SULFANILAMIDE-AZOGLUTEN: A CHROMOPHORIC GLUTEN DERIVATIVE FOR COLORIMETRICALLY ESTIMATING PROTEASE ACTIVITY ON GLUTEN[1]

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ABSTRACT

A chromophoric protein derivative has been formed by reacting red azo dye sulfanilamide with gluten. The sulfanilamide-azogluten is acted on by various proteases and offers a convenient quantitative method for predicting activity of various proteases on gluten. The generated soluble dye-bound peptides are easily separated and can be measured with a simple spectrometer.

Various proteases are currently available for modification of gluten for baked products. Accurate measurement of the activity of the gluten-modifying enzymes is often difficult and time-consuming. Two general approaches have been used for estimating the proteolytic activity of such preparations. First, using either casein or hemoglobin as a substrate, the hydrolytic activity has been estimated by the precipitation of the large peptides with trichloroacetic acid (TCA) and measurement of either the nitrogen remaining in solution or the absorbance of the solution in the ultraviolet range. However, Hanford (1) has reported that the Ayre-Anderson method with hemoglobin as a substrate cannot be correlated with gluten softening. A second approach has been suggested by Kruger (2) using a stretching test for gluten to estimate the degree of proteolytic activity. He observed that the stretching characteristics of gluten are markedly affected by incubation with proteolytic enzymes.

Several workers have reported use of dye protein complexes as model systems for estimation of proteolytic activity.

Oakley *et al.* (3) prepared an insoluble azo dye collagen substrate. The azo collagen was reacted with a collagenase from *Clostridium welchii* and correlated with the activity of the collagenase on collagen and on muscle. Mesh size of the azo-collagen was critical since the collagen was an insoluble substrate. Tomarelli *et al.* (4) used azoalbumin as a soluble substrate for the colorimetric determination of peptic and tryptic activity in gastric juices. Nelson *et al.* (5) have further described dye-bound proteins for specific protease assays: indigo carmine-fibrin for pepsin type proteases and Congo red hide powder for trypsin type enzymes. Both substrates were stable in the pH ranges used. Rowsell and Goad (6) used azoglutenin as a substrate to assay for proteolytic activity while studying the release of β-amylase from glutenin-starch.

Apparently, dye-bound proteins can be conveniently used as substrates for the quantitative measurement of proteolytic activity. However, the substrate selection must be as close as possible to the substrate for which the protein dye complex is being substituted. The present work describes a colorimetric method which combines the convenience of the dye and protein substrate with the need to measure the activity of proteases on gluten. In an attempt to have a substrate

[1]Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

which resembles gluten, a sulfanilamide azogluten was produced and tested with various proteolytic enzymes.

MATERIALS AND METHODS

Azocasein (Pentex, Kankakee, Ill.) and substrate grade hemoglobin (Worthington Biochemicals, Freehold, N.J.) were used. Enzymes used for comparison were MLO (a fungal protease which modifies gluten) (Paniplus Co., Kansas City, Mo.), trypsin, chymotrypsin, pepsin, papain (Worthington Biochemical Co.), carboxypeptidase (Cal Biochem, San Diego, Calif.), and dried wheat flour proteinase prepared according to McDonald and Chen (7).

Sulfanilamide-azogluten (SAG) was synthesized by a modification of the casein dye-binding procedure of Charney and Tomarelli (8). Vital gluten (100 g) (Centennial Mills, Portland, Oreg.) was extracted with 3 × 50 ml of ether at room

