A COMPARISON OF METHODS FOR THE DETERMINATION OF PROTEOLYTIC ACTIVITY

by

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INTRODUCTION

Numerous methods have been used for the measurement of proteolytic activity. However, due to the host of possible substrates and specific enzymes employed, considerable confusion exists as to which method or methods provides the best approximation of activity as related to dough modification during fermentation. Among the substrates used for proteolytic determinations are casein, gelatin, gluten, hemoglobin, and various protein-dye complexes. Methods used to follow changes in the substrate involve viscometric, soluble nitrogen, clotting time, and light absorption measurements.

It is generally agreed that the proteinases in flour, malt supplements and amylase concentrates modify the flour proteins during the baking process. Many of the methods for determining the degradation of protein may indicate little activity for an enzyme which causes drastic changes in dough properties. Hildebrand (14) suggested that the physicochemical methods may give a better measure of proteolysis in doughs than the chemical methods. The latter are assumed to show an increase in low molecular weight compounds rather than a slight alteration of the whole protein molecule.

Several proteolytic methods were studied comparatively by Hildebrand (14, 15). One of these studies (15) indicated that the gelation-rate procedure of Landis and Frey (21), the viscometric method of Koch, Nelson and Ehrnst (19), and the Ayre-Anderson method (5) gave essentailly similar results for the proteolytic activity of cereal products. The results obtained by Sorenson's formol titration procedure using flour or gelatin as a substrate were not well correlated with those obtained by physicochemical methods using gelatin.

Among the methods with which little or no comparative work has been done are many chemical, physicochemical and colorimetric methods. One of these, a modified Ayre-Anderson procedure (Miller, 23) for the determination of

proteolytic activity in flour, is based on the semi-autolytic digestion of the sample in the presence of an auxiliary substrate, Bacto-hemoglobin. The soluble nitrogen released by proteolysis is determined by a Kjeldahl procedure. Both hemoglobin and gluten have been used as a substrate for this method (Abbott, 1). In a collaborative study of this method, Miller (24) found that the results obtained by various workers and duplicate determinations by the same worker showed considerable variation.

The determination of free carboxyl or amino groups released during the digestion of flour, gelatin or casein has been used by a number of workers to show the activity of many proteinases of plant and animal origin. Hildebrand (14), using a formol titration procedure, obtained similar results by employing either flour or gelatin as a substrate. Howe and Glick (17) employed the Linderstrom-Lang acetone method (22) for the titration of amino groups and the Willstatter and Waldschmidt-Leitz alcohol method (28) for the titration of free carboxyl groups released by proteolytic activity in wheat products. These workers found that the increase in free amino groups gave a more reliable measure of proteolysis than the increase in carboxyl groups since there was a non-enzymatic release of carboxyl groups due to hydrolysis of ester linkages. In a study of proteolysis in flours, Balls and Hale (6) used alcohol solutions and alcoholic potassium hydroxide to determine the increase in the number of carboxyl groups during proteolysis.

A number of physicochemical methods are described in the literature for the determination of proteolytic activity. The modification of dough properties as measured by the Farinograph best demonstrates the action of proteolytic enzymes in a dough. Landis (20), who was the first to employ this technic, based the determination of proteinase activity on the decrease in development energy of a standard dough after thirty minutes rest time. The rate of decrease in development

energy of the dough was found to be a linear function of time and of enzyme concentration. The Farinograph method employed by Miller and Johnson (26) involved the measurement of the consistency of a sponge dough containing an excess of alpha-amylase after four hours digestion. The consistency of the dough was noted after 15 minutes mixing, and the difference in consistency between doughs with and without added proteinase was taken as a measure of proteolytic activity. These workers found that the decrease in consistency was directly proportional to the enzyme concentration.

Two other physicochemical methods have been described in the literature. One of these involving the ability of enzymes to clot milk has been used for the measurement of proteolytic activity (Balls and Hoover, 7). The mechanism involves the change of casein to the more insoluble calcium paracaseinate. Balls and Hoover (7) found that the time of clotting was inversely proportional to the enzyme concentration. Another method which involves the change in viscosity of an acidulated flour-water suspension due to proteolysis was used by Cairns and Bailey (8) to show the presence of proteinases in flour. These workers found that the decrease in viscosity paralleled the increase in amino nitrogen as determined by chemical methods.

