AN AUTOMATED FLUOROMETRIC ASSAY FOR PROTEOLYTIC ACTIVITY IN WHEAT[1]

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ABSTRACT

An automated fluorometric method for the determination of proteolytic activity in wheat extracts is described. The method involves the incubation of a suitable protein substrate with enzyme extract at 40° C. A small proportion of proteolytic cleavage products pass through a dialysis membrane into a basic buffer and react with fluorescamine resulting in the formation of fluorescent products. Activity is expressed in terms of the concentration of glycyl glycine (μmol/ml) required to give a fluorescence intensity the same as that of the cleavage products. The method is applicable to large numbers of samples, requires small amounts of sample, is highly reproducible, gives a linear relation between fluorescence and enzyme concentration over a wide range and has high sensitivity.

In the course of studies involving the purification of wheat proteases, it became apparent that the presently accepted methods for determining cereal proteolytic activity were inadequate. Published procedures involving various modifications of the Ayre-Anderson method (1–3) and gelatin viscosity techniques (4, 5) gave erratic results for fractions of low activity. Both methods were tedious and time consuming and thus not generally applicable to multiple-sample analysis. In addition, these methods required relatively large amounts of sample, a requirement which is unacceptable when numerous analyses are needed during enzyme isolations.

Recently, Schwabe (6) developed a relatively simple and reliable assay for cathepsin D utilizing the reagent fluorescamine [4-phenylspiro-(furan-2(3H, 1′-phthalan) - 3, 3′ dione] which reacts with primary amine groups of amino acids, peptides, and proteins resulting in the formation of highly fluorescent complexes (7). In the present paper, an automated fluorometric assay utilizing this reagent is described for the determination of proteolytic activity in wheat. The method is applicable to multiple-sample analysis, has high sensitivity, requires small amounts of sample, and gives reproducible results over a wide-range of enzyme concentration.

MATERIALS AND METHODS

Reagents

Fluorescamine was purchased from Fisher Scientific Company, Hemoglobin Substrate Powder was obtained from Worthington, edestin from Nutritional Biochemicals, and glycyl glycine from Schwarz-Mann. Gluten (pH 5.8 soluble) was extracted from Manitou, a hard red spring (HRS) wheat, by the method of Shogren *et al.* (8). All other reagents were analytical grade.

Extraction of Proteolytic Activity

Manitou wheat (HRS) was germinated for 5 days at 20° C in the dark and then

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freeze-dried to approximately 10% moisture. All steps for extraction of proteases were done at 4°C. Whole seed (100 g) was homogenized in a Vir-Tis Homogenizer with 300 ml of 0.2*M* sodium acetate-acetic acid buffer (pH 4.5) and then stirred for 60 min and centrifuged. The precipitate was resuspended in 200 ml of buffer, stirred for 60 min, and centrifuged. Supernatants were combined. A similar extraction was done for ungerminated wheat. Proteolytic activity was concentrated by the slow addition of solid ammonium sulfate at 4°C. The ammonium sulfate concentration step was included in order to give a wider range of enzyme concentrations to study. The precipitate—from 40–80% saturation—was isolated by centrifugation, dissolved in 30 ml of water, and dialyzed against three 2000-ml vol of 0.05*M* sodium acetate-acetic acid buffer pH 4.5 at 4°C. Following dialysis, the volume of the concentrated protease extract had increased to approximately 60 ml. The concentrated extracts were stored at 4°C until further use.

Dialyzed extracts of five ungerminated wheat varieties were prepared as described above with the exception that they were not concentrated by ammonium sulfate concentration. Two varieties of durum wheat (Leeds and Wacooma), and three varieties of HRS wheat (Manitou, Cypress, and Prairie Pride) were included.

Manual Assay of Proteolytic Activity

Proteolytic activity was assayed by AACC Approved Method 22-62 (3) with the following modifications. The pH of the hemoglobin substrate solution was adjusted to pH 4.0 and 2-mercaptoethanol at an optimal concentration of 0.1% v/v was included in order to optimize activity. Activity was expressed as HUT/ml where 1 HUT is the amount of enzyme that produces in 1 min a hydrolysate whose absorbance at 275 mμ is the same as that of a solution containing 1.10 γ/ml tyrosine in 0.006*N* hydrochloric acid.