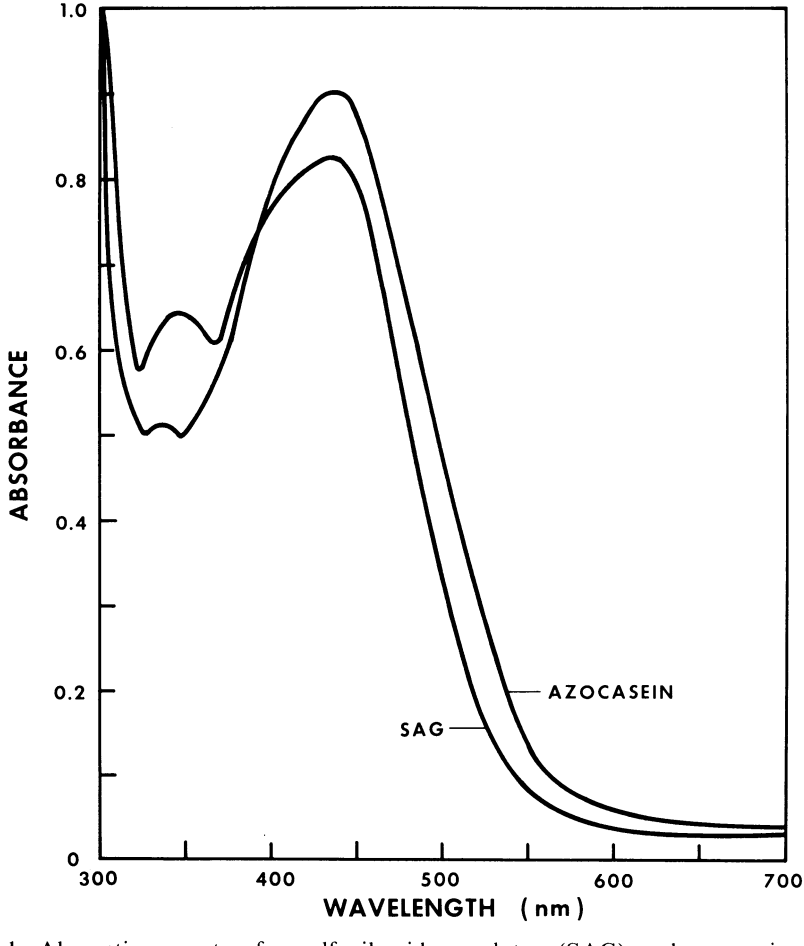

Fig. 1. Absorption spectra for sulfanilamide-azogluten (SAG) and azocasein. One milligram per milliliter protein dye complex in 0.1 *N* NaOH.

temperature and the residual ether removed under vacuum. Fifty grams of extracted gluten was blended in a Waring Blendor for 2 min in 1 l. of 1% $NaHCO_3$ and adjusted to pH 9.0 with 1*N* NaOH.

p-Benzenesulfonamide diazonium chloride (prepared by dissolving 5 g of sulfanilamide (Aldrich) in 200 ml of 0.15*M* NaOH and adding 2.2 g of $NaNO_3$, 18 ml of 5*N* HCl, and after stirring for 2 min, 18 ml of 5*N* NaOH) was added dropwise with stirring over 30 min, the pH was adjusted to 5.0 with 6*N* HCl, and 400 ml of methanol was added. The precipitated SAG was filtered on a Buchner funnel and washed with 3 × 50 ml H_2O and 6 × 50 ml of 95% ethanol. The SAG was then dried overnight under vacuum. Yield: 49.2 g.

Protein dispersibility index (PDI) of SAG and gluten was determined by the AACC method (9).

Various enzymes were added (0.015 to 0.8 mg enzyme nitrogen as determined by micro-Kjeldahl) to 100 mg of substrate in 5 ml of the appropriate solution. The solutions were: papain, 0.06*N* HCl (pH 1.9); trypsin, 0.1*N* phosphate (pH 8.0); and papain, 0.1*N* phosphate (pH 7.25). For MLO enzyme and the freeze-dried wheat flour enzyme, water solutions were used. The pH of the former was

