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Collaborative Evaluation of an Enzymatic Starch Damage Assay Kit and Comparison with Other Methods

T. S. GIBSON,[1] C. J. KALDOR,[1] and B. V. McCLEARY[2]

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ABSTRACT

A commercially available enzymatic assay kit for the measurement of starch damage in wheat flour was compared with current standard methods, and the kit's precision and repeatability were determined in a collaborative study. Starch damage values determined on a range of flours with the assay kit correlated well ($r > 0.96$) with those determined by existing standard enzymatic methods. The precision of the kit was evaluated in a comprehensive interlaboratory study. The kit procedure was found to be highly repeatable (relative standard deviation, 2.94-6.80%) and reproducible (relative standard deviation, 5.00-10.30%).

A proportion of the starch granules in wheat grains is mechanically damaged during the milling process (Evers and Stevens 1985). These damaged granules hydrate rapidly and are susceptible to amylolytic hydrolysis. Consequently, they contribute significantly to the water absorption, rheology, handling properties, and gassing power of a dough and to crumb texture and crust color (Tipples 1969).

The industry standard methods for starch damage measurement are based on the preferential amylolytic digestion of damaged granules with crude commercial preparations of malt (Farrand 1964, Royal Australian Chemical Institute 1988) or fungi (American Association of Cereal Chemists [AACC] 1983, Donelson and Yamazaki, 1962). However, an improved enzymatic assay for starch damage that avoids many of the potential inaccuracies associated with the use of crude enzyme preparations recently was developed (Gibson et al 1992) and is now supplied commercially in kit form.

The aim of this work was to evaluate the reproducibility and repeatability of the proposed new method for starch damage through an extensive interlaboratory study and to correlate values obtained by the kit method on a range of flours with those obtained by standard starch damage assay procedures.

MATERIALS AND METHODS

Starch Damage Assay Kit

Enzymatic assay kits based on the method developed by Gibson et al (1992) were supplied by MegaZyme Pty Ltd., North Rocks,

[1]Biological and Chemical Research Institute, NSW Agriculture, Rydalmere, NSW, 2116 Australia.
[2]MegaZyme (Aust.) Pty Ltd., North Rocks, NSW, 2151 Australia.

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Australia. Each assay kit contained directions for the preparation of the extraction buffer (0.1M acetate, pH 5.0, containing 0.005M $CaCl_2$) and six reagent vials which contained the following items:

1) α-amylase (EC 3.2.1.1) from *Aspergillus niger* (10 ml, 1,000 U/ml, in 3.2M ammonium sulfate) diluted 20-fold with extraction buffer before use.

2) amyloglucosidase (EC 3.2.1.3) from *Aspergillus niger* (4 ml, 200 U/ml, in 3.2M ammonium sulfate) diluted 10-fold with extraction buffer before use.

3) glucose oxidase-peroxidase-4-aminoantipyrine glucose determination reagent (GOPOD) supplied as a freeze-dried powder, sufficient to prepare 1 L of reagent.

4) GOPOD reagent buffer concentrate (50 ml, 1M potassium phosphate, pH 7.4, containing 3.0% *p*-hydroxybenzoic acid and 0.4% sodium azide), sufficient to prepare 1 L of GOPOD reagent buffer. The buffer concentrate is diluted to 1 L with distilled water and mixed with reagent 3 before use. The diluted solution is stable for at least 3 months at 4°C.

5) glucose standard solution (10 ml, 150 μg/ml, in 0.2% aqueous [w/v] benzoic acid).

6) wheat reference flour, with a starch damage level of 6.8%.

All enzymes and the GOPOD reagent in the starch damage assay kit are stable under normal conditions of transportation. α-Amylase and amyloglucosidase were electrophoretically homogenous. Analytical reagent-grade chemicals were used in the preparation of assay reagents. Test flours were provided by the Bread Research Institute, North Ryde, and by the Agricultural Research Center, Tamworth. The reference flour was a commercial flour supplied by George Weston Foods Laboratories, Enfield.