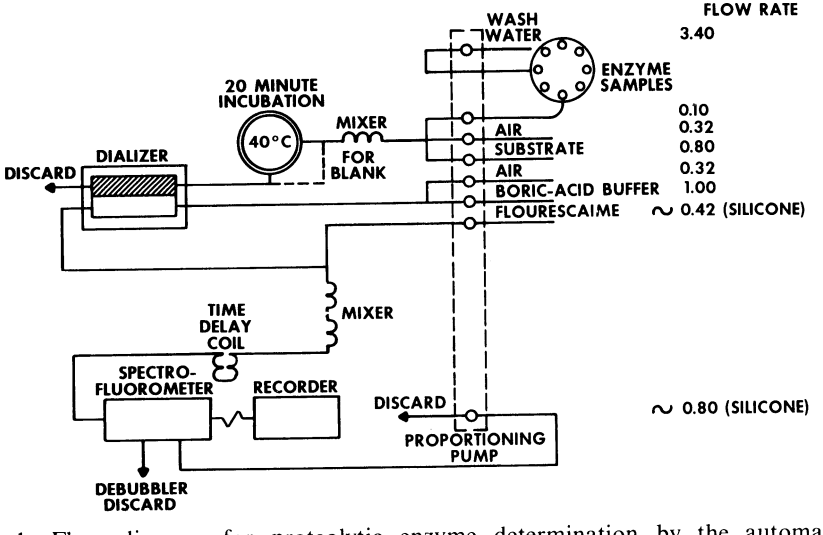

Fig. 1. Flow diagram for proteolytic enzyme determination by the automated fluorometric procedure. The sampling rate was 30/hr with a 1:1 sample to wash ratio.

Automated Fluorometric Assay

The assay system contained the following Technicon AutoAnalyzer™ modules: sampler II; proportionating pump; temperature bath (40° C) equipped with coils giving a flow-through time of approximately 20 min; and a 6-in. dialyzer equipped with a type C membrane (Fisher Scientific). An Aminco-Bowman Ratio Recording Spectrofluorometer equipped with a 0.15-ml quartz flow cell, and a Beckman Model 1005 recorder were used to determine fluorescence intensities. The flow diagram is shown in Fig. 1.

The conditions of the assay, following optimization of buffer pHs and choice of protein substrate (see **Results**) were as follows: the substrate solution was prepared by dissolving 10 g of hemoglobin substrate powder in approximately 300 ml of water. The hemoglobin was denatured by lowering the pH to 1.7 with dilute hydrochloric acid. After stirring 10 min, sufficient sodium acetate was added to give a final concentration of 0.1 *M* and the pH was adjusted to 4.0 with hydrochloric acid. The solution was made to 500 ml with water. Fluorescamine (20 mg/100 ml) was dissolved in acetone. A 0.2 *M* boric acid-sodium hydroxide solution of pH 8.0 was prepared.

As shown in the flow diagram (Fig. 1), hemoglobin substrate solution and sample (30/hr; sample: wash ratio 1:1) were mixed in approximately an 8:1 ratio and passed through a 40° C heating bath coil then through a 6-in.dialyzer. A small proportion of dialyzable amino acids and peptides formed due to proteolysis passed through the dialysis membrane into the boric acid buffer. The boric acid buffer and fluorescamine solution were then mixed (approximately a 3:1 ratio) resulting in the formation of the fluorophores. The solution was then passed through a time-delay coil to allow stabilization of products and then into the spectrofluorometer (ex=390; em=480).

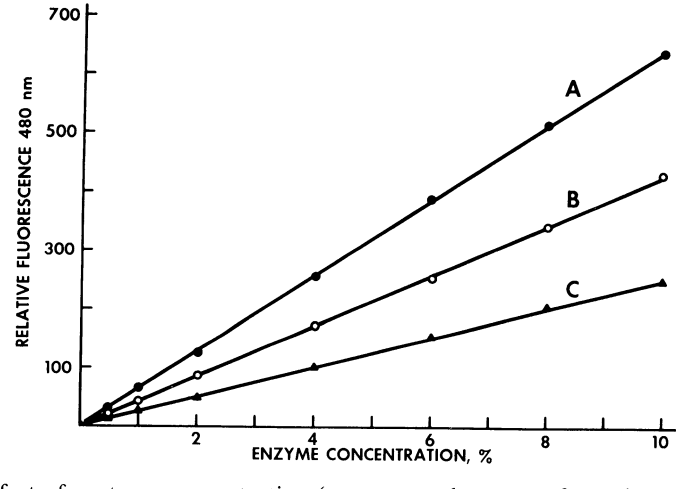

Fig. 2. Effect of protease concentration (concentrated extract of germinated wheat) on fluorescence intensity for different substrates. A = hemoglobin; B = edestin; C = 5.8 soluble gluten. Each substrate solution contained 2% acid-treated protein dissolved in 0.1 *M* or 0.02 sodium acetate-hydrochloric acid pH 4.0 containing 0.1% (v/v) 2-mercaptoethanol.