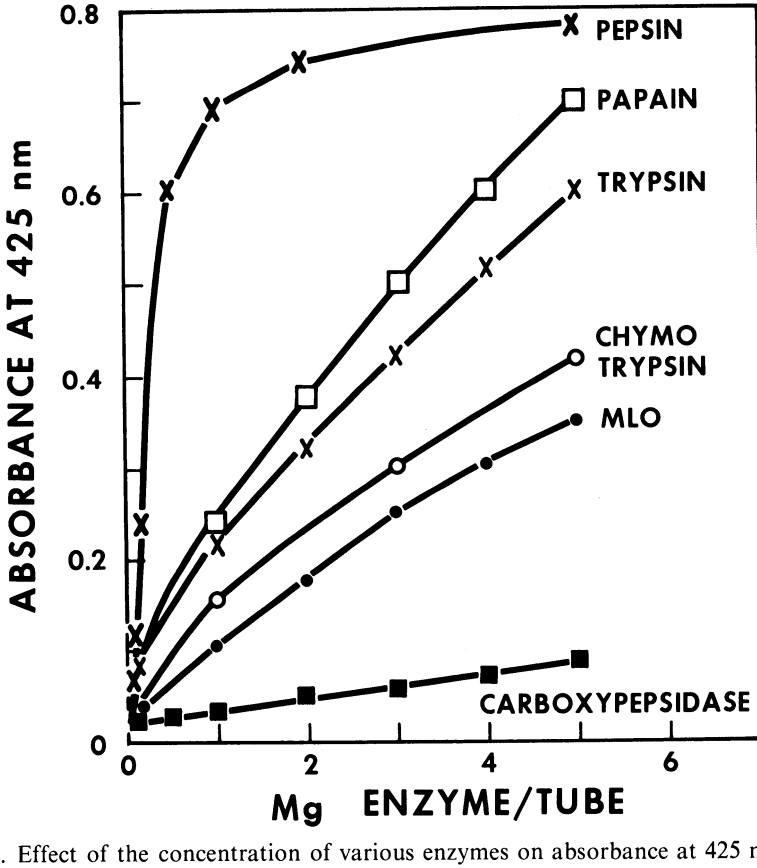

Fig. 2. Effect of the concentration of various enzymes on absorbance at 425 nm after reaction with 100 mg of substrate (SAG) for 30 min.

adjusted to 5.7 and the latter to 5.8 with 0.1*N* HCl. For SAG or gluten, dispersions were prepared with a Waring Blendor (50-ml cup) and were maintained by shaking in test tubes in a gyratory water bath at 150 cpm during incubation with enzymes. It is critical that there is sufficient shaking to maintain dispersion for consistent results.

Enzymes were allowed to react on substrates at 37°C for 5 to 60 min. After reaction, 10 ml of methanol was added and the sample filtered through S & S 576 filter paper. Five milliliters of the filtrate was added to 5 ml of 0.1*N* NaOH.

All spectrophotometric readings were made on a Beckman DK-2 using 1.0-cm cells. Absorbance was read against a blank which contained all reagents including the inactivated enzyme. Absorbances were read for hemoglobin and vital gluten at 280 nm and for SAG and azocasein at 425 nm.

RESULTS AND DISCUSSION

The PDI for SAG and vital gluten from which it was prepared was the same: 96% at pH 1.9, 91% at pH 5.7, 67% at pH 7.25, and 86% at pH 8.0.

Batch-to-batch variation of SAG appeared to be minimal. The blank absorbance compared to water for five batches ranged from 0.012 to 0.023.

In an attempt to demonstrate the similarity of SAG to azocasein, 10 mg of each was dissolved in 10 ml of 0.1*N* NaOH. The spectra for the protein-dye complexes are shown in Fig. 1. Both spectra show a principal absorption maxima at 437 nm. The absorbance per unit weight for SAG is slightly lower than azocasein but is sufficient for reliable measurements of enzyme activity.

The effect of the different enzymes at various concentrations on the hydrolysis of SAG is shown in Fig. 2. The absorbance values were obtained after 30 min of incubation and prepared for reading as described above.

The low activity of carboxypeptidase on the substrate suggests that SAG is not acceptable for use with exopeptidases. The concentration of previous workers on endopeptidases would suggest that dye-bound proteins offer more potential for measurement of endopeptidase activity than for exopeptidases. With exopeptidases it would be necessary for a dye containing amino acid residue to be split off before any apparent activity would be observed.

