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Measuring Cellulase Activity

Application of the Filter Paper Assay to Low-Activity Enzyme Preparations

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Abstract

An approach is presented for obtaining relative filter paper activities for enzyme preparations having activities below that required for application of the traditional International Union of Pure and Applied Chemistry filter paper assay. The approach involves the utilization of protein stabilizers to retard the time-dependent enzyme inactivation that may occur under traditional filter paper assay conditions. Enzyme stabilization allows extended reaction times and the calculation of relative activities based on the time required for saccharification of 3.6% of the traditional substrate, making results proportional to those obtained in the traditional International Union of Pure and Applied Chemistry assay. The assay is demonstrated using a commercial cellulase preparation along with KCl and bovine serum albumin as protein stabilizers.

Index Entries: Assay; cellulase; filter paper.

Introduction

Considerable research is aimed at obtaining novel cellulase enzyme systems for application in the forest products, textile, food, and biomass conversion industries. Microbial enzyme systems capable of catalyzing the degradation of native celluloses include multiple cellulolytic enzymes, either complexed or noncomplexed *(1,2)* that act in concert to solubilize/saccharify crystalline cellulose *(3)*. Assays using cellulose substrates that are somewhat recalcitrant to cellulase-catalyzed hydrolysis are useful for assessing the potential of these enzyme systems because their rates of saccharification are, presumably, dependent on the well-documented synergism that is associated with many cellulase preparations *(4)*. Substrates such as filter paper, microcrystalline cellulose, bacterial cellulose, and cotton are particularly informative because they contain an appreciable amount of crystalline cellulose. The International Union of Pure and

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Applied Chemistry (IUPAC) Commission on Biotechnology has endorsed an assay based on the degradation of filter paper *(5)*. The IUPAC filter paper assay is widely accepted as the "standard" method for measuring the activity of noncomplexed cellulolytic enzyme systems.

The IUPAC assay is based on the identification of the amount of enzyme that will solubilize 2.0 mg of reducing sugar equivalents (RSE) from 50.0 mg of filter paper in a 1-h reaction period. Because of the heterogeneous (amorphous/crystalline) nature of filter paper, the assay is based on the conversion of a specified amount of substrate, i.e., 3.6% (2.0 mg soluble glucose is equivalent to approx 3.6% of the glucose in 50.0 mg dry filter paper) *(6)*. The assay itself requires that a minimum of two enzyme concentrations be tested: one mixture producing slightly more and another mixture producing slightly less than 2.0 mg RSE in the assay. The amount of enzyme preparation containing 0.37 filter paper units (FPU) (i.e., that amount that generates 2.0 mg RSE in 1 h) is then calculated based on the empirical observation that, at enzyme concentrations approaching 0.37 FPU per reaction mixture, the amount of RSE in a given reaction mixture is proportional to the log of the enzyme concentration (Eq. 1).

$$\Delta[P_1] = K_1[\Delta \log(E)] \tag{1}$$

where $[P_1]$ is the product (mg RSE) generated in 1-h filter paper assay, $[E]$ is the enzyme concentration, and K_1 is the constant of proportionality. The value of the proportionality constant is dependent on the reaction conditions, including the source and history of the enzyme. Thus, if one plots the data from a series of reaction mixtures (as total RSE generated vs log enzyme concentration) it will yield a straight line from which the amount of enzyme preparation corresponding to 0.37 FPU can be obtained. The obvious requirement for this assay is that the enzyme preparation must be sufficiently active to generate ≥2.0 mg of RSE in 1 h. This requirement presents a limitation in using the IUPAC assay for the routine analysis of cellulase preparations whose activity is inherently low. In particular, enzyme samples resulting from native microbial environments, novel microbial culture systems, and plant or microbial extracts will often be of relatively low activity. To measure the filter paper activity (FPA) of these "low-activity" samples require that the enzyme sample be concentrated, such that the concentrated preparation will generate sufficient product. In general, enzyme concentration is achieved by ultrafiltration and/or lyophilization techniques, both operations running the risk of enzyme inactivation *(7)*.

The objective of this note is to explain an approach that may be used in conjunction with the 3.6% conversion rationale, as developed for the standard filter paper-based assay, to obtain relative FPAs for "low-activity" cellulase preparations. The approach is based on the use of enzyme stabilizers; enzyme stabilization allows relative FPAs to be determined based on the time taken to generate a specified amount of

RSE (i.e., 2.0 mg as specified in the IUPAC assay). The presented approach, in which time is varied, is in contrast to the traditional assay in which enzyme concentration is varied, whereas time is fixed. Enzyme stabilization allows activity values to be obtained through Eq. 2, which has the same form as Eq. 1.

$$\Delta[P_t] = K_2(\Delta \log t) \tag{2}$$

where P_t is the product (RSE [mg]) generated at time (t), t is the time of reaction, and K_2 is the constant of proportionality.