Details of the assay method are given in Gibson et al (1992). Briefly, α-amylase (1 ml, 50 U, preequilibrated at 40°C) was mixed vigorously with 100 ± 5 mg of flour sample for 5 sec in a 12-ml glass round-bottom test tube and then incubated for exactly 10 min at 40°C without further mixing. The reaction was terminated with sulfuric acid (5.0 ml, 0.2% v/v), and the tubes were centrifuged at 1,000 × *g* for 5 min. Aliquots of the supernatant (0.1 ml) were incubated with amyloglucosidase solution (0.1 ml, 2 U) for 10 min at 40°C. GOPOD reagent (4 ml) then was added to each tube, mixed on a test-tube stirrer, and the incubation was continued for another 20 min at 40°C. The absorbance at 510 nm then was measured. The method in this collaborative evaluation specified a 5-min incubation with amyloglucosidase, which was sufficient time for the reaction to proceed to completion. However, this incubation time has been increased to 10 min (Gibson et al 1992) to compensate for any potential loss in amyloglucosidase activity on extended storage.

Calculation of Starch Damage

Starch damage is measured as anhydroglucose derived from starch in damaged granules and is expressed as starch as a proportion (percentage) of total flour weight on an as-is basis (no correction is made for water content):

$$*E \times F \times 60 \times \frac{1}{1{,}000} \times \frac{100}{W} \times \frac{162}{180} = \frac{*E}{W} \times F \times 5.4$$

where $*E$ is absorbance after amyloglucosidase treatment read against the blank absorbance; F is a factor for conversion of absorbance values to micrograms of glucose (150 μg of glucose/absorbance for 150 μg of glucose); 60 is a volume correction factor (0.1 of 6.0 ml was analyzed); 1/1,000 is a conversion from micrograms to milligrams; W is a weight of sample analyzed, $100/W$ is a factor to express starch damage as a percentage of flour weight; and 162/180 is a factor to convert free glucose to anhydroglucose, as occurs in starch.

Other Assay Methods

Starch damage was determined in 26 soft and hard wheat flours by the method of Farrand (1964) for comparison with values determined (in duplicate) by the kit method. George Weston Foods Laboratories supplied the malt flour for the preparation of α-amylase (1,250 U/g as defined by Farrand 1964). J. R. Donelson of the U.S. Department of Agriculture Agricultural Research Station Soft Wheat Quality Laboratory, Wooster, supplied 21 wheat flours and their respective starch damage values determined by AACC method 76-30A and the Donelson-Wooster method (Donelson and Yamazaki 1962). These flours were analyzed (in duplicate) by the kit method.

α-Amylase activity was determined by the Ceralpha procedure (MegaZyme Pty Ltd.), and amyloglucosidase activity was determined on soluble starch at pH 4.5 and 40°C (McCleary and Sheehan 1987).

One unit (U) of activity is defined as the amount of enzyme required to release one micromole of glucose reducing-sugar-equivalents per minute under the defined assay conditions (Gibson et al 1991).

Design of the Collaborative Study

Assay kits, 10 homogenous test flours, and complete instructions were sent to 36 participating laboratories in a split-level (Youden pairs) experiment designed in accordance with Association of Official Analytical Chemists (1989) guidelines. The test flours supplied to the collaborators ranged in starch damage from 2.3 to 7.3% (based on total flour weight). This is the range to be expected in commercial flours derived from soft and hard wheats when determined with the assay kit. One of the test flours (sample E) was identical to the reference flour sample. Each participant was asked to become familiar with the assay by repeated analyses of the reference flour supplied and then to analyze each sample only once.

Twenty-eight sets of results were received from 24 laboratories, with several laboratories providing repeat determinations, and then analyzed according to Australian Standard 2850-1986 (Standards Association of Australia 1986). This standard is based on standard 5725 of the International Organization for Standardization. Results for the 10 test samples were treated as five pairs, each pair member having a starch damage value similar to the other member (i.e., Youden pairs). Dixon's test for outliers (Standard Association of Australia 1986) was applied to means and differences for each pair of samples. Pairs for which Dixon's test was significant at the 1% level were omitted from the analyses of variance. Components of variance for within-laboratory (S_r^2) and between-laboratory (S_L^2) variation were determined from the analyses of variance of each pair of results and $S_R^2 = S_r^2 + S_L^2$ was calculated. Australian Standard 2850-1986 method defines repeatability (r) as the 95% confidence interval for repeat analyses under identical conditions in the same laboratory (2.83 S_r) and reproducibility (R) as the 95% confidence interval for repeat analyses on identical materials in separate laboratories: $2.83(S_R)^{1/2}$. We also calculated the relative standard deviations (RSD_r and RSD_R) of the S_r and S_R. Several laboratories provided repeat determinations, performed several days apart and/or by different analysts. Therefore, the data provided two mean squares that estimate repeatability. The two estimates were pooled by calculating the average mean square weighted by the degrees of freedom for each mean square. This was considered to be preferable to employing the mean values or to arbitrarily discarding one or more sets of analyses by these collaborators.

