

VISCOMETRIC ASSAY OF THE PROTEASE ACTIVITY OF BATE AND DRUM LIQUOR*

by

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ABSTRACT

A viscometric procedure has been developed for the assay of general (non-specific) proteolytic activity, in particular the proteolytic activity of bate in the drum during the course of the bating operation. The basic features of the assay procedure are as follows: (1) a commercially available gelatin is used as substrate, (2) a clarified sample of drum liquor is added, (3) the resulting mixture is incubated at 40°C for 20 min or 60 min, (4) enzymatic activity is terminated with a stop reagent, and (5) the viscosity of the resulting solution is measured at 40°C. Any one of a variety of instruments may be used to measure the viscosity, including a simple glass viscometer of the Cannon-Fenske type. The dependence of the measured viscosity on the concentration of proteolytic enzyme is well described by a double exponential decay curve. Numerous samples can be run simultaneously (with staggering).

INTRODUCTION

We have previously reported a colorimetric procedure for assay of the proteolytic activity of bate and drum liquor using hide powder azure as substrate¹ and a viscometric procedure for assay of collagenase activity using a pepsin-

treated collagen as substrate.² We herewith report a viscometric procedure for assay of the proteolytic activity of bate and drum liquor using a high molecular weight gelatin as substrate.

The kinetics of the digestion of gelatin, casein, and hemoglobin by pancreatic extract and trypsin were studied viscometrically by Northrop in 1933.³ The gelatin viscosity method was applied to study proteolytic enzymes used in baking⁴ and in a study of reaction mechanisms of proteolytic enzymes.⁵ However, the method appears not to have been widely used, in spite of its simplicity and accuracy,⁴ probably because of the necessity of preparing the substrate, isoelectric (electrolyte-free) gelatin.⁶ With the present commercial availability of high molecular weight gelatins, it seemed to us prudent to develop a procedure using one of these as substrate for viscometric assay of proteolytic activity.

MATERIALS AND METHODS

Equipment

Incubation of enzyme with substrate was carried out in an orbital shaker bath as previously described.² Viscosity measurements were made with a size 100 Cannon-Fenske Routine Viscometer purchased from Cannon Instrument Co., State College, PA. An 8.0 ml aliquot of the sample was introduced into the viscometer. The temperature was

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** Mention of brand of firm name does not constitute an endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

controlled ($\pm 0.03^\circ\text{C}$) by a Cannon model M-1 Constant Temperature Bath.

Solids and Solutions

The substrate, porcine skin gelatin with a Bloom value of 300 (Type A, catalog nr. G-2500), was purchased from Sigma Chemical Co., St. Louis, MO. Trypsin was from bovine pancreas and was also purchased from Sigma (Type I, catalog nr. T-8003); it had a specific activity of 10,200 BAEE units/mg solid. Other reagents were of reagent grade.

Buffer B consisted of 5.0 mM CaCl_2 , 50 mM Tris (chloride), pH 8.25 ± 0.05 (at 25°C). The Substrate Solution contained 70 g/l, unless otherwise specified, of the gelatin described above in warm (ca. 50°C) Buffer B; the solution was adjusted to pH 8.25 ± 0.05 (at ca. 50°C) with 2M NaOH after most of the buffer had been added but before the solution was made up to its final volume. The substrate solution was held overnight in the incubator-shaker at 40.0°C while being rotated at 150 rpm. The stock solution of trypsin contained 1.00 g trypsin/l in 5.0 mM CaCl_2 , 1.00 mM HCl. In a typical run to define the standard curve, the stock trypsin solution was diluted to a concentration of 256 mg trypsin/l with Buffer B and, beginning with the latter, serial 2-fold dilutions were made with Buffer B to yield solutions with concentrations of 128, 64, ..., 2, 1 mg trypsin/l. One ml aliquots were used for the standard curve. The Stop Reagent was 1.00 M glycine HCl.

Sample Preparation

Drum liquor was clarified by centrifugation at 10,000 rpm for 20 min. A 1 ml aliquot of the undiluted supernatant was taken for assay.

For the assay of pancreatic bate, an extract was made by suspending 800 mg of the bate in 10.0 ml Buffer B. The suspension was mixed well and centrifuged at 10,000 rpm for 20 min. A 125 and a 250 μl aliquot of the supernatant were diluted to 10.0 ml with Buffer B. One ml aliquots of the 1:80 and 1:40 dilutions were used in the assay.

