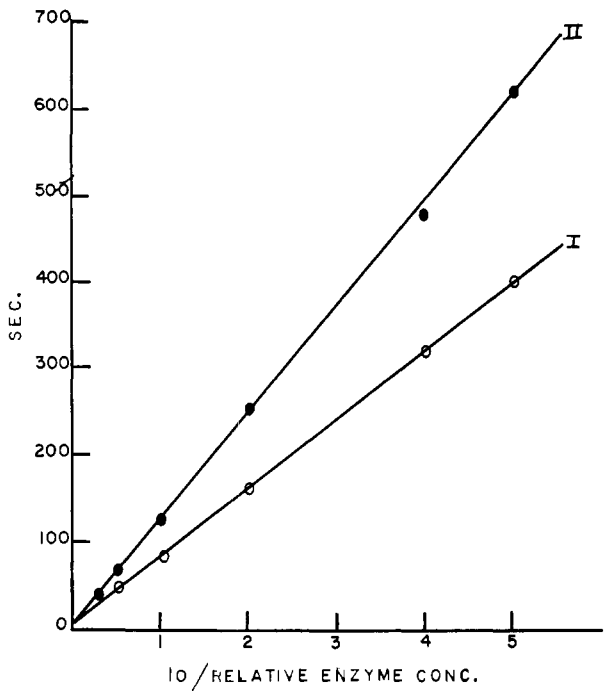
**Linearity of Response to Enzyme Concentration.** If, at constant substrate concentration, the time required for clotting is considered to denote a constant change in the substrate, the reciprocal of the time may be used as a measure of velocity and, if the rate law governing the process does not change with enzyme concentration (*2*), reciprocal time may also be used to investigate the nature of the relationship between enzyme concentration and velocity of reaction.

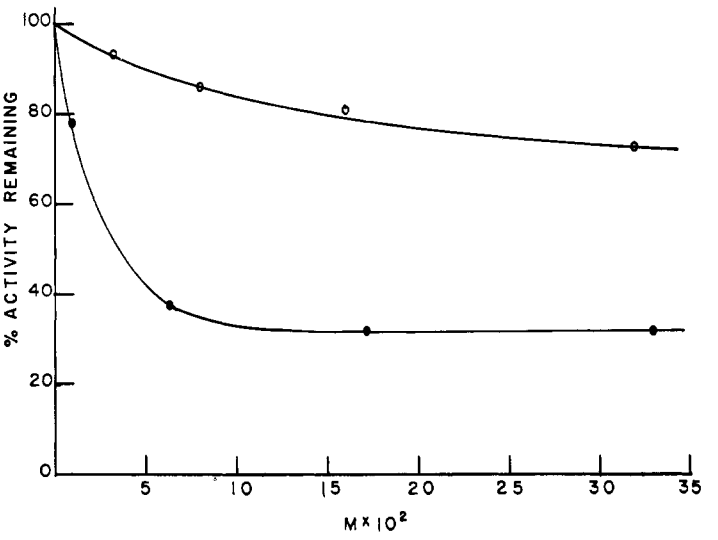
On this basis, curves of velocity *vs.* enzyme concentration are presented in Figure 2. At the higher enzyme concentrations velocities fall off for both the routine substrate concentration (curve I) and the somewhat higher substrate concentration employed in curve II.

Figure 3 presents plots of time of clotting (1/velocity) *vs.* 1/enzyme concentration, derived from the above experiments. Straight lines result, which are similar to Lineweaver-Burk plots (*7*), except that the substrate variable has been replaced by the enzyme variable. This linear relationship suggests that the system may be treated as a Michaelis-Menten zone A system (*5*, *8*) with substrate concentration being held constant and enzyme concentration being varied—viz.

$$\frac{(S_0 - ES)(E_0)}{(ES)} = K_s \qquad (1)$$

where $S_0$ = concentration of added substrate, $E_0$ = concentration of added enzyme, and $ES$ = concentration of enzyme substrate complex. As the velocity, $v$, is equal to $K_3(ES)$ and the maximum velocity, $V$, in this case is equal to $k_3(S_0)$, Equation 1 gives on substitution and rearranging




▲ Figure 4. Per cent activity remaining as function of concentration of inhibiting salts

○ Sodium chloride ● Ammonium sulfate

◀ Figure 3. Clotting times (1/velocity) *vs.* reciprocal of relative enzyme concentration

Curves correspond to those of Figure 2

$$v = \frac{VE_0}{K_s + E_0} \qquad (2)$$

and taking reciprocals

$$\frac{1}{v} = \frac{1}{V} + \frac{K_s}{VE_0} \qquad (3)$$

The assumption that the system is in the zone A conditions, in this case $S_0/K_s \leqq 0.1$ and $E_0 - ES \cong E_0$, could not be tested (*5, 8*) because the data required, based on clotting times of 18 seconds and less to give $v/V$ values $\geqq 0.5$, could not be obtained with any accuracy.