RESULTS AND DISCUSSION

Collaborative Evaluation

Table I lists the laboratories involved in the interlaboratory study. Table II shows the starch damage values determined in the test samples by the collaborating laboratories. The figures in the last column are the means of repeated assays of the samples ($n = 8$) by the NSW Agriculture Laboratory and represent the nominal values for the test samples. The figures in the final column are the starch damage values reported by the collaborators for the reference flour supplied. Repeat determinations reported by collaborators 06, 11, and 16 are listed as separate results. The starch damage values for collaborators 06 and 11 were determined by the same analyst on different occasions. The results for colla-

TABLE I
Laboratories Participating in the Interlaboratory Evaluation

Laboratory	Location	Collaborator
Agricultural Research Institute	Wagga Wagga, NSW, Australia	J. Oliver
Western Australian Department of Agriculture	South Perth, WA, Australia	G. Crosby
George Weston Foods Laboratories	Enfield, NSW, Australia	J. Robertson
Victorian Crop Research Institute	Horsham, Victoria, Australia	J. Panozzo
Bunge Ballarat	Ballarat, Victoria, Australia	G. Walker
Bunge Bioproducts Pty Ltd.	Altona North, Victoria, Australia	R. White
Arnotts Research Centre	Homebush, NSW, Australia	M. O. Andrade
Biocon (Aust.) Pty Ltd.	Boronia, Victoria, Australia	V. Powell
Grain Research Laboratories	Winnipeg, Manitoba, Canada	P. Williams
General Mills Inc.	Minneapolis, MN, USA	R. H. Bowers
U.S. Grain Marketing Research Labs	Manhattan, KS, USA	B. W. Seabourn
North Dakota State University	Fargo, ND, USA	B. D'Appolonia
University of Nebraska	Lincoln, NE, USA	R. Graybosch
ICBD, Heriot-Watt University	Edinburgh, Scotland	A. Lynn
Flour Millers and Bakers Research Association	Chorleywood, UK	A. Evers
Agricultural Canada Research Station	Winnipeg, Manitoba, Canada	O. M. Lukow
Nisshin Flour Milling Co., Ltd.	Tokyo, Japan	H. Waku
North Carolina State University	Raleigh, NC, USA	D. R. Lineback
Goodman Fielder Mills Ltd.	Tamworth, NSW, Australia	I. Brown
University of Minnesota	St. Paul, MN, USA	E. H. Asp
Heygates Ltd.	Northampton, UK	R. J. Keeping
Spillers Milling Ltd.	Cambridge, UK	P. C. Green
The Lord Rank Research Centre	High Wycombe, Bucks, UK	M. Rogers

TABLE II
Collaborative Results for Starch Damage Determinations by the Starch Damage Assay Kit in a Range of Flours