To determine what outflow time would be obtained in the viscosity measurement if the (proteolytic) enzymes in a given sample were inactivated, a control was run by heating 5 ml of the sample at $95 \pm 5^\circ\text{C}$ for 5-10 minutes in a loosely capped tube before taking the (cooled) 1 ml aliquot for assay. To determine what outflow time would be obtained if the substrate gelatin were completely hydrolyzed by proteolytic activity, a 1 ml aliquot of the stock (1 g/l) trypsin solution was taken for assay.

Enzyme-containing solutions were stored on ice until used.

Incubation

Incubation was initiated by adding a 1.00 ml portion of the sample (trypsin, diluted bate extract, clarified drum liquor, or control) to 9.0 ml of the Substrate Solution prewarmed to 40.0°C in a 25 x 150 mm screw-cap test tube. After capping the tube and gently mixing its contents, the solution was incubated for 20.0 or 60.0 min at 40.0°C while rotating at 150 rpm in the incubator-shaker. Incubation was terminated by adding 1.00 ml of the Stop Reagent; this reduced the pH to 3.45 ± 0.05 .²

Measurement of Viscosity

The viscosity (or outflow time) was measured at $40.00 \pm 0.03^\circ\text{C}$. Any one of a variety of viscometers can be used. We used a size 100 Cannon-Fenske Routine viscometer, into which we introduced an 8.0 ml sample. Outflow time was measured automatically by a Jupiter model 821 viscometer timer (Jupiter Instrument Co., Jupiter, FL) attached to the viscometer.

RESULTS AND DISCUSSION

Time Course of Gelatin Hydrolysis

To ensure that the chosen substrate behaves satisfactorily when subjected to proteolysis, the time course of its hydrolysis by trypsin was observed (Fig. 1a). For this purpose, 228 ml of the high molecular weight gelatin at a concentration of 52.5 g/l in Buffer B was mixed with 12 ml of trypsin at a concentration of 20 mg/l in the same buffer. Incubation was as described (Materials and Methods). Two (2) ml of Stop Reagent were added to 20 ml samples of the incubate at times $T = 0, 3, 9, 27, 81,$ and 243 min. Viscosity was measured as described (M&M). Viscosity was assumed to be proportional to outflow time; density differences were neglected. The data were found to be well fitted by the equation²

$$h = h_{\text{inf}} + h_1 \cdot \exp(-k_1 \cdot c \cdot T) + h_2 \cdot \exp(-k_2 \cdot c \cdot T) \quad (1)$$

where c is the enzyme (trypsin) concentration (mg/l), which, in this case, is constant; T is the time of incubation (0-243 min); h_{inf} is the asymptote (min); h is the measured outflow time (min); and $h_1, h_2, k_1,$ and k_2 are parameters. In the present case, h_{inf} was also treated as a parameter, but it is usually determined experimentally as the outflow time for a sample completely hydrolyzed by a high concentration of trypsin (M&M). Details of the curve-fitting procedure are given below in the section on Calculations.

The data and curve of Fig. 1A were normalized² as described under Calculations, yielding Fig. 1B. The normalized curve has the equation

$$h_n = h_{inf\ n} + h_{1n} \cdot \exp(-k_{1n} \cdot c \cdot T) + h_{2n} \cdot \exp(-k_{2n} \cdot c \cdot T) \quad (2)$$

where the suffix n indicates the normalized value of the variable or parameter in equation (1). In effect, normalization effects a change in scale for the ordinate (h): the asymptote is assigned a value $h_n = 0$, and the value of h on the fitted curve at $T = 0$ (or $c = 0$) is assigned a value $h_n = 1$ (Cf. Figs. 1A and 1B).

Normalization of the data for the standard curve and the unknowns partially compensates for differences in conditions obtaining during the processing of different batches of samples. It is therefore useful in the calculation of the enzyme concentration (c) of the unknowns when these are not run simultaneously with the standard curve. In addition, normalization greatly facilitates the comparison of information from different runs.