$S_0$ might be more strictly defined as substrate available to enzyme or as substrate participating in the over-all reaction. This would take into account interaction of the clottable proteins with other components of the system—i.e., calcium ions. However, the constancy of the $S_0$ term in the above treatment is presumably not affected in this work, since only enzyme concentration was varied. Equation 3 can also be written as

$$E = \frac{k}{t - c} \qquad (4)$$

where $t$ is time of clotting in seconds, $c$ is $1/V$ in seconds, and $k$ is $K_s/V$ and is arbitrarily set at 1000 seconds. The enzyme unit is then defined as that quantity of enzyme which under the conditions of the routine assay system gives a $t - c$ value of 1000 seconds. Were the absolute enzyme concentration of the calibrating solutions known, $k$ would be set equal to moles $\times$ liter$^{-1}$ $\times$ seconds and unknown enzyme concentrations would be expressed as moles $\times$ liter$^{-1}$.

For curve I in Figure 3, $c$ is equal to 10 seconds (9.6 seconds by the method of least squares) and Table II presents the units per milliliter of a 1% enzyme solution found at different dilutions.

Enzyme concentrations giving clotting times below 40 and above 600 seconds were not employed in this work. Higher substrate concentrations than those used in the routine system may be employed to extend the useful range to higher enzyme concentrations.

In one experiment with the routine system, the concentration of cysteine was increased $2^1/_2$ times. This led to a decrease in clotting times and a 15% increase in units when calculated by the above formula, using the value of $c$ obtained from the intercept of the curve of clotting time *vs.* $1/E_0$ for this experiment. However, as this would require considerable quantities (20 mg. per tube) of cysteine hydrochloride for routine runs, the lower concentration was employed.

**Effect of Sodium Chloride and Ammonium Sulfate.** In this series of experiments, the effect of the salts used for elution and precipitation experiments was studied.

Table III presents data for the inhibitory effect of sodium chloride (0.00 to 0.32*M*) on enzyme activity over a fourfold range of enzyme concentration. Observed inhibitions are relatively independent of enzyme concentration. This suggests that the inhibition is due

**Table II. Units per Milliliter as a Function of Dilution**

| Dilution[a] | $t$ | $t - c$ | $\frac{1000}{t - c}$ | $\frac{\text{Units}}{\text{Ml.}} = \frac{1000}{t - c} \times \text{diln.}$[b] |
|---|---|---|---|---|
| 5 | 49 | 39 | 25.6 | 128 |
| | 49 | 39 | 25.6 | 128 |
| 10 | 86 | 76 | 13.2 | 133 |
| | 85 | 75 | 13.3 | 132 |
| 20 | 167 | 157 | 6.37 | 128 |
| | 162 | 152 | 6.57 | 132 |
| 40 | 322 | 312 | 3.21 | 128 |
| | 317 | 307 | 3.26 | 130 |
| 50 | 398 | 388 | 2.58 | 129 |
| | 402 | 392 | 2.56 | 128 |

[a] In assay tube of 1% enzyme solution.
[b] Mean = 129.6; av. deviation ± 1.3%.

**Table III. Inhibitory Effect of Sodium Chloride[a]**

| NaCl, M | Units | % Activity Remaining | Units | % Activity Remaining | Units | % Activity Remaining |
|---|---|---|---|---|---|---|
| 0 | 19.8 | 100 | 10.0 | 100 | 5.02 | 100 |
| 0.031 | 17.9 | 90.5 | 9.39 | 93.9 | 4.67 | 93.0 |
| 0.079 | 16.7 | 84.3 | 8.70 | 87.0 | 4.43 | 88.3 |
| 0.16 | 15.3 | 77.2 | 8.10 | 81.0 | 4.09 | 81.5 |
| 0.32 | 13.7 | 69.1 | 7.20 | 72.0 | 3.70 | 73.6 |

[a] Regular test system.

**Table IV. Inhibitory Effect of Ammonium Sulfate[a]**

| $(NH_4)_2SO_4$, M | Units | % Activity Remaining | Units | % Activity Remaining | Units | % Activity Remaining |
|---|---|---|---|---|---|---|
| 0 | 17.5 | 100 | 11.8 | 100 | 5.88 | 100 |
| 0.0095 | 13.5 | 77.1 | 9.90 | 83.9 | 4.38 | 74.6 |
| 0.062 | 6.90 | 39.4 | 3.89 | 33.0 | 2.53 | 43.0 |
| 0.17 | 5.40 | 30.8 | 3.65 | 30.9 | 1.85 | 31.5 |
| 0.33 | 5.50 | 31.4 | 3.70 | 31.4 | | |

[a] Regular test system.

to an alteration of the clottable proteins, probably by binding of chloride anions, and that the enzyme does not compete with the inhibiting agent for the substrate.