Blanks were run by disconnecting the temperature bath coils and running the tubing directly into the dialyzer. Activity was calculated from the increase in dialyzable primary amino groups. Results were expressed in μmol/ml of glycyl glycine required to give a similar increase in fluorescence. In practice, various concentrations of glycyl glycine were inserted at various intervals between samples in order to correct for decreases in the rate of dialysis due to hemoglobin deposition on the surface of the membrane.

RESULTS

Protease Extraction

Approximately 85 to 90% of the total proteolytic activity present in the wheat (ungerminated and germinated) extracts was recovered in the ammonium sulfate concentration step. The calculated activities of the concentrated ungerminated and germinated wheat protease extracts were 0.60 and 3.43 HUT/ml[2], respectively, as determined by the modified AACC Method described. The concentrated protease solutions were used in further studies since this allowed a wider range of enzyme concentrations to be studied.

Optimization of the Automated Fluorometric Assay

1) *Choice of substrate.*—Figure 2 shows the relation between fluorescence emission and enzyme concentration (germinated wheat extract) for three substrate proteins: hemoglobin; edestin; and gluten solubilized at pH 5.8. All

[2]One HUT is equivalent to 6.071×10^{-3} μmol tyrosine/min under the conditions of the standard AACC assay.

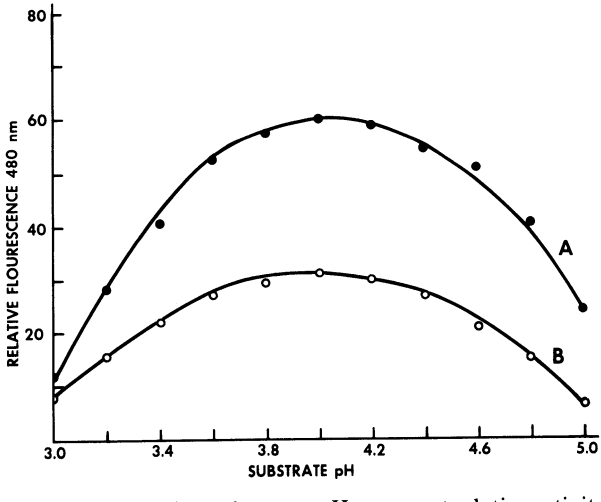

Fig. 3. Effect of hemoglobin substrate pH on proteolytic activity (measured as fluorescence intensity) for germinated and ungerminated concentrated wheat extracts. A = 5% solution of concentrated germinated wheat extract; B = 25% solution of concentrated ungerminated wheat extract. All substrate solutions contained 0.1 *M* sodium acetate-acetic acid and 0.1% (v/v) 2-mercaptoethanol.

substrate solutions contained 2% acid-treated protein dissolved in 0.1*M* (hemoglobin) or 0.02*M* (edestin, gluten) sodium acetate-hydrochloric acid buffer (pH 4.0) containing 0.1% (v/v) 2-mercaptoethanol. As shown in Fig. 2, all substrates gave straight-line relations over a wide range of enzyme concentration. Hemoglobin appeared to be the most effective substrate and was chosen for further studies.

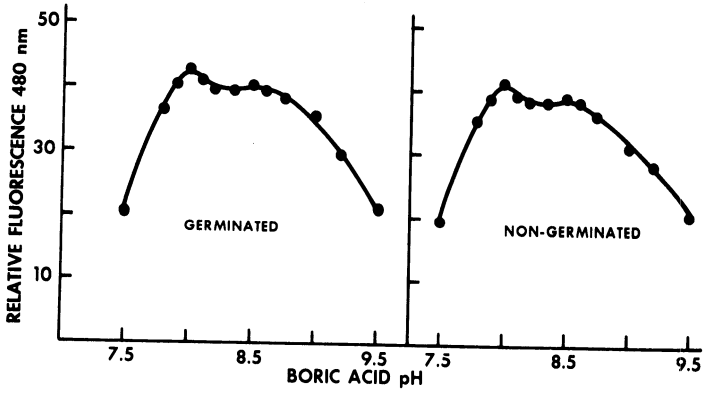

Fig. 4. Effect of boric acid-sodium hydroxide buffer pH on the fluorescence intensity of the reaction of fluorescamine with dialyzable hemoglobin cleavage products due to proteolytic activity. A = germinated wheat extract; B = ungerminated wheat extract.