Several methods have been described employing the absorption of light for the measurement of proteolysis. Anson (3) based his analysis on the digestion of denatured hemoglobin. The color produced by an alkaline solution of digestion products with phenol reagent (Folin and Ciocalteu, 12) was determined as a milliequivalents of tyrosine, which was related by means of a calibration curve to the activity of the particular enzyme involved. A modification of this method was described by Northrop, Kunitz, and Herriott (27) which was based on the absorption of light by tyrosine and tryptophan at 280 mu. Abbott, Miller, and Johnson (1) applied this technic to the analysis of soluble nitrogen from the

Ayre-Anderson digestion. These workers found a high correlation between the increase in total soluble nitrogen as determined by the Kjeldahl procedure and the soluble chromophoric materials released from either hemoglobin or gluten.

A number of colorimetric methods involving protein-dye complexes have been devised. Carroll (9) showed that bovine serum albumin combines with Orange I dye, which is released upon denaturation or hydrolysis of the protein. The colorimetric estimation of the protein remaining in the solution gives a quantitative measure of proteolytic activity. A procedure based on the digestion of protein coupled with a diazotized aryl amine was described by Charney and Tomarelli (10). The digestion of the azoprotein results in the formation of colored compounds which are soluble in trichloracetic acid, and the intensity of color is directly proportional to the proteinase concentration. Greif (13) noted that protein may be completely precipitated from an acid solution by the addition of the indicator, Bromsulphalein. The color intensity of an alkaline solution of the precipitate is proportional to the amount of protein present, and the decrease in color is therefore a measure of proteolytic activity. The method using Bromsulphalein appears convenient and adaptable to colorimetric procedure for routine analysis.

Although the methods for determining proteolytic activity are numerous, few comparative studies have been made. Since interest in and use of proteinases in breadmaking are assuming more and more importance, this work was undertaken to determine which methods give a satisfactory measure of proteolysis in doughs. The methods employed in this study were selected on their adaptability to a wide variety of conditions and to routine determinations.

The methods included the Ayre-Anderson procedure employing either Bacto-hemoglobin or gluten substrate and Kjeldahl as well as spectrophotometric determinations of soluble nitrogen, the Bromsulphalein method, the Farinograph consistency technic, and a method based on the viscosity of an acidulated flour-water suspension, the milk-clotting technic, and a formol titration procedure.

MATERIALS AND METHODS

Enzyme Sources

Enzymes representing plant, animal, fungal and bacterial sources were employed. The following enzyme preparations were used: Rhozyme P-11[1] (fungal), Rhozyme-S[1] (fungal), Protease 26[1] (fungal), Protease 15[1] (bacterial), trypsin, papain, and malted wheat flour. Rhozyme-S and Protease 26 were derived from Aspergillus oryzae but were prepared in different ways.

Enzyme Preparation

The preparation of the enzymes for assay was similar for all the methods with few exceptions. The malted wheat flour was extracted with water for 30 minutes, centrifuged, and filtered through cotton. The other preparations were extracted for 15 minutes and filtered through S and S #507 filter paper. Papain was activated with cysteine (10% by weight of papain). In all cases, the most concentrated enzyme solutions were prepared, and the less concentrated enzyme solutions were made by appropriate dilutions.

Preparation of enzyme extracts for analysis by the Farinograph technic required a variation in procedure due to the extended nature of the technic. The extracts of Rhozyme-S and Rhozyme P-11 were adjusted to a hydrogen-ion concentration of pH 5.5 for greater stability during the course of adding enzyme increments to the dough. Trypsin was weighed separately for each dough and added to the other dry ingredients. Several extracts of Protease 15, Protease 26, and malted wheat flour were prepared at short time intervals to avoid inactivation of the enzymes before adding them to the dough.

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[1]Obtained from Rohm and Haas Co., Philadelphia, Pa.

Farinograph Technic

The procedure employed was essentially that outlined by Miller and Johnson (26). A dough consisting of 490 g of unmalted flour, 2.8 g Arkady[1], 20 ml of 1.0 N acetic acid, and 300 units of proteinase-free amylase, and the proteinase extract in 265 ml water was mixed for two minutes. After four hours digestion at 30° C., 550 g of the dough was transferred to the large bowl of the Farinograph and the consistency noted after 15 minutes of mixing. The difference in consistency between doughs with and without added proteinase was taken as a measure of proteolytic activity.