Table IV presents data for the inhibitory effect of ammonium sulfate. It can be seen that ammonium sulfate exerts its inhibitory effect in a manner similar to that of sodium chloride and that this inhibition is approximately twice that due to the sodium chloride.

Figure 4 presents a plot of the data of Tables III and IV, where the per cent remaining activity (average) is plotted against concentrations of the inhibiting salts. In carrying out routine analyses in the presence of known concentrations of the above inhibitory salts, units obtained were corrected for inhibition by multiplying by the factor, 100/% activity, obtained from the plots.

No attempt was made to investigate either the influence of the inhibitory salts on the value of the constant, $c$, or the possibility of preferential inhibition of one of the enzymes by a given salt.

### Literature Cited


(1) Balls, A. K., Hoover, S. R., *J. Biol. Chem.* **121,** 737 (1937).
(2) Bodansky, O., *Ibid.*, **120,** 555 (1937).
(3) Gawron, O., Cheslock, K. E., *Arch. Biochem. Biophys.* **34,** 38 (1951).
(4) Gawron, O., Draus, F., Division of Biological Chemistry, 122nd Meeting, ACS, Atlantic City, 1952.
(5) Goldstein, A., *J. Gen. Physiol.* **27,** 529 (1944).
(6) Jansen, E. F., Balls, A. K., *J. Biol. Chem.* **137,** 459 (1941).
(7) Lineweaver, H., Burk, D., *J. Am. Chem. Soc.* **56,** 658 (1934).
(8) Straus, O. H., Goldstein, A., *J. Gen. Physiol.* **26,** 559 (1943).



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**MEAT PIGMENTS**

# Factors Affecting the Oxidation of Nitric Oxide Myoglobin


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**The stability of nitric oxide myoglobin, the pigment found in cured, unheated meat, was investigated by measuring its rate of oxidation in solution. The rate of oxidation was first-order and was affected by light, pH changes, temperature variations, nitrite concentration, and certain inhibitors. Three distinct mechanisms were observed: a rapid oxidation by nitrous acid, a slow autoxidation in air, and a photo-oxidation.**


When meat is cured in brine or dry salt mixtures, myoglobin, the major pigment of the fresh meat (*11*), is converted to nitric oxide myoglobin (MbNO). During storage, this characteristic red pigment tends to change to undesirable brown and gray hues on exposed surfaces. The rate of discoloration is increased by light (*5, 8, 14*), but can be retarded by the exclusion of air (*14*), by wrapping the meat in red cellophane to filter out undesirable light (*14*), and by dipping in sodium nitrite after the sulfhydryl groups have been released by heating (*16*).

Kampschmidt (*5*) recently showed that light accelerated the oxidation of nitric oxide myoglobin and suggested that the reaction proceeded by a dissociation followed by an oxidation to metmyoglobin (MMb). Other information concerning the stability of nitric oxide myoglobin has been inferred from studies with nitric oxide hemoglobin, a protein with similar but not identical properties.

To provide more information on the properties of the pigment of cured, unheated meat, studies were undertaken on the influence of various factors on the stability of pure nitric oxide myoglobin in solution.

### Reagents

Buffers above pH 5.8 were mixtures of sodium phosphates; below 5.8, mixtures of sodium acetate and acetic acid. Hydrosulfite reagent was made immediately before use as 0.2% sodium hydrosulfite in 0.04$M$ buffer at pH 6.0. Nitrite reagent was 1% sodium nitrite in water.

Crystalline myoglobin was isolated from horse hearts by the method of Theorell (*12*). Stock solutions (about 0.4m$M$) were prepared by dissolving the crystals in water and dialyzing free of ammonium sulfate. They were stored at 0° C. The total pigment concentrations of these solutions and nitric oxide myoglobin solutions were determined by iron analyses by the modified bipyridine method (*2*), assuming one atom of iron per molecule of myoglobin derivative.

Concentrated nitric oxide myoglobin was prepared immediately before use by combining one part of stock myoglobin, one part of nitrite reagent, and two parts of hydrosulfite reagent.

Standard nitric oxide myoglobin was prepared by diluting concentrated nitric oxide myoglobin to about 0.04m$M$ in a final buffer concentration of 0.048$M$.

Dilute nitric oxide myoglobin solutions were prepared for oxidation rate measurements by dialyzing concentrated nitric oxide myoglobin against 0.04$M$ buffer at 4° C. to reduce the sodium nitrite