The effect of freeze-dried wheat flour proteinase preparation on SAG and gluten is given in Table I. The data show linear absorbance up to 0.6 mg enzyme

TABLE I
Effect of Wheat Protease on SAG and Gluten

Enzyme Concentration mg/ml	Changes in Absorbance due to Treatment[a]	
	SAG	Gluten
0.0	0.002	0.010
0.2	0.051	0.019
0.4	0.099	0.028
0.6	0.150	0.036
0.8	0.174	0.046
1.0	0.187	0.055

[a]SAG absorbance was read at 425 nm and gluten at 280 nm.

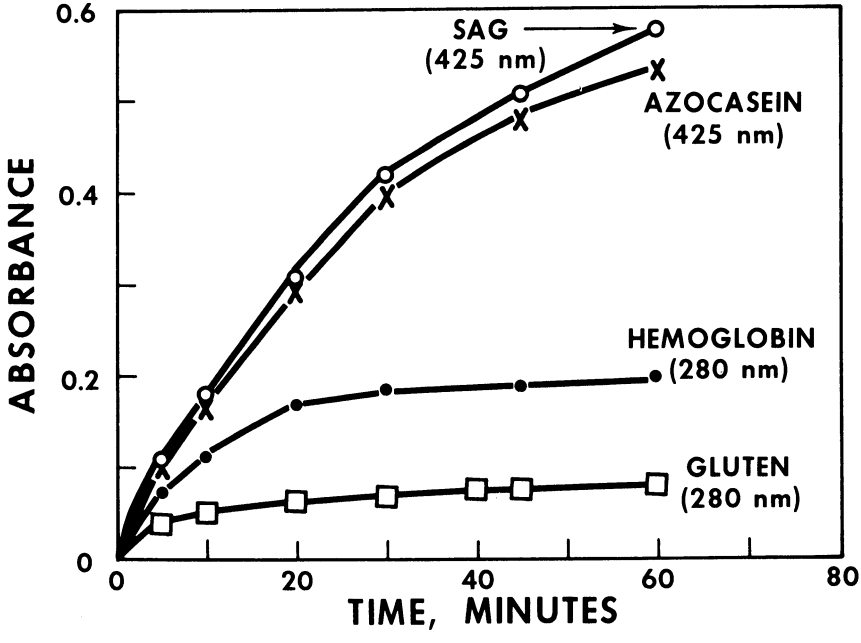

Fig. 3. Effect of time on the final absorbance of substrates treated with trypsin (3 mg/tube).

per ml with SAG and up to 1.0 mg/ml with gluten. The color response with SAG gives more measurable difference in absorbance with increased enzyme concentration than does gluten; however, it is linear over a narrower range of enzyme temperature. The advantage with SAG is the ability to read the absorbance due to the SAG products at 425 nm compared to 280 nm for gluten (visible spectrophotometer *vs.* uv). The absorbance of the SAG products at 425 nm does not change for 4 hr, suggesting excellent stability of the chromophore.

The effect of trypsin on hydrolysis (0–60 min) of hemoglobin, SAG gluten, and azocasein is shown in Fig. 3. The absorbance curves illustrate the greater absorbance using the dye-bound proteins. Under these conditions the reaction rate with gluten is difficult to measure but it appears to level off much sooner than the reaction of trypsin with SAG. Hemoglobin also levels off earlier, which may suggest that shorter reaction times should be employed whenever possible with SAG or azocasein.

In summary, a procedure is given for the preparation of the gluten-dye complex, SAG, whose solubility properties are similar to gluten. This offers a simple method for measuring proteolytic activity on a substrate closely resembling gluten. The degree of hydrolysis can be measured colorimetrically due to the absorbance of the dye-peptide complexes released from the protein by certain proteolytic enzymes.

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[Received September 21, 1974. Accepted February 3, 1975.]