	Test Sample										
Collaborator	A	F	B	J	C	H	D	G	I	E	Reference Flour[a]
01	5.9	5.4	2.3	1.7	3.5	3.2	5.7	5.7	7.3	7.0	6.7
02	5.8	5.3	2.2	2.4	3.4	3.0	5.6	5.5	7.1	6.9	6.9
03	5.9	5.5	2.4	2.6	3.3	3.2	5.7	5.6	7.2	6.9	6.8
04	5.8	5.4	2.2	2.5	3.3	3.1	5.4	5.6	7.1	6.6	6.9
05	5.8	5.1	2.2	2.5	3.3	3.0	5.3	5.6	7.3	6.7	6.8
06/1	5.7	5.3	2.3	3.1	3.3	3.1	5.5	5.6	7.2	6.7	6.7
06/2	5.9	5.3	2.3	4.2[b]	3.4	3.0	5.4	5.5	7.0	6.5	...
06/3	5.3	4.8	2.2	3.6[b]	3.4	3.0	5.5	5.1	9.0	7.3[b]	6.7
07	5.9	5.7	2.6	2.7	3.3	3.3	6.0	6.0	7.3	7.3	7.1
08	5.7	5.5	2.1	2.6	3.2	3.1	5.4	5.6	7.0	6.7	6.8
09	5.8	5.3	2.1	2.3	3.3	2.8	5.5	5.6	7.0	6.5	6.6
10	6.1	5.8	2.6	3.0	3.8	3.5	6.0	6.1	7.5	6.9	7.0
11/1	5.6	5.2	2.2	2.8	3.3	3.1	5.5	5.6	7.1	6.7	6.8
11/2	5.4	5.1	2.1	2.4	3.4	3.0	5.5	5.5	7.2	6.7	6.7
12	5.7	5.1	2.1	2.6	3.4	2.8	5.4	5.2	6.7	6.4	6.8
13	7.2	5.3[b]	2.8	2.6	3.6	3.5	6.7	5.8	7.5	6.9	...
14	5.5	4.9	2.2	2.4	2.9	2.9	5.5	5.1	6.7	6.6	6.7
15	5.6	4.9	2.1	2.2	3.2	2.9	5.2	5.0	6.9	6.7	6.4
16/1	4.7	5.0	2.0	2.3	3.1	2.9	5.2	5.3	7.0	6.4	6.4
16/2	5.1	5.0	2.3	2.2	3.2	3.4	5.1	5.6	7.7	6.1[b]	...
17	5.7	5.0	1.9	2.2	2.9	2.6	5.4	5.6	6.5	7.1	6.8
18	5.1	5.0	2.1	2.3	3.3	3.0	5.0	4.9	6.4	6.4	6.5
19	5.4	4.9	2.2	2.2	3.3	3.0	5.4	5.3	6.6	6.5	6.6
20	5.3	5.1	2.0	2.3	3.1	2.6	5.0	5.4	6.2	6.4	6.6
21	5.5	4.7	2.1	2.2	3.1	2.8	5.0	5.3	6.3	6.2	6.4
22	5.3	4.3	1.8	2.2	2.9	2.6	4.7	5.0	6.3	6.2	5.8
NSW Agriculture[c]	5.9	5.5	2.3	2.6	3.5	3.2	5.7	5.8	7.3	7.0	6.8
Labs retained	25		24		26		26		24		
Outliers removed	3		4		2		2		4		
Mean	5.36		2.33		3.15		5.46		6.80		
r[d]	0.604		0.457		0.361		0.558		0.574		
R[e]	0.918		0.692		0.641		0.984		0.952		
S_r[f]	0.21		0.16		0.13		0.20		0.20		
S_R[g]	0.32		0.24		0.23		0.35		0.34		
RSD_r,[f] %	3.92		6.86		4.12		3.66		2.94		
RSD_R,[g] %	5.97		10.30		7.30		6.41		5.00		

[a] Values reported for reference flour supplied.
[b] Outlier data pairs (1% level) omitted from analysis.
[c] Means of repeated determinations ($n = 8$) by the NSW Agriculture Laboratory.
[d] Repeatability (within laboratory).
[e] Reproducibility (between laboratories).
[f] Repeatability relative standard deviation.
[g] Reproducibility relative standard deviation.

borator 16 were determined by different analysts in the same laboratory.

The Dixon test identified as outliers pair A/F from collaborator 13, pair B/J from collaborators 06/2 and 06/3, and pair E/I from collaborators 06/3 and 16/2 (1% significance level). The test rejected the results for all of the sample pairs of two collaborators. These results are omitted from Table II. One of these collaborators made major modifications to the procedure. Reasons for the poor performance of the other collaborator were not clear. Analysis of the results for the Youden pairs for each collaborator yielded RSD_r values between 2.94 and 6.86% and RSD_R values between 5.00 and 10.30%. These provide an estimate of the within-laboratory coefficient of variation and of the combined between-laboratory and within-laboratory coefficients of variation, respectively.

The results of collaborators who did not strictly follow the directions are included in the analysis. For example, several collaborators did not have access to a vortex mixer and mixed the sample with the enzyme by hand and several used bulb rather than hand-held pipets to dispense the α-amylase. Both of these deviations could reduce the reproducibility of the assay and the efficiency of the mixing, thereby increasing the variance determined for the assay.

