

Rheological Method for Evaluating Endoproteolytic Enzyme Activity

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ABSTRACT

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A rheological method for evaluating endoproteolytic enzyme activity was developed. The decrease in viscosity as a function of time of dispersion of commercially available modified wheat gluten protein in water (15%, w/w) was measured with a rapid visco analyzer (RVA). Measurement of the RVA viscosity signal at a chosen temperature was continuous and reproducible. As described, the method also can be used to determine

the temperature optima of endoproteolytic enzymes at pH values exceeding 5.5 and for partial pH-activity profiles at different temperatures. The method is simple, reproducible, and sensitive and has obvious advantages over more labor-intensive methods in which changes in the rheological properties (elongational viscosity, stretch ratio) of a gluten-starch dough are evaluated as a function of time.

The functionality of gluten in wheat-based food products can be influenced not only by emulsifiers and oxidizing and reducing agents, but also by proteolytic enzymes. Reliable methods for evaluating the hydrolytic action of these enzymes and, more specifically, their impact on the rheological properties of gluten proteins are useful.

Researchers (Hanford 1967, Petit 1974) have shown that the modified Ayre-Anderson method (Ayre and Anderson 1939) is not valid for predicting the gluten-softening effect of proteolytic enzymes, because hemoglobin is used as a substrate. A number of colorimetric (Redman 1971, Skupin and Warchalewski 1971, Finley 1975, Kawamura and Yonezawa 1982, Mathewson et al 1988, Weegels et al 1992) and rheological (Kruger 1971, Redman 1971, Berger et al 1974, Doescher and Hosney 1985, Wu and Hosney 1989, Lin et al 1993) methods have been proposed to measure both endo- and exoproteolytic activity against wheat proteins. Under appropriate experimental conditions, the methods described by Adler-Nissen (1979) and Hosney et al (1979) both can be used for estimating gluten-hydrolyzing enzymatic activity.

In the current study, a procedure to measure the activity of endoproteolytic enzymes was developed. In our method, the decrease in viscosity of commercially available modified wheat gluten protein dispersion is measured with a rapid visco analyzer (RVA). The influence of temperature and reproducibility of the assay were evaluated. The results are compared to those obtained using other rheological methods (Hosney et al 1979, Lin et al 1993).

MATERIALS AND METHODS

Commercial wheat starch (13.1% moisture content [mc]), native gluten, and commercially available modified wheat gluten protein prepared from vital wheat gluten (product code SWP050) were supplied by NV Amylum (Aalst, Belgium). The modification process for SWP050 involves partial deamidation and peptide bond hydrolysis. As a result, the amino acid composition is the same as for vital wheat gluten, except for a larger abundance of glutamic and aspartic acids. The protein contents of the gluten and SWP050 were 76 and 84% (N \times 5.7, dry weight basis, micro-Kjeldahl procedure [Jones 1991]) and 5.9 and 5.5% mc (Approved Method 44-19 [AACC 1995]), respectively. Sulfanilamide used obtained from Sigma-Aldrich (Bornem, Belgium). Enzyme preparations were pronase E from *Streptomyces griseus* (Merck, Darmstadt, Germany); papain from *Carica papaya* (76220 Fluka [Bornem, Belgium]); and protease type XIV from *S. griseus* (P 5147), carboxypeptidase A from bovine pancreas (C 0261), and α -amylase type X-A from *Aspergillus oryzae* (A 6211) (Sigma-Aldrich).

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Rheological Method Developed

A RVA (model 3D, Newport Scientific, Warriewood, Australia) was used to measure RVA viscosity. In the 3D RVA, during measurement a paddle at a constant rate stirs the sample in a cylindrical container. The current required to maintain constant stirrer speed is monitored continuously and converted by an algorithm to RVA viscosity. The term viscosity indicates RVA viscosity (rapid visco units [RVU] [Ross et al 1987], where 1 RVU = 12 mPa sec⁻¹).

SWP050 (15%, w/w) was dispersed in distilled water (pH 6.5). The SWP050 dispersion was stirred (500 rpm) for 30 min with a mechanical stirrer (Janke & Kunkel, IKA-Labortechnik, Staufen, Germany). The mixture was heated for 15 min in a water bath to the viscosity measurement temperature (30°C) under continuous stirring. The substrate dispersion (25.0 g, pH 6.5) was transferred to a RVA cup, and 1.0-mL enzyme solution in deionized water and equilibrated at the same temperature was added. The change in the viscosity of the mixture at a chosen constant temperature was measured for 30 min.

