EVALUATION OF A NEPHELOMETRIC METHOD TO DETERMINE ALPHA-AMYLASE ACTIVITY IN WHEAT FLOURS

by

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INTRODUCTION

Plant breeders, millers, and bakers are interested in rapid, reliable methods to measure alpha-amylase activity in wheat flour because high alpha-amylase activity may indicate that the flour was milled from sprout damaged wheat. While certain levels of alpha-amylase activity appear to be important for the production of good bread, high levels produce breads with a sticky crumb and poor eating quality.

A variety of assay methods for alpha-amylase have been developed for grain and flour. These are based on monitoring different aspects of enzyme action. Methods by Bernfield (1951) and Robyt and Whelan (1968) measure alpha-amylase by measuring the reducing value of a starch solution. However, reducing value methods are not specific for alpha-amylase in the extract. Pretreatment of the extract, either by a short heat treatment at $70^{o}C$ or by the addition of $HgCl_2$, is needed to inactivate beta-amylase. The problem with these pretreatments is that some alpha-amylase may also be inactivated.

The methods of Wohlgemuth (1908), Sandstedt et al. (1939), Briggs (1961) and MacGregor et al. (1971) measure alpha-amylase activity by a decrease in the iodine color of beta-limit dextrin. Iodine-staining methods lack precision and sensitivity, and overestimate alpha-amylase activity at high enzyme concentrations (Barnes and Blakeney, 1974).

The falling number (Hagberg, 1960; Perten, 1964), amylograph method (AACC, 1972; Ranum et al., 1978), and microviscometric method (Tipples, 1969) measure alpha-amylase activity by recording the decrease in the viscosity of a starch solution. The level of starch damage and starch susceptibility greatly influence the results in the viscosity methods. (Barnes and Blakeney, 1974; Mathewson and Pomeranz, 1978a). The falling

number method is somewhat sensitive to particle size distribution (Mallhot, 1980) and not applicable to flour supplemented with alpha-amylase from fungal origins (Campbell, 1980b).

The Phadebas method (Barnes and Blakeney, 1974) and the Cibacron Blue Amylose method (Mathewson and Pomeranz, 1978b) measure alpha-amylase activity as the increase in concentration of soluble dye-labelled products formed during the hydrolysis of an alpha-amylase specific substrate. Damaged starch in the extract decreases the color released from Phadebas tablets because the damaged starch acts as a competitive substrate (Barnes and Blakeney, 1974).

A method by Marchylo and Kruger (1978) uses a fluorometer to measure the increase in intensity of dialyzable fluorescent products formed by the action of alpha-amylase. The technique and instrumentation of the fluorometric method are complex and require a skilled operator.

A recently introduced turbidimetric method to measure alpha-amylase activity uses a prototype Perkin Elmer Model 191 Grain Amylase Analyzer and is based on the nephelometric procedure of Zinterhofer et al. (1973), which is widely used to determine amylase in clinical medicine. The method is based on the decrease in turbidity of a suspension of a commercial substrate as a result of amylase action. The first substrate used in this method was amylopectin. Kruger et al. (1979) found that the use of amylopectin as a substrate provided a technique for measuring concentrations of alpha-, beta-, or total amylase activity in cereals but it could not differentiate between the effects of these two enzymes. By substituting beta-limit dextrin for amylopectin, O'connell et al. (1980) reported this substrate was more reactive than amylopectin and less subject to beta-amylase interference. Campbell (1980a) indicates

that this method discriminately measures alpha-amylase, correlates well with currently used methods, and can measure fungal supplements (Campbell, 1980b).

The objectives of this study were (1) to compare the results of the nephelometric method with those obtained with the falling number and Phadebas methods and (2) to evaluate the commercial substrate packaged in lyophilized form by Perkin-Elmer.

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MATERIALS

Materials

Kansas State hard wheat flour (protein 11.3%, ash 0.43%) having an amylograph viscosity of 1830 BU was used. This flour was supplemented with different levels (0.05% to 3.0%) of barley malt (120 SKB Units/g).

