A TRYPSIN INHIBITOR IN WHEAT FLOUR[1]

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ABSTRACT

Aqueous extracts of wheat flour are shown to inhibit the proteolytic action of trypsin on a gelatin substrate by means of the gelation test. The test is described in detail. The inhibitor is present in wheat flours from widely different sources, is not attributable to chemical treatment or additives, and is not associated with the bran or germ fraction.

The existence of a trypsin inhibitor in soya is well known. A study of its persistence in bread doughs to which enzyme-active soya had been added will be described elsewhere. At an early stage of this work, as already briefly reported (6), such doughs were found to have

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a higher antitryptic activity than their soya content could provide. Control doughs, containing nothing but flour, yeast, salt, and water, proved to have antitryptic power also. The source of this activity was sought in the wheat flour used.

Borchers and Ackerson (1), in an extensive search for antitryptic factors in leguminosae and other seeds, reported wheat to be free from trypsin-inhibiting power. The method of Borchers *et al.* (2) is thought to be less sensitive than the gelation test. Though the use of a gelatin substrate has been criticized[3] as sometimes misleading, it has continued over a number of years in the hands of several different operators to give consistent results in these laboratories. Koch and Ferrari (4) and Baker-Munton[4] have reported satisfaction with its use in a viscometric procedure. The present authors prefer the gelation test because of its simplicity. The opportunity is taken to describe it in some detail as applied in the present experiments.

Materials and Methods

The soya used was prepared by grinding whole raw beans to a fineness such that less than 2% was retained by a 60 British Standard sieve. Commercial wheat flours and commercial raw wheat starch were used. Extracts of these were prepared by suspending the flour (1 g.) or the starch (2 g.) in 100 ml. distilled water at 35°C. and shaking every 5 minutes for 1.5 hours. After centrifugation for 10 minutes at 4,500 r.p.m. the extracts were used immediately.

Gelation Test. The method described by Learmonth (5) was modified to suit the small antiproteolytic activity of wheat flour and the higher optimum pH for trypsin. The reagents were: 8% w/v solution of gelatin (powdered microbiological grade; E. Gurr & Son, London); 2*M* phosphate buffer (pH 7.8) consisting of 191.5 ml. 2*M* disodium hydrogen phosphate A.R. ($Na_2HPO_4 \cdot 2H_2O$) and 8.5 ml. *M* citric acid A.R.; 0.02% w/v solution of powdered trypsin (British Drug Houses Ltd., Poole, Dorset, England). All solutions were made with distilled water.

Gelation tubes (22 × 150 mm.) were prepared in duplicate with the first four ingredients as shown in Table I and brought to 38°C. The trypsin solution at 38°C. was added, the sols thoroughly mixed by repeated inversion of the tubes, and the whole batch incubated together at 38°C. for 60 minutes. The tubes were then cooled under running tapwater (ca. 15°C.) for 30 seconds, placed in an ice bath and gently agitated for 90 seconds, and allowed to remain there with as little disturbance as possible until the sols set. The setting time was

[3] Sandstedt, Rudolph M. Private communication, 5 February 1953.
[4] Baker-Munton, S. H. Private communication, 1 June 1961.

Materials and Methods

Preparation of Samples for Analysis

Oxidant. In a small porcelain dish approximately 10 g. of the periodic acid oxidant solution is weighed, evaporated to dryness on a steam bath, and ashed in a muffle furnace for 16 hours at 600°C. To the cooled sample, 1 ml. concentrated hydrochloric acid is added, and the dish is heated again on a steam bath for 0.5 hour. After cooling, the dish contents are transferred into a 100-ml. heat-resistant volumetric flask with approximately 50 ml. of distilled water.

Solid sodium peroxide is added until the mixture becomes alkaline (pH 10). The solution is then concentrated to approximately 10 ml. by boiling. The sodium peroxide oxidizes the chromium present to dichromate, and boiling the solution in the presence of alkali decomposes any remaining sodium peroxide. The concentrate may have a cloudy appearance. After cooling, 50 ml. of distilled water and enough hydrochloric acid (1:1) are added to acidify (pH 4) the concentrate, whereupon the cloudiness disappears. The volume is adjusted to 100 ml. with distilled water.