One unit of activity (U_{RVA}) was defined as the quantity of enzyme that, under the experimental conditions, resulted in a decrease in the initial viscosity signal by 1% per minute:

$$U_{RVA} (1/\text{min}) = 100 \times (\Delta\eta/\Delta t)/\eta_0$$

where $\Delta\eta/\Delta t$ = the slope of the linear phase of the viscosity time plot (RVU/min), and η_0 = the viscosity of the SWP050 dispersion at the beginning of the reaction (RVU). To determine the slope, linear regression lines were fitted to the RVA readings taken at 5-sec intervals, and the slope was arbitrarily defined as that of a line with $r^2 = 0.950$.

Colorimetric Method

In the colorimetric method tested, sulfanilamide-azogluten (SAG) was synthesized from sulfanilamide, sodium nitrite, and native gluten as described by Finley (1975).

The degree of hydrolysis was determined spectrophotometrically by measuring the absorbance (425 nm) of soluble-dyed peptides released from SAG by endoproteolytic enzymes. One unit of activity (U_{SAG}) was defined as the quantity of enzyme that at pH 6.5 and 30°C resulted in a change in absorbance (425 nm) of 1.000/min.

The colorimetric method was used to calibrate the activity of commercial enzyme preparations. Pronase E (0.01%, w/v) = $8.5 \times 10^{-3} U_{SAG}$ and papain (0.05%, w/v) = $17.5 \times 10^{-3} U_{SAG}$.

Rheological Method Based on Modified Spread Ratio

The rheological method of Hosney et al (1979), based on a spread ratio that was modified slightly, was tested. Gluten-starch (15/85, w/w, 14% mb) mixtures were prepared in a 100.0-g National Manufacturing (Lincoln, NE) pin-mixer. Absorption level (52%) and mixing time (126 sec) were determined with the mixograph. When enzymes were used, appropriate dilutions were made in 1.0 mL of water (pH 6.5) and included at the beginning of mixing.

After mixing, doughs were sheeted in two passes to 0.635 cm thick, molded, and placed on a glass plate in a fermentation cabinet (30°C, 85% rh) for 30 min. The spread ratio (SR), calculated as width (W) over height (H) of molded dough (spread [W/H]), was measured before (SR₀) and after (SR₃₀) 30 min of fermentation. A higher SR indicated more proteolytic activity.

Rheological Method Based on Elongational Viscosity

Using the rheological method described by Lin et al (1993), based on elongational viscosity, gluten-starch doughs were prepared as described above and transferred to a fermentation cabinet (30°C, 85% rh) for 30 min. Doughs were subsequently sheeted in two passes to 1.588 cm thick. Five pieces were cut out of each dough with a circular (2.9 cm) cookie cutter. Doughs were uniaxially compressed to a sample height of 0.3 cm with a UTS Universal Testing Machine 5 (UTS Testsysteme, Ulm-Eisingen, Germany) at a cross-head speed (V_z) of 1.0 cm/min. Elongational viscosity (EV) was calculated as

$$EV(N \times \text{min}/\text{cm}^2) = 2F h/r^2 V_z$$

where F = the total force (N) on the sample, h = the sample height after compression (0.3 cm), and r = the sample radius (cm) after compression. A lower EV indicated more proteolytic activity.

RESULTS AND DISCUSSION

Rheological Evaluation of Proteolytic Enzyme Activity

Figure 1 shows the effect of increasing concentrations of pronase E on the relative viscosity (%) of a SWP050 dispersion as a function of time at 30°C. A progressive decrease in viscosity was found with increasing enzyme concentrations.