METHODS

Purification of Alpha-Amylase

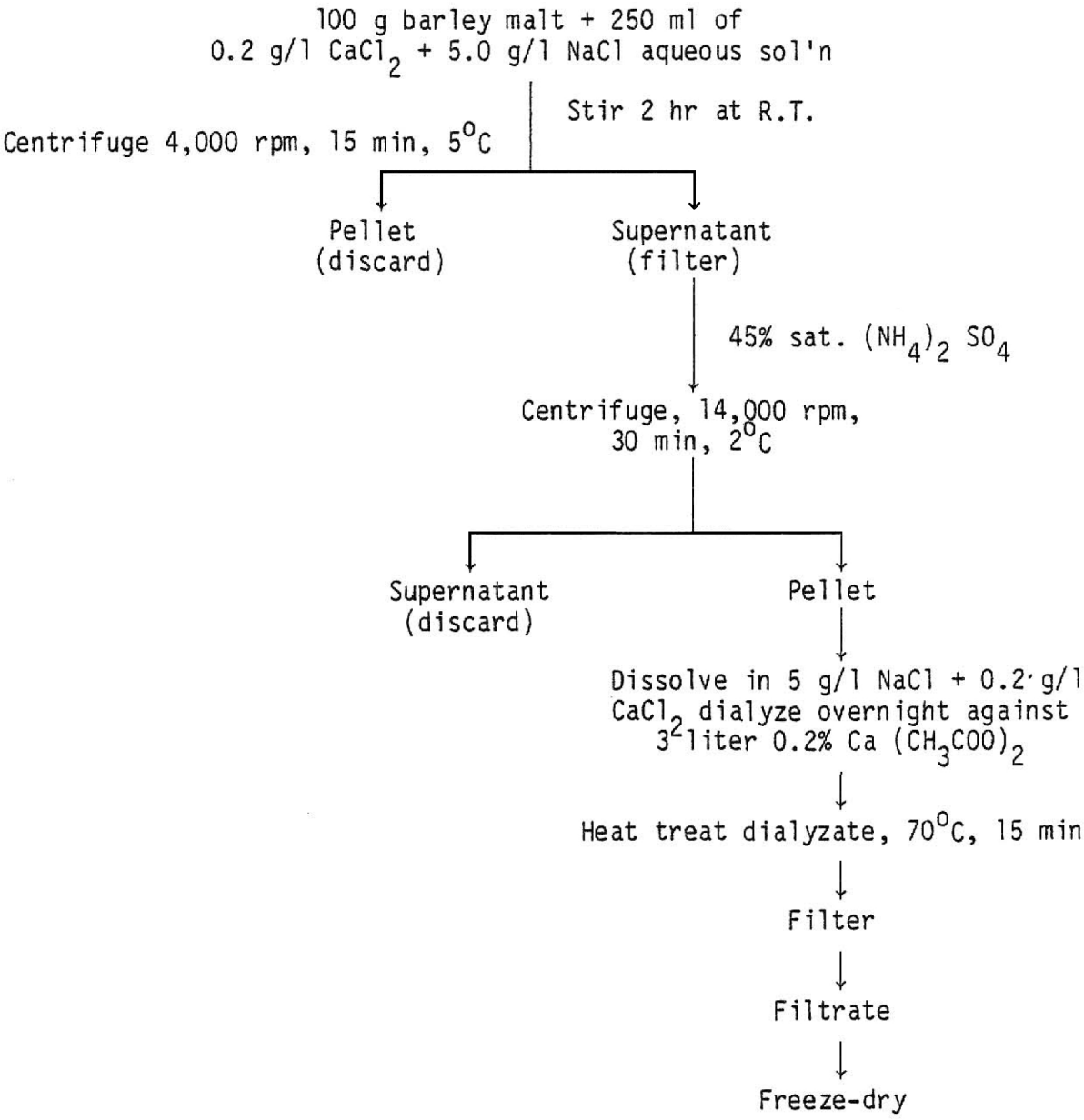
Alpha-amylase was purified from barley malt according to the procedures of Greenwood and Milne (1968) with slight modifications as shown in Figure 1.

Alpha-Amylase Activity

Reducing value method. The activity of purified alpha-amylase was determined by the method of Robyt and Whelan (1968) but the temperature was maintained at 37°C or 50°C instead of 25°C. Reducing sugars were determined by Nelson's colorimetric copper method (1944). Enzyme activity is expressed as μmoles of apparent maltose produced per ml of alpha-amylase solution per min.

Nephelometric method. The procedure for determining alpha-amylase with the Model 191 Grain Amylase Analyzer (Perkin-Elmer, Oak Brook, IL) was as described by Campbell (1980b). The substrate, Grain Alpha-Amylase Reagent (Perkin-Elmer, Oak Brook, IL), is a lyophilized beta-limit dextrin in citrate buffer, pH 5.5, containing trace amounts of sodium azide. The reagent is reconstituted with 80 ml of deionized water, heated in a boiling water bath for 10 min., and slowly cooled to 30°C by allowing it to stand at room temperature. In this study alpha-amylase activity was

Figure 1. Procedure for purifying alpha-amylase from barley malt.