Dependence of Viscosity on Enzyme Concentration

A standard curve relating outflow time to enzyme concentration, determined as described (M&M and Calculations),

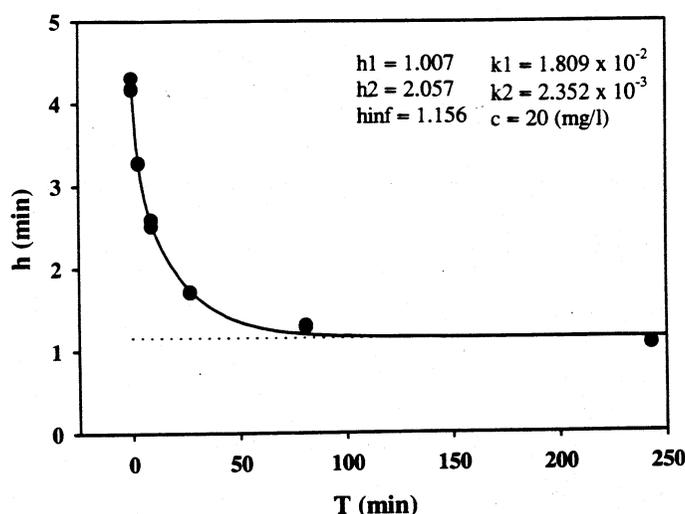


FIGURE 1A

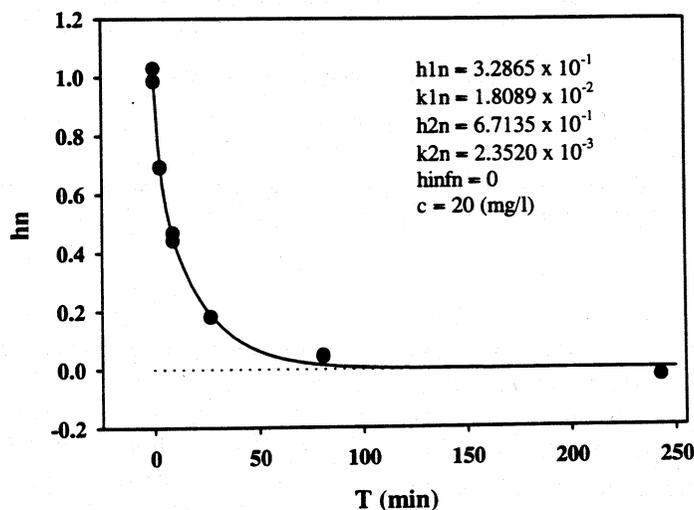


FIGURE 1B

FIGURE 1. — Time course of gelatin hydrolysis by trypsin. A) The raw data and the curve fitted to the data by non-linear least-squares analysis. The outflow time (h) is plotted against the incubation time (T). c is the concentration of trypsin. h₁, h₂, k₁, and k₂ are parameters of the fitted curve. (See equation (1) and associated text). The dotted line is the asymptote, $h = h_{inf}$. B) The normalized data and the normalized curve: normalized viscosity (h_n) vs incubation time (T). h_{1n} , h_{2n} , k_{1n} , and k_{2n} are parameters of the normalized curve. (See equation (2) and associated text.) The dotted line is the asymptote $h = h_{infn} = 0$.