The proteinase-free amylase was prepared according to Miller and Johnson (25). The proteinase in a 0.2% calcium chloride solution of Rhozyme-S (2mg/ml) was inactivated by heating at 50° C. for 30 minutes at a hydrogen-ion concentration of pH 10.0. A decrease in dough consistency of approximately 80 Brabender units resulted from the use of 60 ml of this preparation.

Viscosity Technic

This method was based on the work of Cairns and Bailey (8). Since their procedure was quite laborious, the procedure for the apparent viscosity of an acidulated flour-water suspension as outlined in Cereal Laboratory Methods (2) was adopted. For each determination 20 g of flour (14% moisture basis) was weighed into a 250 ml Erlenmeyer flask, and 100 ml of enzyme and water was added. The hydrogen-ion concentration of this mixture was pH 5.8. During the four hours digestion at 30°C. each flask was shaken every 15 minutes. After digestion, several drops of octyl alcohol were added to reduce foaming, and the slurry

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[1]Commercial yeast food containing dough oxidant.

transferred to a MacMichael viscosimeter. The viscosity was noted after the addition of 1, 3, 5, and 7 ml of 1.0 N lactic acid and the maximum value recorded. The decrease in viscosity between determinations with and without enzyme was taken as a measure of proteolytic activity. Enzyme concentrations were chosen to give a decrease in viscosity no greater than 100° MacMichael.

Milk-clotting Technic

The milk-clotting method of Balls and Hoover (7) was used. Twenty grams of dry, whole milk[1] and 10 ml of acetate buffer (pH 4.2) were diluted to 100 ml with water. The hydrogen-ion concentration of the resulting solution was pH 5.5. One ml of enzyme solution was added rapidly to 10 ml of this milk solution at 40° C., and the clotting time noted. The end-point was taken as the time when the milk showed a thickening on tilting the test tube. The concentrations of each enzyme were selected to give a clotting time range of 0.5-10 minutes. Activity was expressed as the reciprocal of the time of clotting.

Ayre-Anderson Procedure

The procedure as outlined in Methods of Analysis (4) was modified to provide enough filtrate for spectrophotometric determinations. For each determination 2.67 g of Bacto-hemoglobin were weighed into each of two 125 ml Erlenmeyer flasks and pumice added. Enzyme solution and acetate buffer (pH 4.7) to make 50 ml total volume were added, and the mixtures thoroughly shaken. A 10 ml portion of 36% trichloroacetic acid was added to one flask immediately and to the second flask after 5 hours digestion at 40° C. After the addition of trichloroacetic acid the flasks were shaken for 30 minutes and filtered through S and S #597 filter paper.

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[1]The Borden Company, New York.

Aliquots (10 ml) of the filtrate were used for the Kjeldahl determinations of nitrogen. Activity was expressed as increase in titration value of 0.0714 N sodium hydroxide.

For the digestion of gluten, 3 g of undenatured gluten was used as a substrate instead of Bacto-hemoglobin. After the mixtures were precipitated with trichloroacetic acid, they were centrifuged and filtered using Watman #5 filter paper. Solutions which were cloudy after centrifugation were boiled 5 minutes and the evaporated water replaced before final filtration. The rest of the procedure was identical with that used for the hemoglobin digestion method.

Spectrophotometric Determination of Soluble Nitrogen

Aliquots of the filtrate obtained from the Ayre-Anderson digestions of hemoglobin or gluten were diluted (1:10) and the optical density determined at 275 mu with a Beckman spectrophotometer. An appropriate dilution of trichloroacetic acid and buffer was used as a blank for standardization of the instrument. The difference in optical density between the sample and the corresponding blank was taken as a measure of proteolytic activity.

Formol Titration Procedure

The basic procedure for this method was outlined by Koch (18). The digestion time, hydrogen-ion concentration and other conditions were selected arbitrarily to conform to the other methods. Bacto-hemoglobin (10 mg/ml) was dissolved with dilute acetate buffer (pH 4.7) for 30 minutes, centrifuged and filtered. Equal volumes of substrate and enzyme solution were mixed and digested for one hour at 30° C. Before and after digestion, 20 ml aliquots were added to 15 ml of a neutralized (pH 8.5) 10% formalin solution, and the resulting mixture titrated to pH 8.5 with standard sodium hydroxide. Beckman glass electrode equipment was

employed for these titrations. The titration values were calculated as ml of 0.05 N sodium hydroxide and the activity was expressed as the difference in titration values of the mixture before and after digestion. Enzyme concentrations were employed which gave a range of at least 1.20 ml difference in titration.