This study is the first to subject an enzymatic starch damage assay to a detailed interlaboratory evaluation of precision, although several have evaluated the intralaboratory precision (repeatability) of different assays (Williams and Fegol 1969, Williams and LeSeelleur 1970, Dodds 1971, Finney et al 1988). The reproducibility data in this study (S_r and RSD_r), derived from analyses by a number of laboratories, are comparable to the corresponding data reported in these earlier studies, which are derived from the analyses of one laboratory and probably by one or two analysts. Also, the analysts in several of the laboratories in this study were not familiar with the small volumes and additional care required for the kit assay. It is probable that the RSD_r values obtained would be improved with experience in using the assay. We consistently achieve RSD_r values of less than 4%.

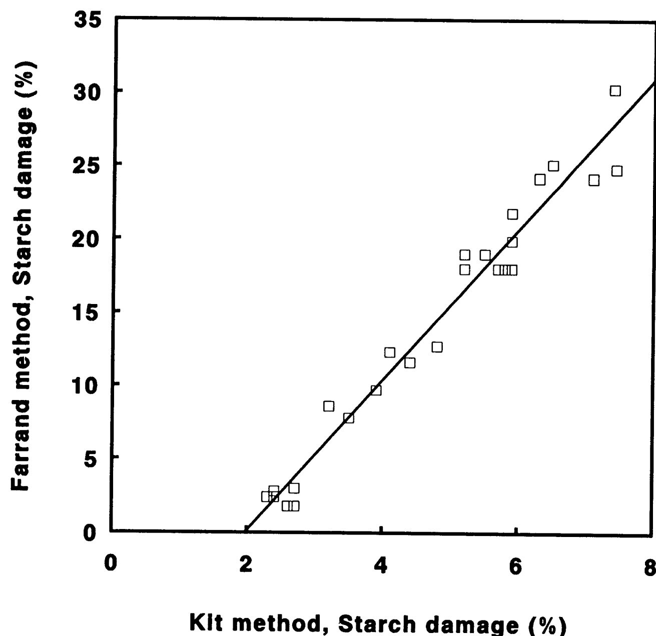

Fig. 1. Relationship between starch damage in a range of flours determined by the starch damage assay kit and determined by the Farrand (1964) method. Data points are means of duplicate determinations.

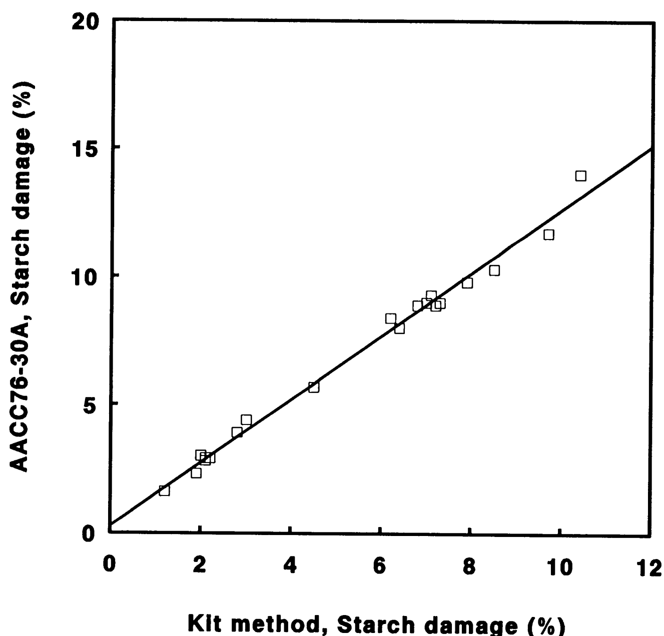

Fig. 2. Relationship between starch damage in a range of flours determined by the starch damage assay kit and determined by AACC method 76-30A (data provided by J. R. Donelson). Data points for the kit method are means of duplicate determinations.

Correlations with Standard Methods

Figures 1 and 2 show the relationship between the starch damage (percentage) levels in a range of wheat flours (which covers the entire range likely to be experienced in commercial soft and hard wheat flours) determined by the starch damage assay kit and by the Farrand method (Farrand 1964) ($n = 26$, $RSD_r = 5.36\%$) or by AACC method 76-30A ($n = 21$), respectively. In both cases, the correlation was high ($r > 0.98$). Differences in the absolute values given by the different methods are due to differences in flour-enzyme ratios, differing reagent purity, and differing procedures for measuring the reaction products. The regression equations for these methods, the Donelson-Wooster method (a modification of AACC method 76-30A) ($n = 21$), and the Barnes (1978) method ($n = 45$) (see Gibson et al 1992) are reported in Table III. The large disparity between the absolute starch damage values obtained by the Farrand method and those obtained by the other methods is due to the derivation of the Farrand starch damage units. They are defined in relationship to arbitrary limits and are increased by mathematical treatment (Farrand 1964, Evers and Stevens 1985).