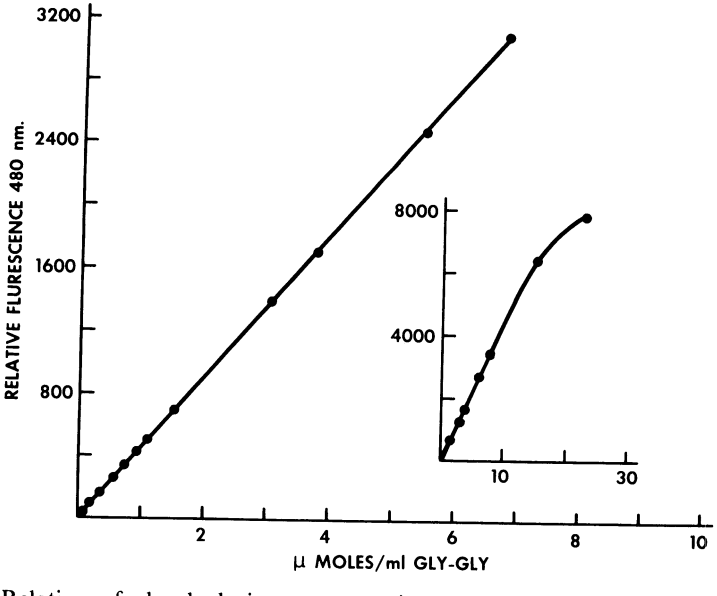

Fig. 5. Relation of glycyl glycine concentration and fluorescent intensity under the standard conditions of the assay.

2) *Effect of substrate pH.*—Figure 3 shows the relation between proteolytic activity (measured as fluorescence intensity) and the pH of the hemoglobin substrate solution. For diluted samples of both germinated (5%) and ungerminated (25%) concentrated wheat extracts, the optimum substrate pH was near pH 4.0. This compares to an optimum pH of 3.8 reported by McDonald and Chen (9) for ungerminated wheat extracts using a modified Ayre-Anderson method with denatured hemoglobin. The difference in optimum pH was probably a result of differences in the methods rather than representing differences in the properties of the enzymes.

3) *Effect of boric acid buffer pH.*—Previous studies (7) have shown that the fluorescence intensity of products formed by the reaction of fluorescamine with primary amino groups of amino acids, peptides, and proteins was pH dependent. Figure 4 shows the relation of fluorescence intensity and pH of the boric acid buffer for ungerminated and germinated wheat. For both extracts two peaks were evident with the most intense peak at pH 8.0. However, it should be noted that the actual optimum pH was near pH 7.7 since the pH of the boric acid buffer is lowered by the passage of low pH substrate buffer through the dialysis membrane.

The optimum pH of 7.7 would seem to indicate that the proteolysis of hemoglobin by wheat proteases produced mainly peptides. Peptides generally give optimum fluorescence near pH 7–8 whereas amino acids give optimum fluorescence near pH 9.0 when reacted with fluorescamine (7).

4) *Standard.*—Glycyl glycine was chosen as a standard due to its availability and its optimum fluorescence with boric acid buffer at a pH similar to that of the enzyme cleavage products. A typical standard curve obtained for glycyl glycine under the conditions of the assay is shown in Fig. 5. A straight-line relation was obtained for the concentration range—0 to 10 μmol/ml. These values actually

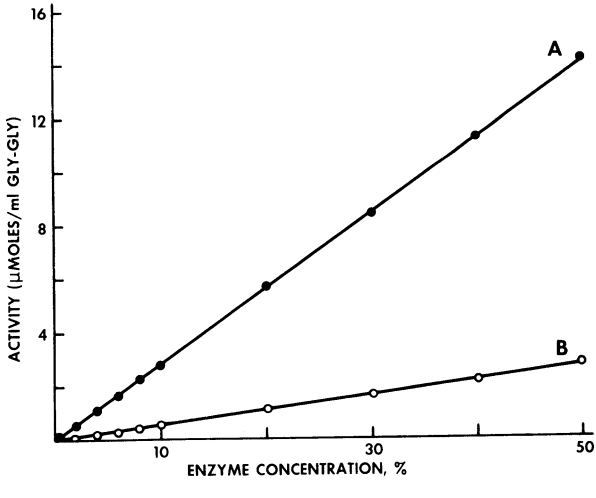

Fig. 6. Relation of protease concentration and activity (expressed as μmol gly-gly/ml) for concentrated germinated (A) and ungerminated (B) wheat extracts.