100 g barley malt + 250 ml of
0.2 g/l $CaCl_2$ + 5.0 g/l NaCl aqueous sol'n
Stir 2 hr at R.T.
Centrifuge 4,000 rpm, 15 min, 5°C
Pellet
(discard)
Supernatant
(filter)
45% sat. $(NH_4)_2 SO_4$
Centrifuge, 14,000 rpm,
30 min, 2°C
Supernatant
(discard)
Pellet
Dissolve in 5 g/l NaCl + 0.2 g/l
$CaCl_2$ dialyze overnight against
3 liter 0.2% Ca $(CH_3COO)_2$
Heat treat dialyzate, 70°C, 15 min
Filter
Filtrate
Freeze-dry

reported in Amylase 1 Units on the Grain Alpha-Amylase Analyzer.

Phadebas method. The Barnes and Blakeney method (1974) is based on the colorimetric measurement of the products released from a cross-linked dye-labelled potato starch substrate (Pharmacia, Piscataway, NJ). Alpha-amylase activity is determined as the amount of soluble dye-labelled products produced during a 15 min. reaction time at 50°C. Results are expressed in mEU/10 ml as defined by Robyt and Whelan (1968).

Falling number method. AACC Method 56-81B(1972) was used to determine the falling number (F.N.). It is defined as the time in seconds required to stir and allow the stirrer to fall a measured distance through a hot aqueous flour gel undergoing liquefaction.

Gel permeation chromatography. Portions of the prepared commercial beta-limit dextrins were applied to a Sepharose 2B Column (2.6 x 70 cm) and eluted with 0.01 N NaOH containing 0.02% NaN_3. Carbohydrate in the fractions was determined by the phenol sulfuric acid method (Dubois et al., 1956). Debranched substrate was fractionated on the same column. Fractions eluting after the Vo of the column were combined and concentrated and then applied to a Bio-Gel P-10 column (2.6 x 54 cm).

Average chain length. Pullulanase (Enterobacter aerogenes, Sigma Chemical Co., St. Louis, MO) was used to debranch the beta-limit dextrin. A portion of the prepared substrate was incubated with pullulanase (160 units/ml) at 37°C for 24 hours. The average unit-chain length ($\overline{C.L.}$) was determined according to Gunja-Smith et al. (1971).

Beta-amylolysis limits. Susceptibility of the prepared beta-limit dextrin substrate to beta-amylolysis was according to Whelan (1964). Portions of the substrate were incubated with sweet potato beta-amylase (crystalline, type I-B, Sigma Chemical Co., St. Louis, MO) at 37°C for

24 hours. The maltose liberated was determined using Nelson's colorimetric copper method (1944).

RESULTS AND DISCUSSION

Comparisons With Falling Number and Phadebas Method

The nephelometric calibration curve is shown in Figure 2. This curve was prepared using known concentrations of purified alpha-amylase from malted barley and the commercial beta-limit dextrin substrate. The vertical line in the figure indicates the standard deviation among means of replicates, which ranged from 3% to 16%. A linear relationship exists between alpha-amylase concentration and Amylase 1 Units up to 660. Beyond 660 Amylase 1 Units, the readings deviate from linearity. Campbell (1980a) and O'Connell et al. (1980) reported linearity up to 700 and 720 Amylase Units for the Grain Alpha-Amylase Calibrator (Perkin-Elmer, Oak Brook, IL) and sprouted soft white wheat, respectively. Undoubtedly, there will be differences in standard curves for different enzymes sources and this is one drawback of the method; a new curve must be prepared for each type of grain analyzed. O'Connell et al. (1980) suggested that lack of linearity above 720 Amylase 1 Units may be due to substrate depletion or product inhibition.

The Phadebas calibration curve prepared by using purified alpha-amylase from malted barley is shown in Figure 3. The standard deviation of means of replicates ranged from 2% to 8%. A good linear relationship existed over the range of concentrations analyzed which included absorption values up to 1.6.

The effect of malt addition on alpha-amylase activity as determined by the nephelometric, Phadebas, and falling number methods is shown in Table I. The random error in the nephelometric method ranged from 6% to 21% while it was only 4% to 14% for the Phadebas method and 2% to 6% for

Figure 2. Nephelometric calibration curve using purified alpha-amylase from malted barley.