Dialdehyde Starch. Sample preparation is similar to that for the oxidant. Five grams of starch with a known moisture content are weighed into a small porcelain dish, and 2 ml. of concentrated sulfuric acid are added. After careful charring, the sample is ashed carbon-free and processed as described for the oxidant.

Chromium. The reagent solution is prepared by dissolving 100 mg. of diphenylcarbazide in 10 ml. of glacial acetic acid and adding 90 ml. of absolute ethyl alcohol.

A portion of the sample solution containing 10 to 100 γ of chromium is pipetted into a 100-ml. volumetric flask. One milliliter of reagent is added, and the sample is slowly acidified with 2 ml. of concentrated hydrochloric acid. The sample is diluted to 100 ml. with distilled water and mixed thoroughly. The red color produced by the reagent will develop in 10 minutes. A blank must be prepared at the same time as the sample.

A Coleman Universal Spectrophotometer, Model 14, was used to measure color intensity. The instrument has an arrangement for reading by null balance. This method of reading increases the accuracy of the measurement. The colored sample was contained in a Coleman C cuvet, 3/4 in. o.d. by 4 in. long. These cuvets require at least 10 ml. of solution.

Lead. Commercial-grade diphenylthiocarbazone (dithizone) is used for the reagent preparation. It is purified by dissolving 1 g. in 50 ml.

of chloroform and extracting the solution three times with 75-ml. portions of 1% ammonium hydroxide. The extracts containing the dithizone are combined and acidified with hydrochloric acid. This compound is extracted with three 20-ml. portions of chloroform and recovered by evaporating the chloroform at a temperature not exceeding 50°C. A solution of 10 mg. of the purified dithizone in 100 ml. chloroform is used for the color reaction.

After a preliminary approximation, a portion of the 100-ml. sample solution containing 2 to 20 γ of lead is pipetted into a 50-ml. glass-stoppered separatory funnel. Ammonium hydroxide (1:1) is added to the funnel to raise the pH of the contents above 10. One milliliter of 5% potassium cyanide solution is added to the funnel, followed by 10 ml. of the dithizone reagent. The funnel is shaken vigorously for 30 seconds, and the layers are allowed to separate. The bottom layer, which contains the red-colored complex of lead, is drawn off, and the color intensity is measured as described for the chromium. A blank is prepared simultaneously with the sample.

Results and Discussion

Measurement of Chromium and Lead. Figure 1 shows the results of the measurement of the absorbances of the colored dichromate and

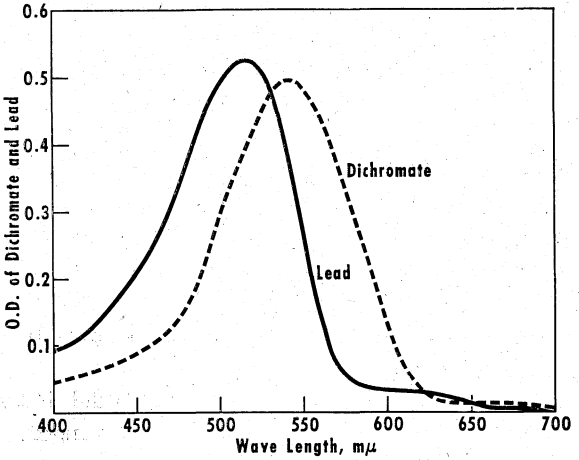

Fig. 1. Adsorption maximum for dichromate (543 mμ) and lead (518 mμ).

lead solutions at wave lengths from 400 to 700 mμ. A peak was observed at 543 mμ (1) for dichromate and 518 mμ for lead. These wave lengths were selected for determining standard curves. Standard solutions were prepared from NBS potassium dichromate and CP lead

nitrate. With these, the absorbances of colored solutions containing known concentrations of metal over a range from zero to 1 γ per ml. for dichromate and from zero to 2 γ per ml. for lead were determined. The absorbances were plotted against a concentration and the least-squares slope calculated. For dichromate, the slope was 2.02 p.p.m. per absorbance unit with a standard deviation from regression of 0.028; whereas for lead, the equivalent statistics were 3.57 and 0.048.