Materials and Methods

Materials

All chemicals were reagent grade unless specified otherwise. General reagents were obtained from commercial suppliers. Bovine serum albumin (BSA), fraction V, was obtained from Sigma Chemical Co. (St. Louis, MO). The substrate used for all assays was Whatman No. 1 filter paper (Whatman LabSales, Inc., Hillsboro, OR). Fifty milligram substrate strips were weighed to 1.0 mg accuracy. The cellulase preparation (Cellulysin) was purchased from Calbiochem Corp. (San Diego, CA) and used without modification. Enzyme stock solutions were prepared by dissolving enzyme preparations in distilled-deionized water immediately before use.

Activity Assay

Traditional filter paper assays were done according to IUPAC specifications *(5)*. Modified assays for the measurement of "low-activity" enzyme preparations were done according to IUPAC specifications with the following exceptions:

1. Reaction mixtures were supplemented with stabilizers at the concentrations given in Table 1.
2. Where indicated in the text, reaction times were extended beyond the 1 h assay period until a time at which ≥2.0 mg RSE could be detected in the reaction mixture.
3. Stabilized reaction mixtures were made 0.02 *M* in sodium azide to prevent microbial growth over the prolonged reaction period. Soluble RSE were determined using the dinitrosalicylic acid assay *(8)*.

Results and Discussion

Classical enzyme theory dictates that for a specified set of reaction conditions the amount of product generated in any of a series of reaction mixtures differing only with respect to enzyme concentration will be entirely dependent on the product of the enzyme concentration of the

Table 1
Solutes and Combinations of Solutes Tested for Ability to Alleviate Time-Dependent Inactivation of Commercial Cellulase Preparations Under Standard Filter Paper Assay Conditions

Component	Concentration
Glycerol	1.1 *M*
Sorbitol	1.1 *M*
Mannitol	1.1 *M*
Inositol	1.1 *M*
Ascorbic acid	1.9 m*M*
Potassium chloride	1.0 *M*
Potassium chloride	3.0 *M*
Magnesium sulfate	1.0 *M*
BSA	1.0 mg/mL
BSA	3.0 mg/mL
Combinations	
Mineral oil and degassed buffer	200 μm layer
BSA and calcium chloride	1.0 mg/mL, 1.0 *M*
BSA and glycerol	1.0 mg/mL, 1.1 *M*
BSA, glycerol, and ascorbic acid	1.0 mg/mL, 1.1 *M*, and 1.9 m*M*
BSA, mannitol, and ascorbic acid	1.0 mg/mL, 1.1 *M*, and 1.9 m*M*
BSA, glycerol, and potassium chloride	1.0 mg/mL, 1.1 *M*, and 2.0 *M*
BSA and potassium chloride	1.0 mg/mL, 2.0 *M*
BSA and potassium chloride	1.0 mg/mL, 1.0 *M*
BSA and potassium chloride	1.0 mg/mL, 0.4 *M*

reaction mixture and the time of the reaction *(9)*. This general statement may be expressed as in Eq. 3.

$$[E] \times t = f[(P_t)] \tag{3}$$

where [*E*] is the enzyme concentration, *t* is the time of reaction, and [P_t] is the product concentration generated at time *t*. In terms of the IUPAC filter paper assay, Eq. 3 indicates that the point on a progress curve corresponding to 2.0 mg of RSE will be directly related to the product of enzyme concentration and time ([E] × *t*). The equation also suggests that FPA can be determined by using a single enzyme concentration and noting the time required to obtain 2.0 mg of RSE. The activity of a cellulase preparation would then be calculated as in Eq. 4.

$$FPA = \frac{1(h)}{t_2}(0.37) \tag{4}$$

where FPA is the filter paper activity and t_2 is the time in hours, required for generation of 2.0 mg RSE. As Eq. 3 must be valid for Eq. 4 to be applied, its validity must be determined. The validity of Eq. 3 is easily tested by