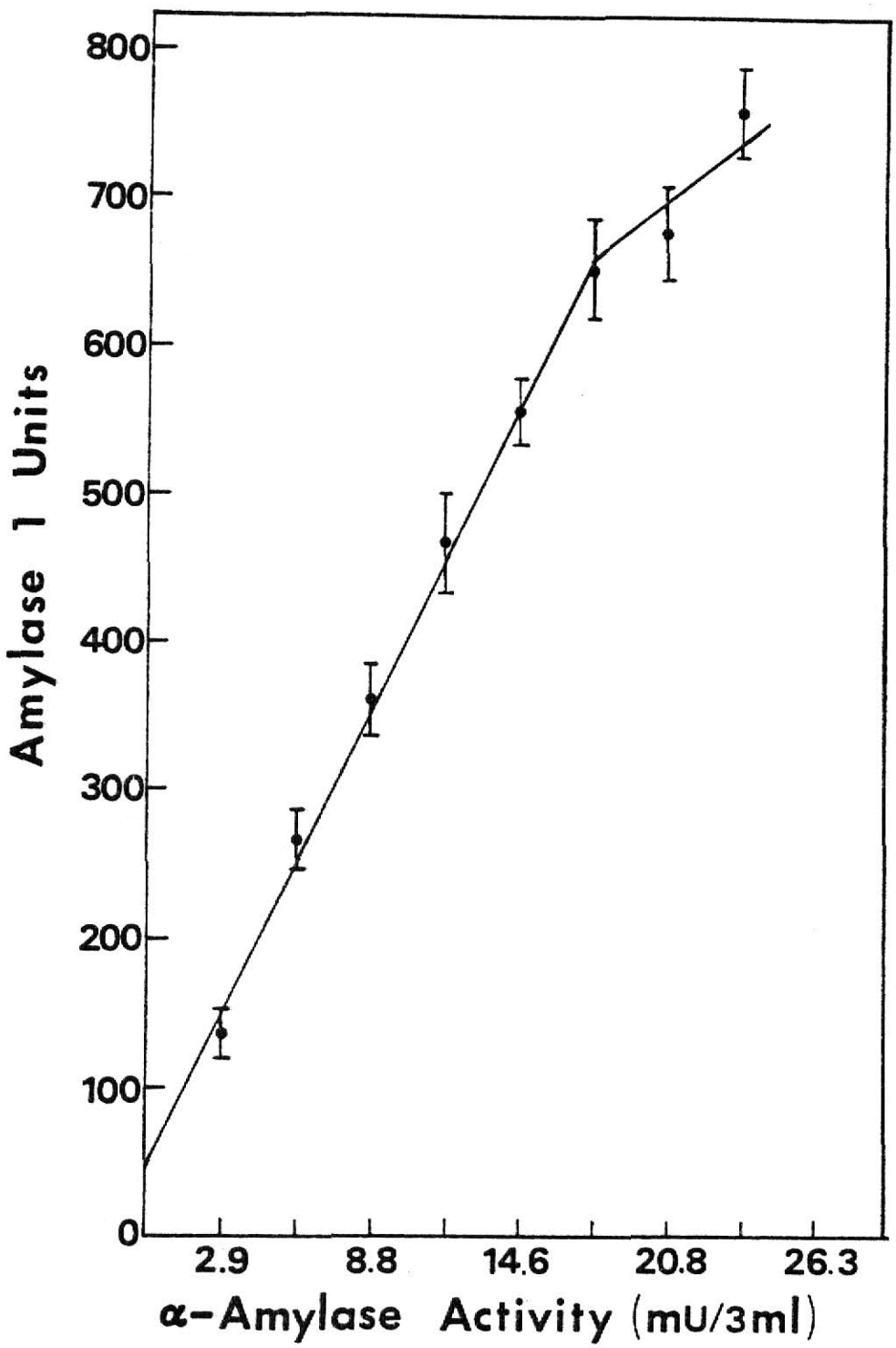

800
700
600
500
400
300
200
100
0
Amylase 1 Units
2.9
8.8
14.6
20.8
26.3
α-Amylase Activity (mU/3ml)

Figure 3. Phadebas calibration curve using purified alpha-amylase from malted barley.