Bromsulphalein Method

Several modifications of the procedure outlined by Greif (13) were necessary to use hemoglobin as a substrate. Preliminary experiments were performed to determine a suitable substrate concentration, and the concentration of Bromsulphalein, the time of digestion, and the hydrogen-ion concentration necessary for maximum precipitation of the substrate.

Bacto-hemoglobin (10mg/ml) was dissolved in acetate buffer (pH 4.7) for 30 minutes, centrifuged, and filtered. The Bromsulphalein[1] solution was prepared by diluting 5 ml of a 5% aqueous solution of the indicator to 100 ml with citrate-phosphate buffer (McIlvaine, pH 3.0). One ml of the enzyme solution was added to one ml of substrate in a short-tapered centrifuge tube, and the mixture digested for one hour at 30° C. The reaction was stopped by the addition of 2 ml of the Bromsulphalein solution. The tubes were centrifuged for 10 minutes at 1800 rpm, and the excess liquid removed by suction. The precipitate was washed with 5 ml of mixed buffer (2 parts citrate-phosphate, 1 part acetate, 1 part water), centrifuged and the liquid again removed by aspiration. The precipitate was then dissolved in 2 ml of 10% sodium hydroxide, transferred quantitatively to a volumetric flask and diluted to 200 ml. The optical density of the resulting solution was determined on a Beckman spectrophotometer at 570 mu. The difference between the optical densities of zero and one hour digestion times was taken as a measure of proteolysis.

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[1]Disodium phenoltetrabromphthalein. Obtained from Hynson, Wescott and Dunning, Inc., Baltimore, Md.

EXPERIMENTAL

The regression equations expressing the relationship between enzyme concentration and activity for each enzyme preparation analyzed by the various methods are presented in Table 1. For purposes of statistical comparisons of the data for the various methods, the relationships between enzyme concentration and activity were assumed to be linear, although in a few instances curvilinearity was indicated.

The data obtained by the Bromsulphalein method were curvilinear for all the enzyme systems investigated. The papain analysis data required a square transformation to produce a linear relationship between enzyme quantity and its activity. A transformation to the 3/2 power was required for Rhozyme P-11, Rhozyme-S, Protease 26, and malted wheat flour. No suitable transformation was found for the bacterial proteinase, Protease 15.

Analysis of the data obtained by each method indicated that there were no real differences between the slopes of the regression lines for analyses performed on different days. Differences which did occur may be due to slight variations in weighing or diluting the enzyme preparations, or to changes in the temperature of digestion. Two different levels of activity were shown by analyses performed on different days with the Farinograph technic and in a few cases with other methods. This variation may be due to a variation in substrate or instruments from day to day.

The relative precision of the several procedures may be seen by comparing the variation between replicate determinations and the variation about the regression line. The coefficients of variation (C. $V._e$) for each method based on the standard deviation of the average activity values for a given enzyme concentration are presented in Table 2. These data indicate the variability between replicate

Table 1. Summary of regression equations for the relationship between enzyme concentration and activity for all enzyme preparations and methods employed.

Enzyme	Method		
	Ayre-Anderson; Kjeldahl determinations		
	Hemoglobin substrate	Gluten substrate	Formol
Rhozyme P-11	-0.133 + 4.1186 X	0.2125 + 3.657 X	1.0767 + 0.8102 X
Rhozyme-S	1.134 + 3.8009 X[3]	0.6341 + 1.179 X[1]	0.3337 + 1.3689 X
Protease 26	-0.370 + 0.0858 X	0.4684 + 0.068 X	
Malted wheat flour	0.344 + 0.0062 X[3]		0.3635 + 0.0029 X
Papain	-0.890 + 2.0930 X	0.2425 + 1.388 X[1,2]	
Protease 15	-0.678 + 4.2205 X	0.5441 + 3.334 X	
Trypsin	-1.500 + 0.1662 X	0.3838 + 0.048 X	

	Bromsulphalein	Farinograph[1]	Milk-clotting
Rhozyme P-11	0.8182 X	19.875 + 2.2875 X	0.019 + 0.0603 X
Rhozyme-S	0.5890 X	3.751 + 2.133 X	0.004 + 0.0146 X
Protease 26	0.0104 X	0.000 + 0.0355 X	0.035 + 0.0035 X[1]
Malted wheat flour	0.00125 X	1.550 + 0.0043 X	
Papain	0.1321 X[4]		-0.087 + 0.1646 X
Protease 15		8.050 +11.300 X[2]	0.024 + 0.1041 X
Trypsin		16.850 + 0.4730 X	0.0000+ 0.0068 X