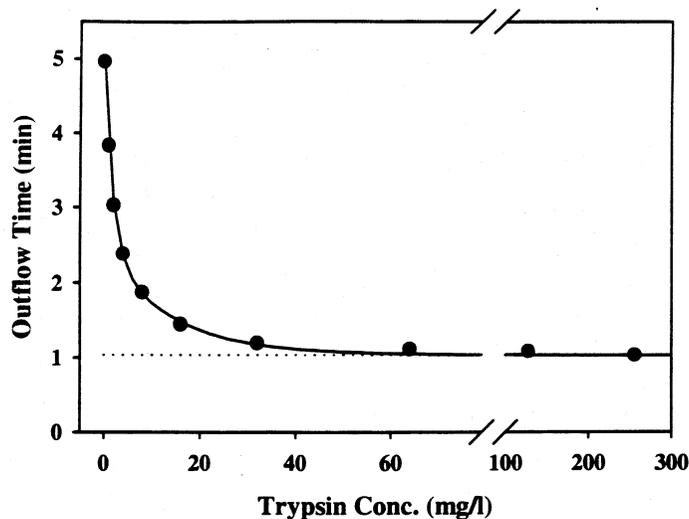


FIGURE 2A

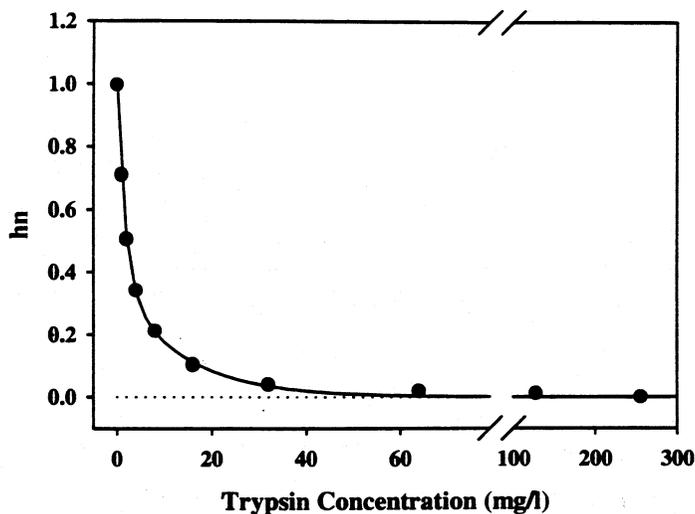


FIGURE 2B

FIGURE 2. — Dependence of viscosity on protease concentration. A) The initial standard curve: outflow time (h) vs trypsin concentration (c). B) The normalized standard curve: normalized viscosity (hn) vs trypsin concentration (c).

is shown in Fig. 2A; the constraining equation is equation (1). The corresponding normalized viscosity curve (equation (2)) is shown in Fig. 2B. Here the asymptote (1.0384 min in Fig. 1A) was determined experimentally, as usual, using a sample with a trypsin concentration of 1000 mg/l; this point is not shown on the graph. The standard deviation of error for the normalized viscosity h_n (Fig. 2B) was 0.011, i.e., 1.1 percent of full scale. Note that more than 80 percent of the drop in viscosity occurs in the range 0-10 mg trypsin/l, indicating that the procedure is sensitive enough to assay for very low levels of protease activity.

Preliminary Application of the Assay Procedure

As a test of the proposed assay procedure (M&M), it was applied to drum liquor (DL) samples taken during the bating of hides in our pilot plant and to samples taken from 3 drums during the bating of hides in a commercial tannery, Garden State Tanning in Williamsport, MD. The results for the bating in our facility, shown in Fig. 3A, indicate that bate activity held up well for the first hour of bating, and then decreased to ca. half of the original level after 90 min. The results for the commercial bating operations are shown

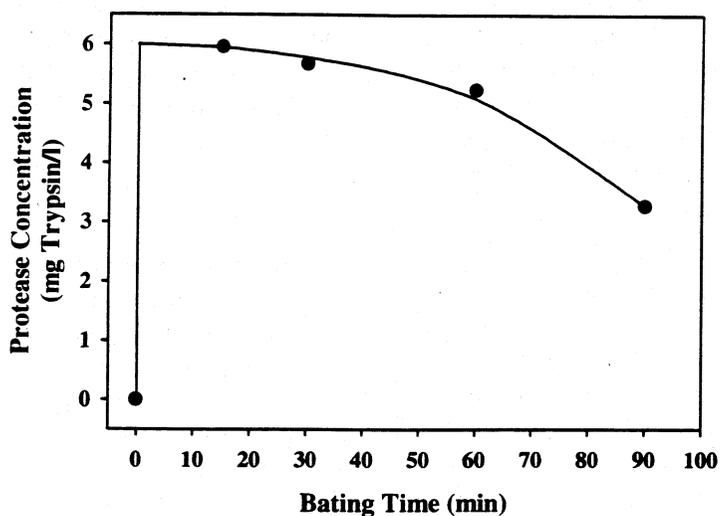


FIGURE 3A

Protease Conc.
c (mg/l)

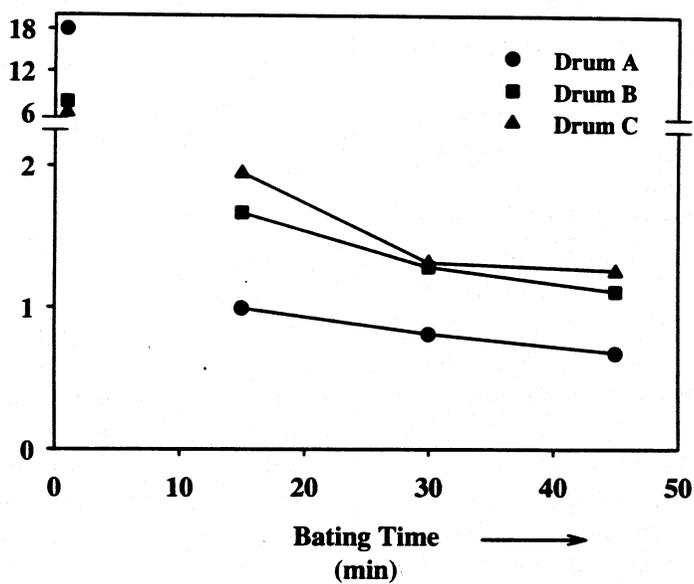


FIGURE 3B

FIGURE 3. — Proteolytic activity of drum liquor during bating. A) For hides processed in a pilot plant at the authors' site. B) For hides processed in 3 drums at a commercial tannery. Protease concentration is expressed in mg/l, the specific activity being 10,200 BAEE units per mg.