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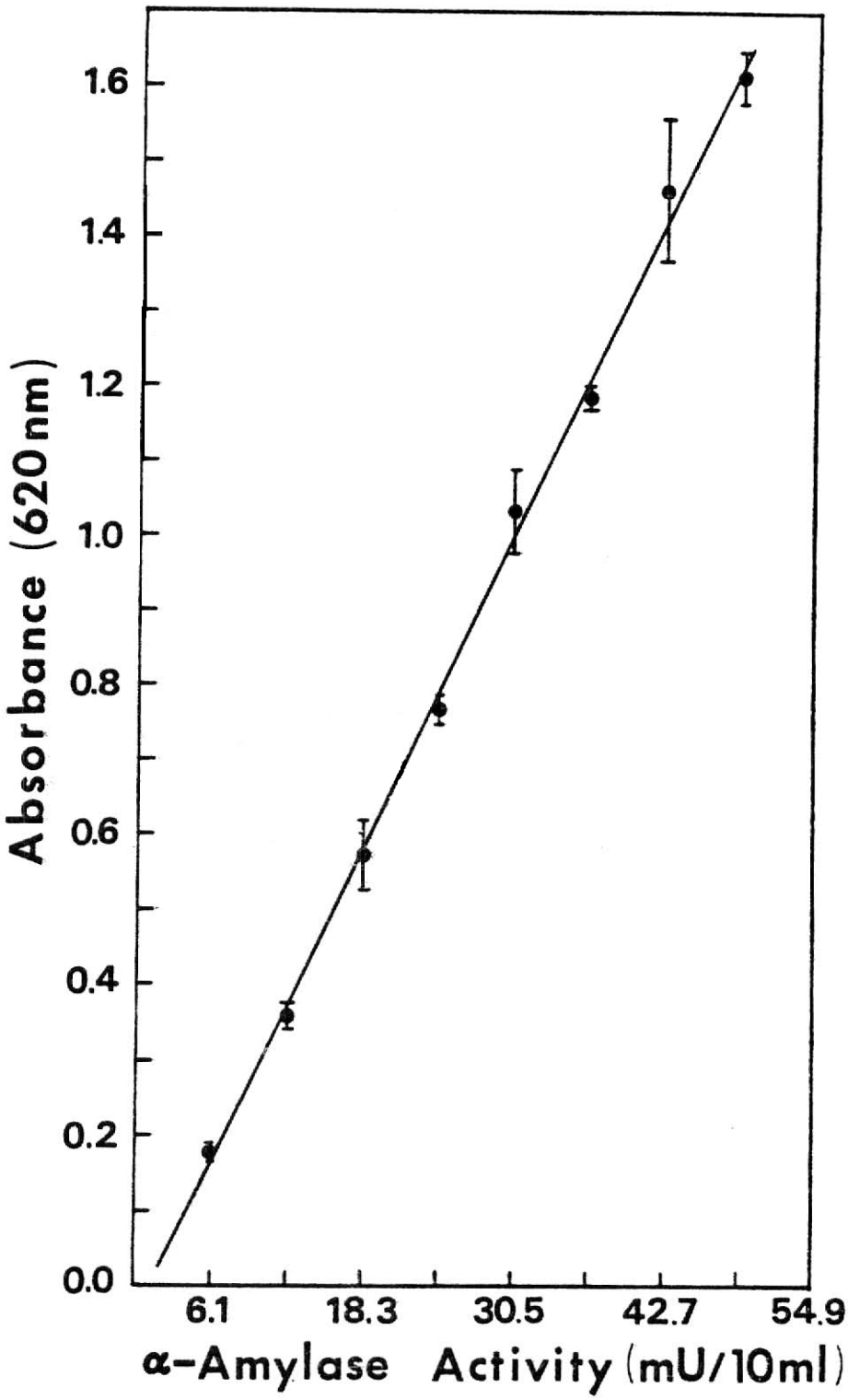

Absorbance (620nm)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
6.1
18.3
30.5
42.7
54.9
α-Amylase Activity (mU/10ml)

Table I. Effect of barley malt supplementation on alpha-amylase activity as measured by three methods.[a]

% Barley Malt	Nephelometer (Amylase 1 Units)	F.N. (Sec.)	Phadebas (mU/10 ml)
none	63 ± 8	448 ± 23	10 ± 1
0.05	186 ± 40	332 ± 14	18 ± 2
0.10	277 ± 27	281 ± 16	25 ± 2
0.15	417 ± 61	258 ± 5	34 ± 1
0.20	549 ± 77	235 ± 5	47 ± 4
0.35	762 ± 63	199 ± 10	135 ± 16
1.00	2193 ± 129	137 ± 3	1087 ± 61
2.00	5051 ± 601	98 ± 4	2157 ± 244
3.00	7289 ± 905	82 ± 2	3870 ± 554

[a]Values represent the mean ± the standard deviation from the mean.

the falling number method. The correlation coefficients among the three methods and levels of barley malt addition are shown in Table II. All showed high linear correlations with each other. Campbell (1980b) showed that the nephelometric method had a correlation of -0.996 with falling number and a correlation of 0.994 with the percentage of sprout-damaged wheat samples. O'Connell et al. (1980) found a correlation of 0.973 between the nephelometric method and liquefaction number in sprouted soft white wheat. No correlations between the nephelometric method and the Phadebas method have been reported.