	Ayre-Anderson; Beckman determinations		
	Hemoglobin substrate	Gluten substrate	Viscosity
Rhozyme P-11	0.022 + 2.157 X[1,2]	0.0734 + 0.1770 X	-3.660 + 1166.25 X[1,2]
Rhozyme-S	0.058 + 0.1822 X	0.1857 +0.0344 X	4.575 + 245.50 X
Protease 26	0.019 + 0.0038 X	0.0956 + 0.0035 X	-1.320 + 21.426 X[1,2]
Malted wheat flour	0.041 + 0.0003 X		8.55 + 0.127 X
Papain	0.005 + 0.1003 X	0.0678 + 0.0299 X	
Protease 15	-0.027 + 0.2132 X	0.0632 + 0.1230 X	4.75 + 134.00 X
Trypsin	-0.097 + 0.0108 X	0.0671 + 0.0032 X	5.85 + 20.90 X

[1] Intercepts differ significantly between analyses on different days.
[2] Regression coefficients differ significantly between analyses of different days.
[3] Data curvilinear.
[4] Transformed to the 2nd power.

samples. The large variability for the Farinograph and Bromsulphalein technics indicate that more subsamples should be employed for a level of precision comparable with the other methods. The coefficients of variation (C. $V._r$) based on standard deviation from regression also are presented in Table 2. These statistics indicate the fit of the data to the regression lines. The large value for the Bromsulphalein method is due primarily to the regression line being forced through the origin. Although the variation for the Bromsulphalein method was larger than that for the other methods, this method is considered valuable because of its simplicity and speed of analysis.

Table 2. Summary of statistical calculations showing the precision of the various methods.

Method	C. $V._e$	C. $V._r$
Farinograph	15.5	7.1
Hemoglobin-Kjeldahl	4.1	6.2
Hemoglobin-Beckman	4.1	7.4
Bromsulphalein	16.0	17.0
Formol titration	6.0	3.5
Gluten-Kjeldahl	9.3	5.5
Gluten-Beckman	7.0	2.8
Milk-clotting	6.0	4.6
Viscosity	10.9	5.4

The variability of the several enzyme preparations are presented in Table 3. The largest variation between duplicate determinations (C. $V._e$) was exhibited by papain and Rhozyme-S. Malted wheat flour and papain showed greater variation about the regression line (C. $V._r$) than the other preparations.

Table 3. Summary of statistical calculations showing the variability of each enzyme averaged over all the methods.

Enzyme	C. V.$_e$	C. V.$_r$
Rhozyme P-11	7.2	6.6
Rhozyme-S	10.2	5.6
Protease 26	8.1	6.9
Malted wheat flour	9.5	10.5
Papain	12.6	8.2
Protease 15	7.0	3.4
Trypsin	9.4	5.9

The regression equations in Table 1 have been utilized as a source of basic data to facilitate comparison of the various methods. The regression coefficients between enzyme concentration and activity for each enzyme preparation analyzed by the various methods are presented in Table 4. The milligrams of each enzyme which possess an arbitrary level of activity were calculated from the regression equations for each method and are presented in Table V.

Table 4. Coefficients of regression between enzyme concentration and activity determined by several methods.

Method	P-11	Rho-S	P-26	MWF	Papain	P-15	Trypsin
Farinograph	2.29	2.130	0.036	0.0043		11.30	0.473
Hb-Kjeldahl	4.12	3.080	0.086	0.0062	2.09	4.23	0.166
Hb-Beckman	0.22	0.182	0.004	0.0003	0.10	0.21	0.011
Bromsulphalein	0.82	0.589	0.010	0.0012			
Formol	0.80	1.369		0.0029			
Gluten-Kjeldahl	3.66	1.179	0.068		1.39	3.33	0.048
Gluten-Beckman	0.18	0.034	0.004		0.03	0.12	0.003
Milk-clotting	0.06	0.015	0.004		0.17	0.10	0.007
Viscosity	1166.0	245.5	21.43	0.127		134.0	20.9

Table 5. Quantities of enzyme showing equivalent activity for a given method of analysis.