in Fig. 3B. The protease levels of the very early (1min) samples are much higher than those of later samples. Since the samples were taken from the same port as that through which the bate was added, it is assumed that the bate in the drum had not yet dispersed uniformly into the DL one minute after it was added. The protease levels 15-45 min into the bating operation suggest (again) that bate activity is reasonably stable during at least the first 3/4-hr of bating.

Calculations

For tannery work, where errors of 10-15 percent can be tolerated, the initial standard curve (cf. Fig. 2A) and the normalized standard curve (cf. Fig. 2B) can be drawn to fit the data by eye, and the enzyme concentration (c) of an unknown can be estimated by locating, on the normalized standard curve, the value of c for which the normalized viscosity (h_n) is equal to that calculated for the unknown (see below). For research work, where substantially better precision is required (≤ 5 percent), the parameters of equation (1) should be determined by statistical procedures which minimize error, the parameters of equation (2) are calculated from those of equation (1) (see below), and the enzyme (protease) concentration (c) of the unknown should be calculated by plugging its normalized viscosity (h_n) into equation (2) and solving for c . While conceptually straightforward, these calculations are, in practice, so slow and tedious as to be impractical unless done by computer with suitable software. We have found Axum (5.0) (Mathsoft, Inc., Cambridge, MA) to be excellent for determination of the initial standard curve by non-linear least-squares analysis of the data. The Axum program uses the Marquardt-Levenberg algorithm to fit the curve to the data, and supplies the values of the parameters for equation (1), as well as a plot of the data and the fitted curve (Fig. 2A). After the normalized parameters have been evaluated (as later described), the concentration (c) of an unknown is readily calculated (Fig. 4A) with the "root" function of Mathcad (also from Mathsoft, Inc.), using equation (2) and the predetermined value of h_n for the unknown (see below). If several unknowns have been assayed, their concentrations can be obtained simultaneously with Mathcad using the "find" function (Fig. 4B). If the h_n values are supplied as a vector (H_n), the corresponding enzyme concentrations can be output as the second column of a matrix (M) in which the first column is the vector of h_n values.

For calculation of the normalized viscosity (h_n) of an unknown, three data values are needed: (1) the outflow time (h) for the sample in which the gelatin substrate (S) was incubated with the unknown, (2) the outflow time (h_c) for the control, i.e., for a sample in which S was incubated with

the heat-inactivated unknown, and (3) the outflow time (h_{inf}) for a sample in which S was incubated with a very high concentration of trypsin. These three samples should be run simultaneously. We then obtain

$$h_n = \frac{h - h_{inf}}{h_c - h_{inf}} \quad (3)$$

Normalization of initial standard curve data is done in a similar way. In this case, the role of a heat-treated control is replaced by the point on the initial standard curve for $c = 0$. From equation (1), the ordinate (outflow time) for this point is $h_0 = h_{inf} + h_1 + h_2$. The normalized viscosity for any one of the standard curve data points is then given by

$$h_n = \frac{h - h_{inf}}{h_0 - h_{inf}} = \frac{h - h_{inf}}{h_1 + h_2} \quad (4)$$

In particular, the normalized value of h_0 $h_{0n} = 1$, and the normalized value of h_{inf} $h_{inf n} = 0$, as desired.

The normalized parameters are

$$h_{1n} = \frac{h_1}{h_1 + h_2} \quad (5a)$$

$$k_{1n} = k_1 \quad (5b)$$

$$h_{2n} = \frac{h_2}{h_1 + h_2} \quad (5c)$$

$$\text{and} \quad k_{2n} = k_2 \quad (5d)$$

For our system, the specific viscosity can be defined (cf. ref. nr. 7, p. 700) as $h_{sp} = (h - h_{inf}) / h_{inf}$. Comparing this to equation (4) it is apparent that $h_n = [h_{inf} / (h_0 - h_{inf})] h_{sp}$, i.e., h_n differs from h_{sp} by a constant factor. Our preference for h_n for our work is explained above.