A comparison of alpha-amylase activity per gram flour as determined by nephelometric and Phadebas methods is shown in Table III. The data are expressed in standard enzyme units per gram of flour. The nephelometric method yields higher values than the Phadebas method at the same level of barley malt addition. This was especially true at low levels of barley malt (0.05% to 0.20%). A better idea of the differences between the two methods at low concentrations of alpha-amylase can be seen in Figure 4. The slope of the alpha-amylase curve is much greater for the nephelometer than for the Phadebas method. This suggests that at equal malt additions, the former method is more sensitive to alpha-amylolysis. The nephelometer yields higher readings for alpha-amylase than the Phadebas method probably because of differences in reaction conditions, i.e. pH and temperature (Table IV). The optimum temperature for alpha-amylase from barley malt is 35^{o}C and the optimum pH is between 4.0 and 5.8 (Robyt and Whelan, 1968). The conditions for the nephelometric method more closely follow those optima than do the reaction conditions for the Phadebas method. Furthermore, the beta-limit dextrin may be more susceptible to hydrolysis than the Phadebas substrate. Lineback and Ponpipon (1977) reported that

Table II. Linear correlation coefficients for three alpha-amylase methods.

	Nephelometer	L.N.[a]	Phadebas	% Barley Malt
Nephelometer	—	0.997**	0.993**	0.999**
L.N.	—	—	0.995**	0.998**
Phadebas	—	—	—	0.994**

[a] L.N. = Liquefaction No. = $\frac{6{,}000}{F.N. - 50}$

**Significant at $P < .05$ level.

Table III. Alpha-amylase activity per gram of flour as determined by the nephelometer and Phadebas methods.[a]

% Barley Malt	Nephelometer (mU/g)	Phadebas (mU/g)
none	8 ± 6	8 ± 1
0.05	97 ± 29	14 ± 1
0.10	162 ± 20	20 ± 2
0.15	264 ± 44	27 ± 1
0.20	358 ± 56	38 ± 3
0.35	512 ± 45	108 ± 13
1.00	1545 ± 93	870 ± 49
2.00	3603 ± 435	1726 ± 196
3.00	5221 ± 653	3096 ± 444

[a]Values represent the mean ± the standard deviation from the mean.

Figure 4. Comparison of nephelometric (•——•) and Phadebas methods (*——*) at low levels of malt addition.