Method	Activity base	P-11	Rho-S	P-26	MWF	Papain	P-15	Trypsin
Farinograph	50 B. U.	13.16	21.68	1492.5	11267		3.71	70.08
Hb-Kjeldahl	3 ml.	0.76	0.49	39.3	428	1.86	0.87	27.08
Hb-Beckman	0.2 O. D.	0.83	0.78	47.6	530	1.94	1.07	27.50
Bromsulphalein	0.2 O. D.	0.24	0.34	19.3	160	1.51*		
Formol	2 ml	1.14	1.22		564			
Gluten-Kjeldahl	2 ml	0.49	1.15	22.5		1.29	0.46	33.70
Gluten-Beckman	0.2 O. D.	0.71	0.50	29.5		5.77	1.09	40.00
Milk-clotting	0.5 l/t	7.98	33.97	132.9		3.56	4.57	73.53
Viscosity	50° MacM.	0.05	0.19	2.3	330		0.34	2.13

*Transformed to the second power.

DISCUSSION

There are several ways in which the data in Table 4 and Table 5 may be compared to show the relationship between methods. The only direct comparison of methods which could be made was that involving the change in soluble nitrogen as determined by the Kjeldahl and spectrophotometric procedures. The correlations between increased titration and optical density values were highly significant for each enzyme when either hemoglobin or gluten was used as substrate. The coefficients of correlation and the regression coefficients are presented in Table 6. These results corroborate the work of Abbott, Miller and Johnson (1).

Table 6. Summary of the relationship between optical density and soluble nitrogen for several different enzyme preparations acting on two substrates.

Enzyme source	Substrate: Hemoglobin b	Hemoglobin r*	Gluten b	Gluten r*
Rhozyme P-11	0.0515	0.983	0.0474	0.984
Rhozyme-S	0.0484	0.995	0.0298	0.964
Protease 26	0.0437	0.872	0.0503	0.971
Malted wheat flour	0.0495	0.995		
Papain	0.0479	0.998	0.0203	0.937
Protease 15	0.0468	0.982	0.0366	0.983
Trypsin	0.0587	0.905	0.0701	0.979

*All are significant at the 0.1% level.

Indirect comparisons of the data involving the other methods must be employed, since the levels of enzyme used varied widely among the methods and the units of activity have no common basis. The activities of each enzyme determined by the various methods are compared with the activity of Rhozyme P-11 in Tables 7 and 8 by dividing the activity for each enzyme by the activity of Rhozyme P-11 for each method. A constant ratio of activities for a given enzyme for several methods indicates that these methods measure the same type of activity or give corresponding results for the enzymes involved.

Table 7. Ratios of regression coefficients for several enzymes compared with Rhozyme P-11.*

Method	P-11	Rho-S	P-26	MWF	Papain	P-15	Trypsin
Farinograph	1	0.93	0.016	0.0019		4.97	0.206
Hb-Kjeldahl	1	0.92	0.021	0.0015	0.51	1.03	0.040
Hb-Beckman	1	0.84	0.018	0.0014	0.46	0.99	0.051
Bromsulphalein	1	0.72	0.013	0.0014			
Formol	1	1.71		0.0036			
Gluten-Kjeldahl	1	0.32	0.019		0.38	0.91	0.013
Gluten-Beckman	1	0.19	0.020		0.17	0.695	0.019
Milk-clotting	1	0.25	0.058		2.73	1.72	0.116
Viscosity	1	0.21	0.018	0.0001		0.115	0.018

*Based on data in Table 4.

Table 8. Ratios of activity for several enzymes compared with Rhozyme P-11.*

Method	P-11	Rho-S	P-26	MWF	Papain	P-15	Trypsin
Farinograph	1	1.65	113.4	856		0.28	5.32
Hb-Kjeldahl	1	0.64	51.6	563	2.44	1.14	35.58
Hb-Beckman	1	0.94	57.7	642	2.36	1.29	33.33
Bromsulphalein	1	1.39	79.0	656	6.19**		
Formol	1	1.07		495			
Gluten-Kjeldahl	1	2.35	45.9		2.57	0.90	68.77
Gluten-Beckman	1	0.70	41.6		8.13	1.53	56.34
Milk-clotting	1	4.26	16.7		0.45	0.57	9.21
Viscosity	1	4.07	50.5	7253		7.43	46.68

*Based on data in Table 5.
**Transformed to the second power.