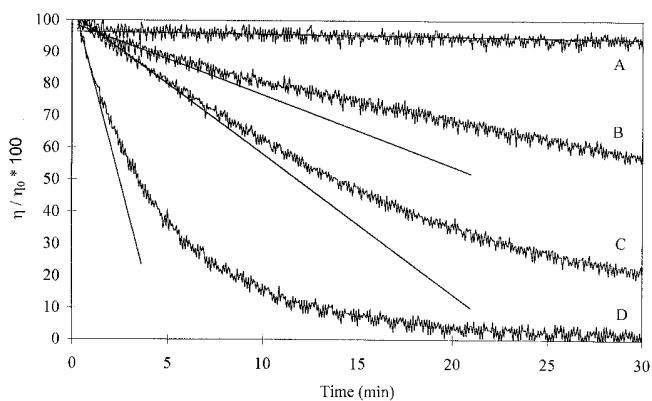


Fig. 1. Effect of increasing concentrations of pronase E (A: 0.0 U_{SAG} ; B: $8.5 \times 10^{-3} U_{SAG}$; C: $2.1 \times 10^{-2} U_{SAG}$; D: $8.5 \times 10^{-2} U_{SAG}$) on relative viscosity (%) of a SWP050 dispersion as a function of time (pH 6.5, 30°C, 30-min incubation time).

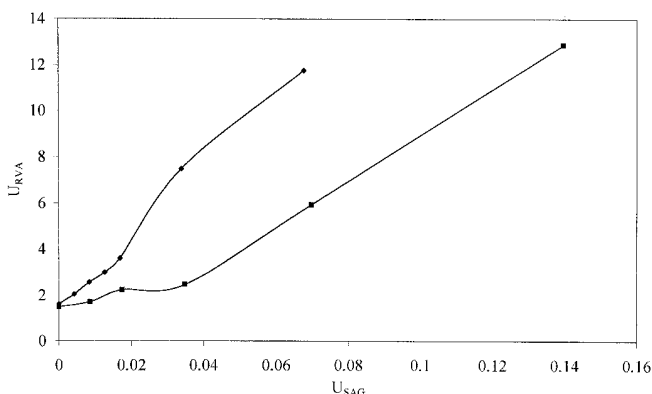


Fig. 2. Activities (U_{RVA}) of pronase E (◆) and papain (■) as a function of concentration (pH 6.5, 30°C, 30-min incubation time).

The activities of two proteolytic enzyme preparations (pronase E and papain) at various concentrations are shown in Fig. 2. Increasing concentrations of pronase E resulted in an almost linear increase in activity. In the case of papain, measurements at higher enzyme concentrations indicated that the plot is curvilinear, as was shown previously by a farinograph technique (Kruger 1971).

Neither α -amylase (20.0 mg/mL) nor carboxypeptidase A (12.0 mg/mL) decreased the viscosity of the SWP050 dispersion (results not shown). Under our experimental conditions, the rheological method only measured endoproteolytic activity.

Pronase E activity was evaluated as a function of temperature. The resulting curve is shown in Fig. 3. The profile shows activity between 30 and 65°C, which agrees with an earlier report (Nomoto and Narahashi 1959) that found pronase E to be very active over a 37–57°C temperature range.

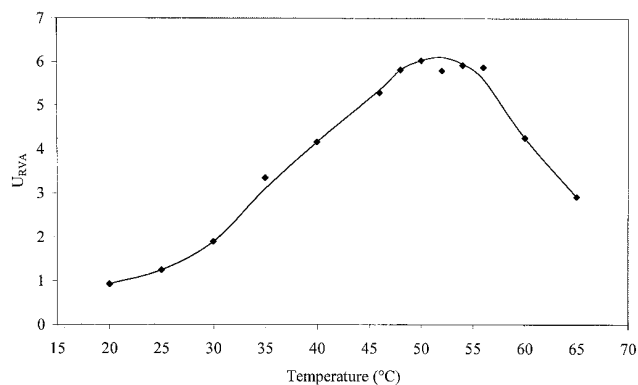


Fig. 3. Activities (U_{RVA}) of pronase E ($8.5 \times 10^{-3} U_{SAG}$) as a function of temperature (pH 6.5, 30-min incubation time).