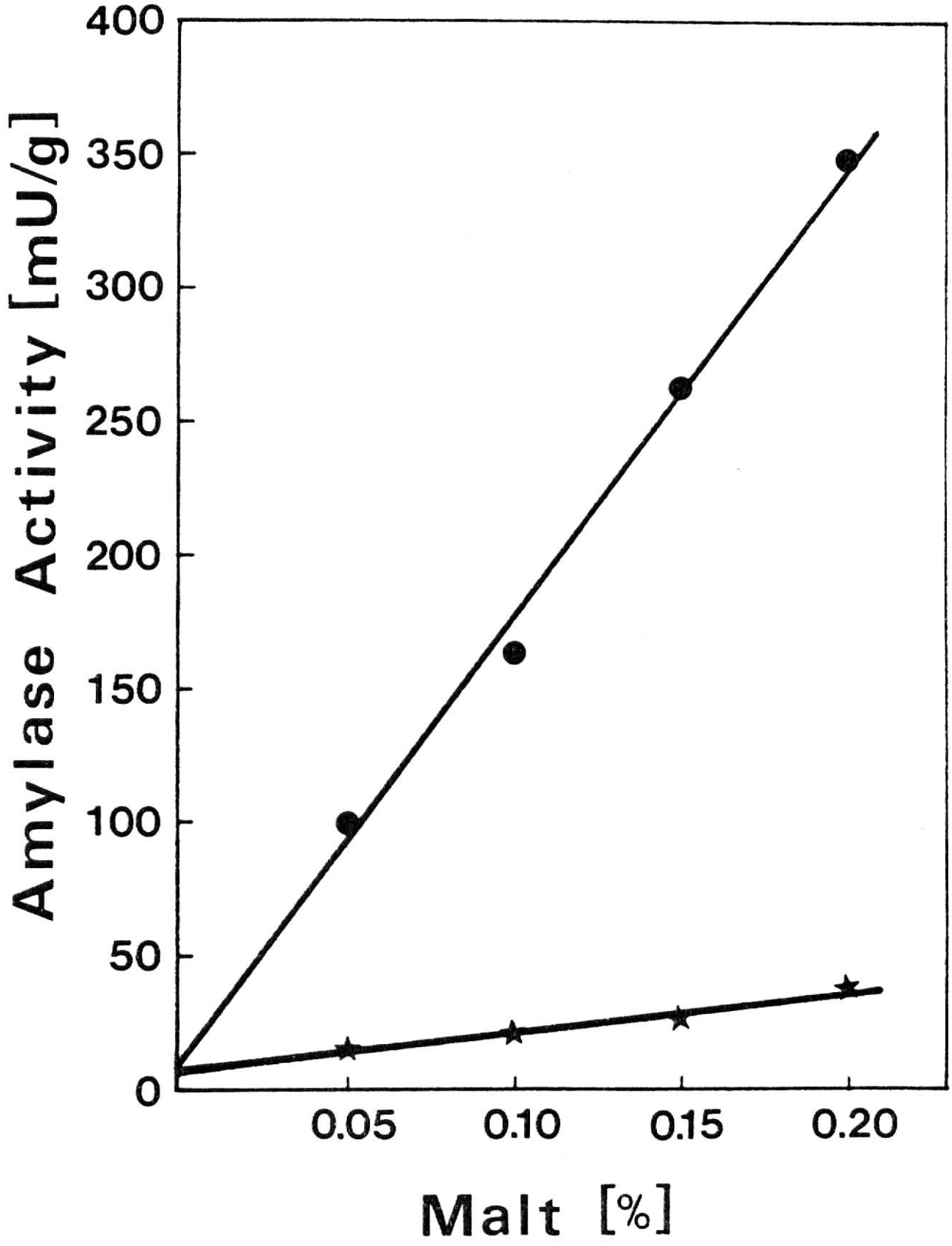

Amylase Activity [mU/g]
400
350
300
250
200
150
100
50
0
0.05
0.10
0.15
0.20
Malt [%]

Table IV. Reaction conditions for the nephelometric and Phadebas methods

Method	Temperature (°C)	pH	Time (min.)
Nephelometric	37	5.5	1
Phadebas	50	7.0	15

based on SEM data, millet alpha-amylase appeared to be very active; this high activity was not reflected in the reading obtained using the Phadebas substrate. They suggested that the Phadebas substrate may not be applicable for assaying alpha-amylase from that grain. Such problems with this synthetic substrate may also apply to malted barley alpha-amylase.

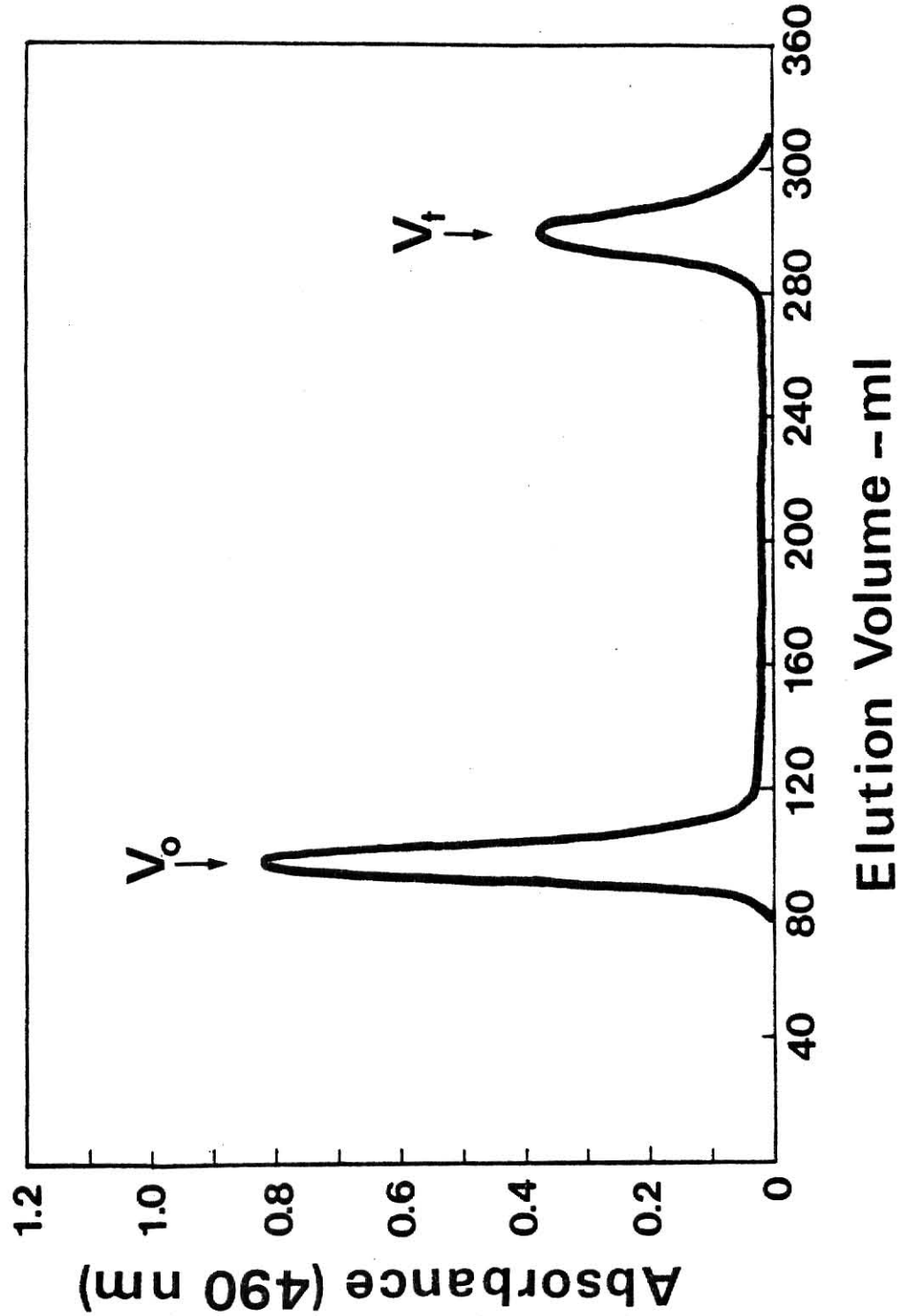
One factor which may contribute to the lessened sensitivity of the Phadebas method compared to the nephelometer is the fact that the extracted sample used in the test contains soluble starch which can act as a competitive substrate for alpha-amylase. The nephelometric method uses only 200 ul of the extract per 3 ml of substrate (compared to 5 ml for the Phadebas method); such a small aliquot does not add significant levels of soluble starch to the reaction mixture.