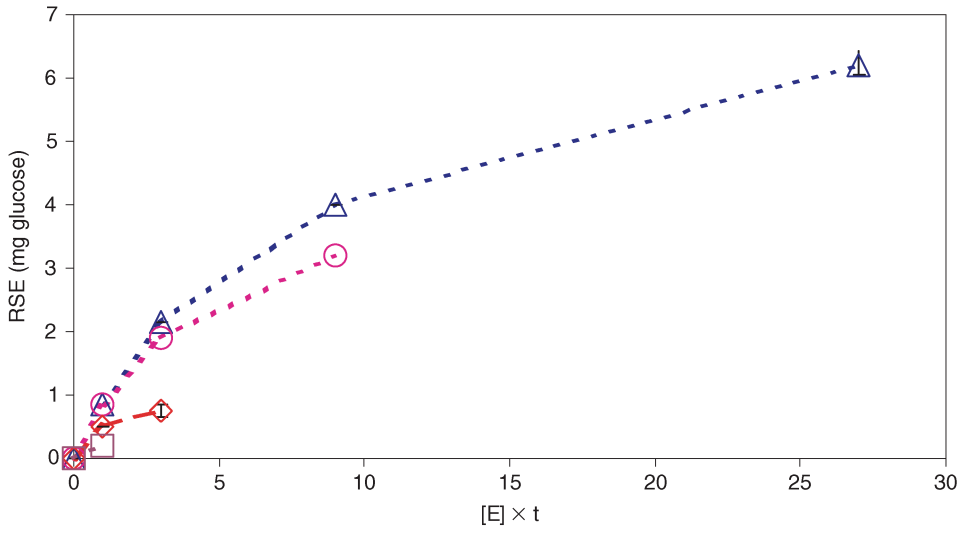

Fig. 1. Progress curves of cellulase reaction plotted in terms of soluble RSE vs reaction time (h) multiplied by the amount of enzyme added to the reaction mixture (expressed as the fold dilution of a stock solution). Reaction conditions were as described for the IUPAC filter paper assay. The stock cellulase preparation for this experiment contained 0.14 FPU/mL. Dilutions: 27-fold (□), ninefold (◊), threefold (○), and undiluted (△).

comparing progress curves (as $[P_t]$ vs [E] × t) for reaction mixtures containing different enzyme concentrations *(9)*. The progress curves are expected to overlay each other if Eq. 3 is obeyed. And Eq. 3 will be obeyed if the activity of the enzyme is stable over the assay period, or if changes in enzyme activity are a consequence of the percent of substrate converted (such as is expected with a heterogeneous amorphous/crystalline substrate like cellulose) or classical product inhibition. If such theoretical behavior is observed (Eq. 3 is valid), then Eq. 4 may be applied directly. However, if the progress curves are not well behaved, as is the case for the commercial enzyme preparation depicted in Fig. 1, then Eq. 4 is not directly applicable. The nature of the time-courses in Fig. 1 indicate that there is a time-dependent enzyme inactivation that occurs over the course of the assay, and this change in activity is not a simple function of the extent of substrate conversion. Thus, under standard IUPAC conditions (as was used in the experiment generating the data in Fig. 1), one cannot interchange reaction time and enzyme concentration.

Equation 4 may be correctly applied to the enzyme preparation of Fig. 1 only if the time-dependent inactivation is effectively alleviated. Previous studies suggest that the observed inactivation is owing to proteolytic degradation *(10)* and/or thermal inactivation *(11–13)*. Hence, approaches to stabilize the enzyme preparation should likely focus on these mechanisms of inactivation. A number of potential stabilizers, alone or in