These correlation coefficients are similar to those reported in other comparative studies of starch damage assay methods (Williams and LeSeelleur 1970, Dodds 1971, Finney et al 1988). Therefore, the starch damage assay kit provides a simple and reliable alternative to the current standard methods for the measurement of starch damage in wheat flour. It has the advantage of incorporating standardized, purified enzymes and reagents,

TABLE III
Regression Equations and Correlation Coefficients for the Kit Method Compared with Standard Procedures

Regression Equation			Correlation Coefficient (*r*)
Starch damage (%) (Farrand)[a]	=	5.2 × starch damage (%) −10.3 (starch damage assay kit)	0.98
Starch damage (%) (AACC)[b]	=	1.4 × starch damage (%) −0.09 (starch damage assay kit)	>0.99
Starch damage (%) (Wooster)[c]	=	1.2 × starch damage (%) +0.5 (starch damage assay kit)	>0.99
Starch damage (%) (Barnes)[d]	=	1.5 × starch damage (%) +0.44 (starch damage assay kit)	0.96

[a] Farrand 1964.
[b] American Association of Cereal Chemists 1983.
[c] Donelson and Yamazaki 1962.
[d] Barnes 1978.

which avoids the possible contaminating activities in commercial preparations of fungal α-amylase or malt amylase extracts. Also, the method is rapid, allowing the analysis of 20 samples in duplicate in 2 hr.

The starch damage assay kit provides all of the necessary reagents and a reference flour and employs only one standard solution (glucose), which is readily prepared and stabilized. Both the AACC and Farrand methods determine reducing groups on the degradation products of damaged starch granules by the alkaline ferricyanide method, which requires standardization of two reagents and a titration step, and employs several unstable and corrosive reagents.

CONCLUSIONS

The availability of an assay kit for the determination of starch damage in flour offers a convenient and simple alternative to the current standard methods. Starch damage determinations with the assay kit are highly correlated to those of the standard methods, but the kit procedure is standardized and more rapid. With the kit procedure, 40 samples can be analyzed in 2 hr. The kit is therefore applicable for use in situations where there are large sample numbers, such as in the monitoring of millstream runs and in wheat breeding programs. The starch damage assay kit is now available commercially from MegaZyme (Aust) Pty Ltd.

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Distribution of Polyphenol Oxidase in Flour Millstreams of Canadian Common Wheat Classes Milled to Three Extraction Rates[1]

D. W. HATCHER and J. E. KRUGER

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ABSTRACT

Polyphenol oxidase (PPO) levels were determined on individual and pooled millstreams of five cultivars representative of five different classes of Canadian wheat. The wheats were milled on a pilot mill to extraction rates of approximately 75 (conventional), 80, and 85%. Enzyme activity in individual streams ranged widely but increased with increasing bran contamination in the millstreams. With the exception of the soft spring wheat cultivar, PPO levels, as a percentage of total activity, were similar for the different wheats at similar cumulative flour yields. Less than 10% of the total PPO activity was present in cumulative flour streams corresponding to 70% extraction, after which the amount of the enzyme rapidly increased. Slightly more PPO activity appeared in the lower ash streams if the wheats were milled to a higher extraction using this mill flow. PPO activity was linearly correlated with ash content (up to 2.0% ash) and flour grade color figure (up to 5 units).

Polyphenol oxidase (PPO) has been implicated in enzymatic browning reactions in whole wheat flours used for making *chapati* (Abrol et al 1971, Singh and Sheoran 1972). The enzyme is located mainly in the bran of milled wheat and is not readily extractable with common buffers (Marsh and Galliard 1986). A number of studies have elaborated on the chemical and physical properties of the enzyme, as well as its multiplicity (Tikoo et al 1973; Taneja and Sachar 1974; Kruger 1976; Interesse et al 1980, 1981, 1983). Wheat enzyme levels are dependent on wheat class (Lamkin et al 1981).

A number of end products are commonly prepared from flours milled to higher extraction rates in which the presence of PPO

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