The Farinograph technic has been used as a standard for comparison of the methods in Tables 9 and 10. The ratio of the regression coefficients (Table 9) expresses the rate of change in activity measured by a specific method for each unit change measured by the Farinograph. The data in Table 10 expresses the ratio of an arbitrary level of activity of the various enzymes determined by a given method to a different arbitrary level of activity determined by the Farinograph. A constand ratio for all the enzymes analyzed by a given method indicates that the method gives similar results and measures the same type of activity as measured by the Farinograph technic.

Table 9. Ratios of regression coefficients for enzyme activity measured by different methods compared with that measured by the Farinograph.*

Method	P-11	Rho-S	P-26	MWF	P-15	Trypsin
Farinograph	1	1	1	1	1	1
Hb-Kjeldahl	1.79	1.780	2.42	1.44	0.375	0.351
Hb-Beckman	0.09	0.086	0.107	0.07	0.019	0.023
Bromsulphalein	0.35	0.281	0.293	0.29		
Formol	0.35	0.643		0.67		
Gluten-Kjeldahl	1.60	0.553	1.914		0.295	0.101
Gluten-Beckman	0.08	0.016	0.099		0.011	0.007
Milk-clotting	0.03	0.007	0.099		0.009	0.015
Viscosity	509.	115.	605.0	29.5	11.7	44.2

*Based on data in Table 4.

Table 10. Ratio of enzyme activity for several methods compared with that obtained by the Farinograph technic.*

Method	P-11	Rho-S	P-26	MWF	P-15	Trypsin
Farinograph	1	1	1	1	1	1
Hb-Kjeldahl	0.058	0.023	0.026	0.038	0.235	0.386
Hb-Beckman	0.063	0.036	0.032	0.047	0.287	0.392
Bromsulphalein	0.018	0.016	0.013	0.014		
Formol	0.087	0.056		0.050		
Gluten-Kjeldahl	0.037	0.053	0.015		0.124	0.481
Gluten-Beckman	0.054	0.023	0.020		0.294	0.571
Milk-clotting	0.606	1.570	0.089		1.230	1.050
Viscosity	0.004	0.009	0.002	0.029	0.091	0.030

*Based upon data in Table 5.

The comparative data (Tables 7-10) show that the results for several methods parallel those for the Farinograph technic. The data obtained for malted wheat flour and the fungal concentrates indicate that the Kjeldahl or spectrophotometric determinations of soluble nitrogen products from hemoglobin digestion, the Bromsulphalein method, and the formol titration procedure all measure the same degree of activity as that indicated by Farinograph consistency measurements. These relationships also indicate that the Bromsulphalein indicator and trichloroacetic acid each precipitate similar fragments from the protein digestion mixture. Since these results are correlated with changes in dough consistency, it is likely that these precipitants remove only the protein or larger protein fragments.

The comparative data (Tables 7-10) also indicate a similarity of action between trypsin and Protease 15. These results provide further evidence of the similarity between bacterial and animal enzymes which was noted previously by Fischer and de Montmollen (11). These enzymes appear to behave in a similar manner when analyzed by the Farinograph, the milk-clotting, and the hemoglobin and gluten digestion technics.

A comparison of the analyses obtained with methods involving gluten digestion, milk-clotting and viscosity measurements shows no consistent trends for these procedures. The importance of substrate in proteolytic determinations is shown by the differences in the digestion of hemoglobin and gluten by the Ayre-Anderson procedure. The failure of the viscosity and the milk-clotting technics to be related to the other methods may be attributed to a number of factors, including substrate differences, hydrogen-ion concentration, and type of activity being measured.

No single relationship can be defined relating the results of a particular method to those of the Farinograph for all proteinase preparations. However, by

dividing the preparations into two groups, one including the plant and fungal enzymes and the other including animal and bacterial preparations, the results for several methods appear to be sufficiently well correlated with the Farinograph technic to give a good estimate of activity in doughs. These methods include the Ayre-Anderson digestion of hemoglobin with Kjeldahl or spectrophotometric determinations, the Bromsulphalein method, and the formal titration procedure. The Ayre-Anderson procedure was applicable to all the proteolytic preparations employed and the spectrophotometric modification makes this procedure fairly simple. The Bromsulphalein method is both rapid and simple, and may be employed for most proteolytic preparations. The formol titration procedure, although very rapid and easy, was not suitable for the determination of preparations with low activity.