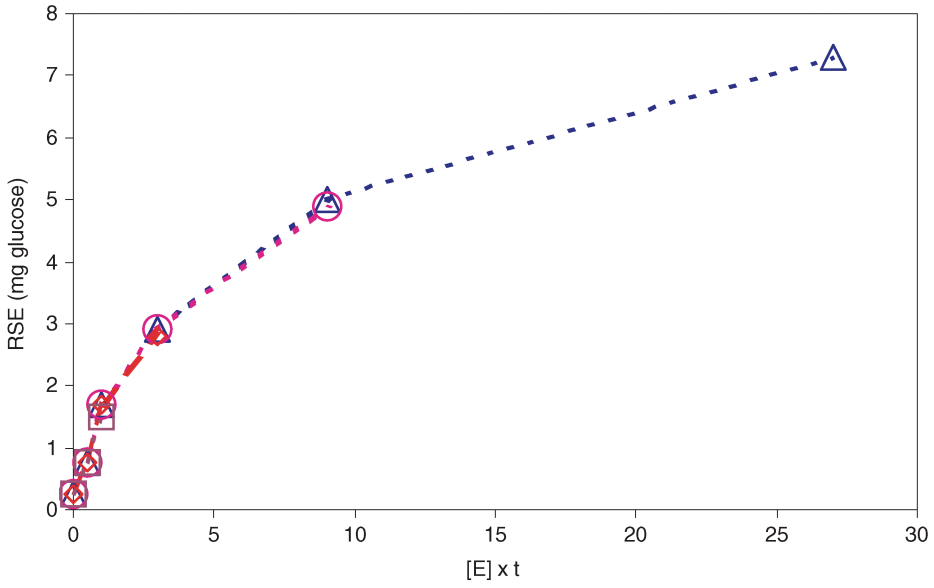

Fig. 2. Progress curves of cellulase reaction plotted in terms of soluble RSE vs reaction time (h) multiplied by the amount of enzyme added to the reaction mixture (expressed as the fold dilution of a stock solution). Reaction conditions were as described for the IUPAC filter paper assay with the exception that reaction mixtures were supplemented with enzyme stabilizers (reaction mixtures contained 1.0 mg BSA/mL and were 1.0 *M* in KCl). The stock cellulase preparation for this experiment contained 0.35 FPU/mL. Dilutions: 27-fold (□), ninefold (◊), threefold (○), and undiluted (△).

combination, were tested in this study (Table 1), including glycerol, sugar alcohols, salts, BSA, ascorbic acid, and the exclusion of dissolved gases (particularly oxygen; achieved by degassing the buffer and covering the reaction mixture with mineral oil). In the present case, optimal stability was obtained when reaction mixtures were made 1.0 *M* in KCl and 1.0 mg/mL in BSA. The salt is expected to stabilize the protein through its preferential hydration *(14)*. The BSA may stabilize the system by its hydration properties as well as its ability to act as a competitive substrate for proteolysis. The addition of the stabilizers resulted in an enzyme preparation whose activity was stable for up to 20 h, as evidenced by the overlaying progress curves of Fig. 2 (compare with the analogous curves of Fig. 1). Thus, Eqs. 2–4 appear to be applicable, and a relative FPA can be calculated based on Eqs. 2 and 4 when the stabilizers are used.

An unfortunate consequence of the added stabilizers is that they tend to decrease the measured FPA relative to equivalent enzyme preparations acting in the absence of stabilizers. The "stabilized" enzyme system described earlier, when tested in the standard IUPAC assay with sufficient enzyme to generate 2.0 mg RSE in 1h, had approx 60% of the activity observed for the same enzyme preparation in the absence of stabilizers.

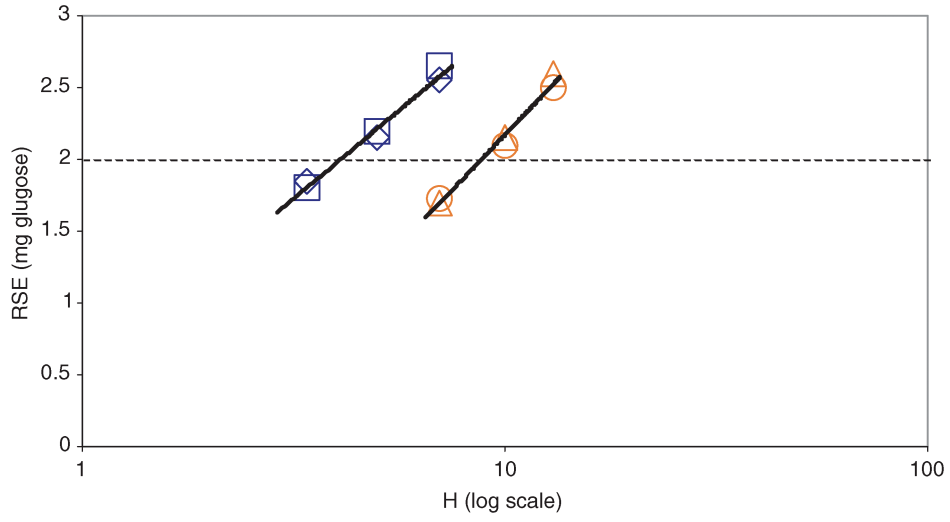

Fig. 3. Semilog plot of filter paper-derived soluble RSE vs time for two "low-activity" enzyme preparations. Reaction conditions were as described for the IUPAC filter paper assay with the exception that enzyme stabilizers were added to reaction mixtures (as described in Fig. 2). The enzyme preparations were prepared by diluting approx 0.9 FPU/mL stock enzyme solution sevenfold (□,◊) and 14-fold (○,△).