The concentration of dichromate ion and lead in the original samples may be calculated by the following equations:

$$\text{Dichromate (wave length 543 m}\mu\text{)} \quad \frac{2.02 \times \text{absorbance} \times 10^4}{W \times A} = \text{p.p.m.}$$

$$\text{Lead (wave length 518 m}\mu\text{)} \quad \frac{3.57 \times \text{absorbance} \times 10^3}{W \times A} = \text{p.p.m.}$$

W = sample weight (starch on m.f.b.).
A = volume in ml. of the portion of the 100 ml. solution used in the color reaction.

Accuracy. The accuracy of both methods was determined by adding given amounts of standard solutions of dichromate and lead to either a freshly prepared periodic acid solution or regular corn starch. The oxidant solution was prepared by dissolving 25 g. of periodic acid in 250 ml. of distilled water and adjusting the pH to 1.8 by adding a saturated solution of sodium hydroxide.

The intercepts of the regression lines of the accuracy data given in Tables I and II did not differ significantly from the origin, so that calculations of slope (b), and standard deviation from regression(s), were made by using this fact. The errors of these data were compared with the error due to the analytical procedure alone as previously noted. To make this comparison, the previously given errors (0.028

TABLE I

DATA OF TESTING ACCURACY OF DICHROMATE METHOD IN OXIDANT AND DIALDEHYDE STARCH

OXIDANTS (p.p.m. Dichromate)		DIALDEHYDE STARCH (p.p.m. Dichromate)	
Added	Found	Added	Found
24	24	24	22
48	48		
74	76	48	34
98	96	72	68
122	122	244	242
1,316	1,308	732	728
		1,220	1,212
Slope (b)	= 0.994	Slope (b)	= 0.993
Standard deviation from regression(s)	= 0.4	Standard deviation from regression(s)	= 5.8

TABLE II

DATA OF TESTING ACCURACY OF THE LEAD METHOD FOR OXIDANT AND DIALDEHYDE STARCH

OXIDANTS (p.p.m. Lead)		DIALDEHYDE STARCH (p.p.m. Lead)	
Added	Found	Added	Found
42	42	62	60
82	74	82	78
124	108	104	96
164	164	164	168
206	186	186	174
246	242	206	186
310	282	310	290
370	340	412	386
Slope (b)	= 0.929	Slope (b)	= 0.939
Standard deviation from regression(s)	= 8.1	Standard deviation from regression(s)	= 6.1

and 0.048) in the analytical procedure are multiplied by 200 to give 5.6 for dichromate and 9.6 for lead. The close agreement between these errors and those for the equivalent analyses in Tables I and II indicates that the precision of both analyses is not decreased by the procedure for preparing the samples for analysis. There was, however, a lack of complete recovery of lead, as the slightly low values of the slope for lead indicate. The bias, however, is not great enough to be important to the investigation for which these analyses were developed. Obviously the preparative procedure is adequate for both oxidant and starch samples, and the normally interfering iodate ion has been effectively eliminated.

In the lead analysis, the purity of the reagent dithizone is important. When impure reagent is used, the highly colored blank formed makes analysis impossible. In the chromium analysis, the decomposition of sodium peroxide by boiling before acidification is very important. Failure to accomplish complete decomposition will result in reduction of chromate to chromium ion by acidic hydrogen peroxide and will give an erroneous result.

The best accuracy is achieved when absorbance of the solution is between 0.2 and 0.8. Greater sensitivity can, of course, be obtained by taking larger samples for analysis.

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