SUMMARY

A number of methods suitable for the determination of proteolytic activity have been investigated using enzyme preparations from different sources. The widely different experimental conditions of the various methods made direct comparisons of the data impossible. However, from the statistical analyses of the data and from indirect comparisons of methods and enzyme preparations, the following information has been obtained:

1. The relationships between enzyme concentration and activity were linear for all enzyme preparations analyzed by the Farinograph technic, the Ayre-Anderson digestion of hemoglobin or gluten with either Kjeldahl or spectrophotometric determinations, the viscosity technic, the formol titration, and the milk-clotting technic. The few exceptions were assumed to be linear to facilitate comparisons of the methods.

2. The data for all enzymes analyzed by the Bromsulphalein method were curvilinear. The papain data required a square transformation to produce linearity between enzyme concentration and activity. The data for Rhozyme-S, Rhozyme P-11, Protease 26, and malted wheat flour required a transformation to the 3/2 power. No suitable transformation was found for the analysis data of Protease 15.

3. The slopes of the regression lines relating enzyme concentration and activity were constant from day to day with very few exceptions.

4. Significantly different levels of activity for analyses performed on two days were shown for all preparations analyzed by the Farinograph technic and for some preparations analyzed by several other of the methods.

5. For the milk-clotting, Farinograph, viscosity, and Bromsulphalein technics, and spectrophotometric analysis of hemoglobin digestion products, the intercepts on the Y-axis were not significantly different from zero.

6. The enzyme preparations showed marked differences in variability. Papain, Rhozyme-S, and malted wheat flour showed the most variation.

7. A high correlation was found between soluble nitrogen determined by Kjeldahl and spectrophotometric procedures using either hemoglobin or gluten as a substrate.

8. The large variation between duplicates for the Farinograph and Bromsulphalein methods indicate that more subsamples must be employed with these methods to obtain a level of precision equal to that of the other methods.

9. The enzyme preparations employed appear to fall into two groups according to their activity. Trypsin and the bacterial proteinase, Protease 15, compose one group, while the fungal preparations and malted wheat flour compose another group.

10. For both groups of enzymes, the Farinograph technic, the Ayre-Anderson digestion of hemoglobin with either Kjeldahl or spectrophotometric determinations, the formol titration procedure and the Bromsulphalein method gave similar results.

11. The methods involving gluten digestion, milk-clotting and viscosity measurements showed no consistent trends among the enzymes.

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A COMPARISON OF METHODS FOR THE DETERMINATION OF PROTEOLYTIC ACTIVITY

by

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Since interest in and use of proteinases in breadmaking are assuming more and more importance, this work was undertaken to determine which methods of analysis give a satisfactory measure of proteolysis in doughs.

The following proteolytic methods were investigated: The Ayre-Anderson procedure employing either Bacto-hemoglobin or gluten substrate and Kjeldahl as well as spectrophotometric determinations of soluble nitrogen, the Bromsulphalein method, the Farinograph consistency technic, and a method based on the viscosity of an acidulated flour-water suspension. The enzyme preparations investigated included malted wheat flour, papain, trypsin, a bacterial and three fungal concentrates.

The relationships between enzyme concentration and activity were assumed to be linear for all methods except the one employing Bromsulphalein. Suitable linear transformations of the data from the latter method were found for all enzyme preparations except the bacterial concentrate. A comparison of the relative variation of the methods indicated that the Farinograph and Bromsulphalein technics require more subsamples to provide a level of precision equal to that of the other methods. The analyses based on replications of different enzyme levels showed marked differences in variability. Papain, Rhozyme-S and malted wheat flour exhibited the greatest variation.

The enzyme preparations appear to classify into two groups according to the similarity of their action on the different substrates. One group includes the animal and bacterial preparations. The other group includes the fungal preparations and malted wheat flour.

The Farinograph consistency technic was used as the standard procedure for estimating proteolysis in doughs. Indirect comparisons of the results obtained by the Ayre-Anderson digestion of hemoglobin using either Kjeldahl or spectrophotometric determinations of soluble nitrogen, the Bromsulphalein method, and

the formol titration procedure indicate that these methods give results essentially similar to those obtained with the Farinograph. The failure of the technics involving gluten digestion, the clotting of milk, and viscosity measurements to give results similar to those obtained with the other methods emphasizes the importance of substrate, hydrogen-ion concentration and type of activity measured by different proteolytic methods.