represent a concentration range of approximately 0 to 50 nmol/ml when sample dilution and dialysis efficiency are considered. Concentrations of less than 0.005 μmol/ml were easily detectable. At higher concentrations (>15 μmol/ml) fluorescent quenching was apparent.

5) *Assay of protease activity.*—Under the conditions of the assay, protease activity (expressed as μmol/ml of glycyl glycine) was directly proportional to enzyme concentration over a wide range of enzyme concentration as shown in Fig. 6. Activities with concentrations as low as 0.1% of the concentrated germinated wheat extract and 0.5% of the concentrated ungerminated wheat extract were easily measured. This represents an activity of approximately 3×10^{-3} HUT/ml (1.8×10^{-5} μmol/tyrosine/min/ml) using standard AACC units. At high concentrations of concentrated germinated wheat extract (>70%), fluorescent quenching took place. Quenching effects could be corrected by comparison with glycyl glycine standards which gave similar quenching effects at high fluorescence intensities.

Over the range of enzyme concentrations used, high reproducibility was obtained. For example, triplicate samples each of glycyl glycine standards containing 0.5, 1.0, and 3.0 μmol/ml and concentrations of germinated wheat extracts (ammonium sulfate concentrated) of 1, 5, and 50% gave variations in fluorescence intensity of less than 2% under the conditions of the assay.

6) *Assay of unconcentrated wheat extracts.*—Dialyzed ungerminated extracts of three HRS wheat varieties and two durum varieties all gave straight-line relations between enzyme concentration and fluorescence intensity under the conditions of the assay. Extracts of the HRS varieties Manitou, Cypress, and Prairie Pride gave activities of 0.48, 0.45 and 0.80 μmol gly-gly/ml, respectively, while values of 0.90 and 0.50 μmol gly-gly/ml were determined for the durum varieties Wacooma and Leeds. Studies are now being carried out to determine if these differences are due to varietal or environmental differences.

DISCUSSION

The automated fluorometric assay described in this paper, based upon the formation of highly fluorescent fluorophores by the reaction of dialyzable hemoglobin cleavage products with fluorescamine, shows promise as a routine method for the determination of proteolytic activity in wheat and related cereals. At present this method is being used extensively in our laboratory for determining activity profiles from chromatographic separations of proteases in ungerminated and germinated wheat extracts and in the routine analysis of flour extracts. This method gives highly reproducible results, is readily applicable to large numbers of samples, and has high sensitivity. The last-mentioned factor is important when activity profiles following fractionations of sound wheat extracts of low activities are analyzed. Since activity can be determined over a wide range of enzyme concentration, extracts differing widely in protease activity can be directly compared. In contrast, the commonly accepted procedures for determining cereal proteolytic activity (1–4) give reliable results over relatively small ranges of enzyme activity, necessitating either dilution steps to decrease activity, or concentration steps and longer incubation times to increase sensitivity. Extracts of low activity usually cannot be measured due to high blank

values and/or inherent low sensitivities associated with the methods.

Although the proteolytic enzyme assay described in this paper was developed for wheat, it should also have applications for the determination of other proteolytic enzymes requiring protein substrates. In contrast to methods measuring an increase in tyrosine or tryptophan (3), nitrogen (1), or the release of protein-bound dyes (10, 11), the fluorescamine based method measures directly the increase in free primary amine groups. It has the advantage over ninhydrin of increased sensitivity (12) and the fluorescamine reaction does not require heating. A previously published fluorescamine based proteolytic assay (6) had the disadvantages of high blank values when unmodified protein substrates were used. The dialysis step in the present assay removes high-molecular-weight proteins and thus overcomes these problems. However, sources of error in the present method may include differences in peptide dialysis efficiency due to differences in the physical and chemical properties of substrate cleavage products, and differences in reactivity and fluorescent properties of peptide, and/or amino acid-fluorescamine reaction products.

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