Hence, the activity measured under stabilizing conditions cannot be directly interpreted in terms of absolute IUPAC FPU. However, this does not limit the use of the presented approach for obtaining relative FPAs. If absolute FPUs are to be calculated for enzyme preparations assayed in the presence of stabilizers, then a correction factor, based on separate kinetic experiments, must be determined. Such correction factors are not necessary for the determination of relative FPAs.

The application of stabilizers in the filter paper assay is illustrated in Fig. 3 and Table 2. In this case, two low-activity enzyme solutions were prepared by diluting (7-fold and 14-fold) an enzyme solution known to contain 0.95 FPU/mL, based on the traditional IUPAC assay. Hence, the diluted enzyme solutions contained approx 2.5- and 5-fold less activity than the minimum required for the traditional assay (0.37 FPU/mL). The test solutions were then assayed in the presence of stabilizers. Total RSE was determined at selected time-points over extended assay periods until at least 2.0 mg RSE could be detected. Times corresponding to the targeted 2.0 mg RSE end point were determined based on Eq. 2 (data presented in Fig. 3), and subsequently, FPAs were calculated based on Eq. 4. The measured activities were found to be directly proportional to the fold dilution used to prepare the test samples (Table 2). Hence, the assay with the incorporated stabilizers correctly measured the relative FPA of the enzyme preparations; these values could not have been obtained using the traditional IUPAC assay because of their relatively low activities.

Table 2
Measured Vs Theoretical Activities for a Series of Diluted Enzyme Preparations[a]

Enzyme preparations[b]	Measured activity[c]	Theoretical activity[d]	Measured activity/ theoretical activity
Standard solution	0.56 (0.010)	–	–
Sevenfold diluted	0.078 (0.009)	0.080	0.98
14-fold diluted	0.039 (0.001)	0.040	0.98

[a]Activity determined as a function of time required to produce 2.0 mg soluble RSE in modified filter paper assay.

[b]The standard solution was prepared from a commercial cellulase preparation and assayed "as prepared," other enzyme preparations were prepared by the indicated dilution of the standard solution. All reaction mixtures contained 1.0 mg BSA/mL and were 1.0 *M* KCl, as described in Fig. 2.

[c]Mean (standard error of mean).

[d]Obtained by dividing the activity of the standard solution by the fold dilution.

It is informative to consider the assay approach described in this note to previously suggested permutations of the filter paper assay. The assay, as adopted by IUPAC, was discussed in some detail in 1976 *(6)*. The emphasis at that time was to fix the extent of conversion of the substrate and the time of the reaction. The chosen extent of conversion was deemed appropriate because 3.6% was considered well past the extent of conversion that would be expected for an incomplete cellulase. In choosing this value, and the corresponding 1-h reaction time, it set a limit as to the minimum activity to which this assay could be applied. This limitation has been addressed in different ways over the years. The "low-activity" assay of Chan et al. *(15)* is based on the conversion of approx 1.5% of the filter paper substrate, the activity then being calculated in Forintek units. Assays based on either Forintek or FPUs are expected to show similar trends with respect to enzyme activity; but Forintek and FPUs are not expected to be directly proportional owing to the two assays being based on different extents of substrate conversion. Another permutation that under certain circumstances may allow the analysis of lower activity samples is the inclusion of supplemental β-glucosidase in the reaction mixture *(16)*. The cited paper was actually addressing the need for a better measure of the extent of saccharification (as have others, *[17]*); but inclusion of β-glucosidase may serve to enhance the rate of filter paper saccharification by those enzyme preparations inherently low in this enzyme and thus, inadvertently make the assay more amenable to lower activity enzyme preparations. However, it is clear that a β-glucosidase-supplemented activity will not be particularly relevant for some applications. A similar argument may be made about the present work that a "stabilized" FPA will not be particularly relevant for some applications. All of the suggested assay permutations that directly or indirectly accommodate low-activity samples have some limitations. We think the method presented here is a

noteworthy addition to the field in that it addresses the mechanistic basis underlying the limitation of the filter paper assay for low-activity samples and it allows one to obtain relative FPA values that were previously unattainable. The merit of any such values, as is the case with activity values obtained with the standard IUPAC assay, is dependent on the particular application of the enzyme system *(18)*.

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