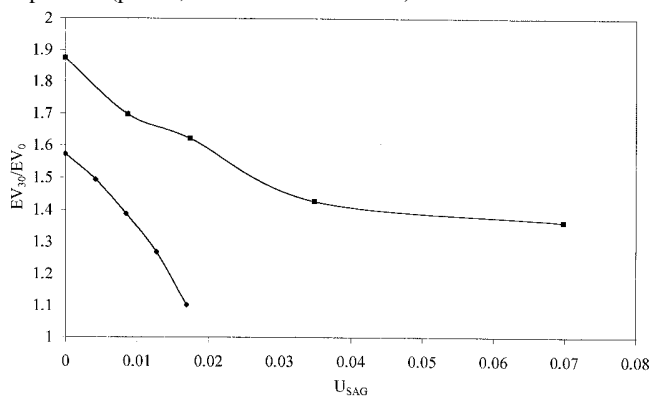


Fig. 4. Decrease in relative elongational viscosity (EV_{30}/EV_0) as a function of pronase E (◆) and papain (■) concentration (pH 6.5, 30°C, 30-min fermentation time).

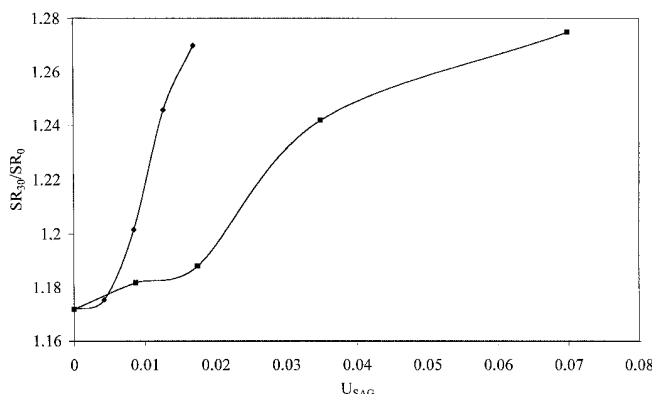


Fig. 5. Increase in relative spread ratio (SR_{30}/SR_0) as a function of pronase E (◆) and papain (■) concentration (pH 6.5, 30°C, 30-min fermentation time).

In a typical experiment, the sample mean (X , $n = 3$) of the activity of protease type XIV from *Streptomyces griseus* (0.5 mg/mL) was $7.75 U_{RVA}$, with a standard deviation of $0.21 U_{RVA}$. The sample mean (X) and standard deviation for $n = 10$ were 7.74 and $0.21 U_{RVA}$, respectively, showing that the method is highly reproducible. Similar results were obtained with pronase E and papain.

Rheological Methods for Evaluating Gluten-Hydrolyzing Enzyme Activity

Changes in EV or SR of gluten-starch doughs as a result of proteolytic action were evaluated. Pronase E and papain enzyme preparations were examined using both methods. A decrease in the relative EV (EV_{30}/EV_0) and an increase in the relative SR (SR_{30}/SR_0) of a gluten-starch dough as a result of increasing enzyme concentrations were found (Figs. 4 and 5). Pronase E caused an almost linear decrease in EV with increasing enzyme concentrations, whereas the decrease in EV with papain was stronger at lower concentrations of added enzyme.

Low concentrations of enzyme had only a small effect on the relative SR of the gluten-starch dough (Fig. 5). At higher concentrations, increasing SR were found, but in general, the effect of proteolytic enzymes on SR was small.

Both the rheological test described by Lin et al (1993) and the spread test described by Hosoney et al (1979) are more labor-intensive than the RVA method developed in our study. The rheological RVA method is able to show SWP050 hydrolysis in a relatively short time, even when small concentrations of proteolytic enzyme preparations are used, whereas longer reaction times are necessary for the other two methods.

RVA is an elegant method for evaluating the temperature and pH profiles of proteolytic enzymes. In both gluten-starch rheological methods tested, it is more difficult to change the pH and temperature conditions.

CONCLUSIONS

We developed a rheological method for evaluating the activity of gluten-hydrolyzing enzymes. The decrease in viscosity of a SWP050 dispersion was measured as a function of time with an RVA. The method is simple, reproducible, and sensitive. Small enzyme concentrations and short incubation periods suffice for evaluating the effects of proteolytic enzyme activities on gluten proteins, possibly due to the fact that proteolytic enzymes react more readily with gluten proteins in a SWP050 dispersion than in a dough system. Proteolytic activities can be measured over a broad temperature range and at pH values higher than 5.5 without dough-handling problems.

One limitation of the RVA method is that proteolytic enzyme activity cannot be determined at pH values lower than 5.5 because of the poor dispersibility of the modified wheat gluten proteins below such values. However, for most applications of wheat flour and gluten, such as soft wheat and yeast-leavened products, this is not a problem.

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