Specific viscosity is generally defined as $h_{sp} = (h - h_s) / h_s$, where h_s is the viscosity of the solvent (ref. nr. 7, p. 700). The dependence of h_{sp} on time or enzyme concentration is frequently used when enzymatic reactions are monitored viscometrically,^{8,9} it being assumed that $h_{inf} = h_s$, i.e., that the viscosity of the solution (h) approaches the viscosity of the solvent (h_s) (and therefore $h_{sp} \rightarrow 0$) as the substrate is exhausted. This, in turn, assumes that the products of the reaction do not contribute to the viscosity of the solution. These assumptions are not justified. We therefore determine h_{inf} experimentally, and use it instead of h_s in our equations; see, for example, equation (1).

PROTEOLYTIC ACTIVITY DURING BATING

$h_{1n} = 0.3587$ $k_{1n} = 3.615 \cdot 10^{-3}$
 $h_{2n} = 0.6413$ $k_{2n} = 2.799 \cdot 10^{-2}$
 $T = 20 \text{ (min)}$

Expt'l 0

$h_n = 0.8055$
 $f(c) = h_{1n} e^{(-k_{1n} T c)} + h_{2n} e^{(-k_{2n} T c)}$
 $g(c) = f(c) - h_n$
 $c = 0.58 \text{ (mg/L)}$ An estimate
 $c_0 = \text{root}(g(c), c)$ $c_0 = 0.5869$
 $f(c_0) = 0.8055$ As required

FIGURE 4A

$H_n =$	0.5091	Given $h_n = h_{1n} e^{-k_{1n} T c} + h_{2n} e^{-k_{2n} T c}$ $h_{1n} = 0.3587$ $k_{1n} = 3.615 \cdot 10^{-3}$ $h_{2n} = 0.6413$ $k_{2n} = 2.799 \cdot 10^{-2}$ $T = 60 \text{ (min)}$	Find c :	<table border="0" style="display: inline-table;"> <tr> <td style="padding-right: 10px;">H_n</td> <td style="padding-right: 10px;">C</td> <td></td> </tr> <tr> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0.5091</td> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0.6925</td> <td style="padding-left: 10px;">S_0</td> </tr> <tr> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0.4555</td> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0.8397</td> <td style="padding-left: 10px;">S_0</td> </tr> <tr> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0.5396</td> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0.6208</td> <td style="padding-left: 10px;">S_{15}</td> </tr> <tr> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0.5420</td> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0.6155</td> <td style="padding-left: 10px;">S_{30}</td> </tr> <tr> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0.4443</td> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0.8747</td> <td style="padding-left: 10px;">T_0</td> </tr> <tr> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0.4759</td> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0.7800</td> <td style="padding-left: 10px;">T_0</td> </tr> <tr> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0.5730</td> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0.5500</td> <td style="padding-left: 10px;">T_{15}</td> </tr> <tr> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0.3649</td> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">1.1857</td> <td style="padding-left: 10px;">T_{30}</td> </tr> <tr> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0.3874</td> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">1.0840</td> <td style="padding-left: 10px;">T_{60}</td> </tr> <tr> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0.3656</td> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">1.1824</td> <td style="padding-left: 10px;">T_{90}</td> </tr> <tr> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">1</td> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0</td> <td style="padding-left: 10px;">Cnt</td> </tr> <tr> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">0</td> <td style="border-left: 1px solid black; border-right: 1px solid black; padding: 2px;">∞</td> <td style="padding-left: 10px;">Dig</td> </tr> </table>	H_n	C		0.5091	0.6925	S_0	0.4555	0.8397	S_0	0.5396	0.6208	S_{15}	0.5420	0.6155	S_{30}	0.4443	0.8747	T_0	0.4759	0.7800	T_0	0.5730	0.5500	T_{15}	0.3649	1.1857	T_{30}	0.3874	1.0840	T_{60}	0.3656	1.1824	T_{90}	1	0	Cnt	0	∞	Dig
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FIGURE 4B

FIGURE 4. — Calculation of enzyme concentration from normalized viscosity. A) Solving for the enzyme concentration of a single sample. B) Solving for several enzyme concentrations simultaneously. The column to the right of matrix M contains the sample designations. Note: not all steps in the protocols are shown.

CONCLUSION

A viscometric procedure for the assay of general proteolytic activity, such as the bating activity of drum liquor, is presented (Materials and Methods). Viscosity is measured after termination of the enzymatic activity with a Stop Reagent. Procedures for efficient analysis of assay results to minimize error are described (Results & Discussion – Calculations). The assay procedure is simple and provides results with excellent precision.

ACKNOWLEDGMENT

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