Evaluation of the β-Limit Dextrin

We thought that problems with the beta-limit dextrin may have caused the large standard deviation obtained with the nephelometric method so we studied the chemical characteristics of the substrate in detail. O'Connell et al. (1980) found that Amylase 1 Units above 720 were not reliable and suggested that this may be related to the characteristics of the substrate. We determined reducing sugars (as maltose) in a number of different bottles of the substrate; the values for four bottles are shown in Table V. The high concentration of reducing sugars in the substrate may cause product inhibition and thus explain the lack of reliability of nephelometric readings above 720.

Because the nephelometric method measures light scattered by particles in suspension, the reproducibility of the substrate suspension is the most important condition to be fulfilled in nephelometric work. The Sepharose 2B Cl elution diagram of beta-limit dextrin is shown in Figure 5. The first

Figure 5. Elution diagram of beta-limit dextrin on Sepharose 2B Cl column.


Absorbance (490 nm)
1.2
1.0
0.8
0.6
0.4
0.2
0
40
80
120
160
200
240
280
300
360
Elution Volume - ml
V_o
V_t

peak, eluting at the void volume, consists of particles having a molecular weight greater than 20 x 10^6. The second peak, eluting at the Vt of the column, had a molecular weight of 10,000 or less. The second peak had a high content of reducing sugars as determined by Nelson's method (1944).

The molecules eluting at the V_0 of the Sepharose 2B Cl column are responsible for the turbidity of the substrate. We debranched that fraction with pullulanase and rechromatographed the sample on a Bio-Gel P10 column. The elution pattern is shown in Figure 6. A major portion of the molecules had a molecular weight of 10,000, but some chains had molecular weights of 5,000 or less. The bottle to bottle variability in the average chain length ($\overline{C.L.}$) of the debranched beta-limit dextrin is shown in Table V. The $\overline{C.L.}$ ranged from 26 to 32 glucose units. Although the standard deviation ($\pm$ 3) suggests that there is significant variability in $\overline{C.L.}$ from bottle to bottle, it is difficult to say whether those differences would greatly affect the turbidity of the solution during amylolysis.

If the substrate is to be specific for alpha-amylase, then it should not be susceptible to attack by beta-amylase. If the average chain length of the β-limit dextrin is 26 to 32 glucose residues, then one might expect that the substrate would be susceptible to β-amylolysis. To determine the susceptibility of the commercial beta-limit dextrin to beta-amylase, beta-amylolysis limits were determined using sweet potato beta-amylase as described by Whelan (1964). β-amylolysis limits were found to be consistent from bottle to bottle and averaged 22% (Table VI). These data do indicate that the substrate is susceptible to sweet potato beta-amylase. O'Connell et al. (1980) compared the rates of amylolysis of beta-limit dextrin by sprouted wheat extracts using $HgCl_2$ as a beta-amylase inhibitor. They

Figure 6. Elution pattern of debranched beta-limit dextrin on Bio-Gel P10.

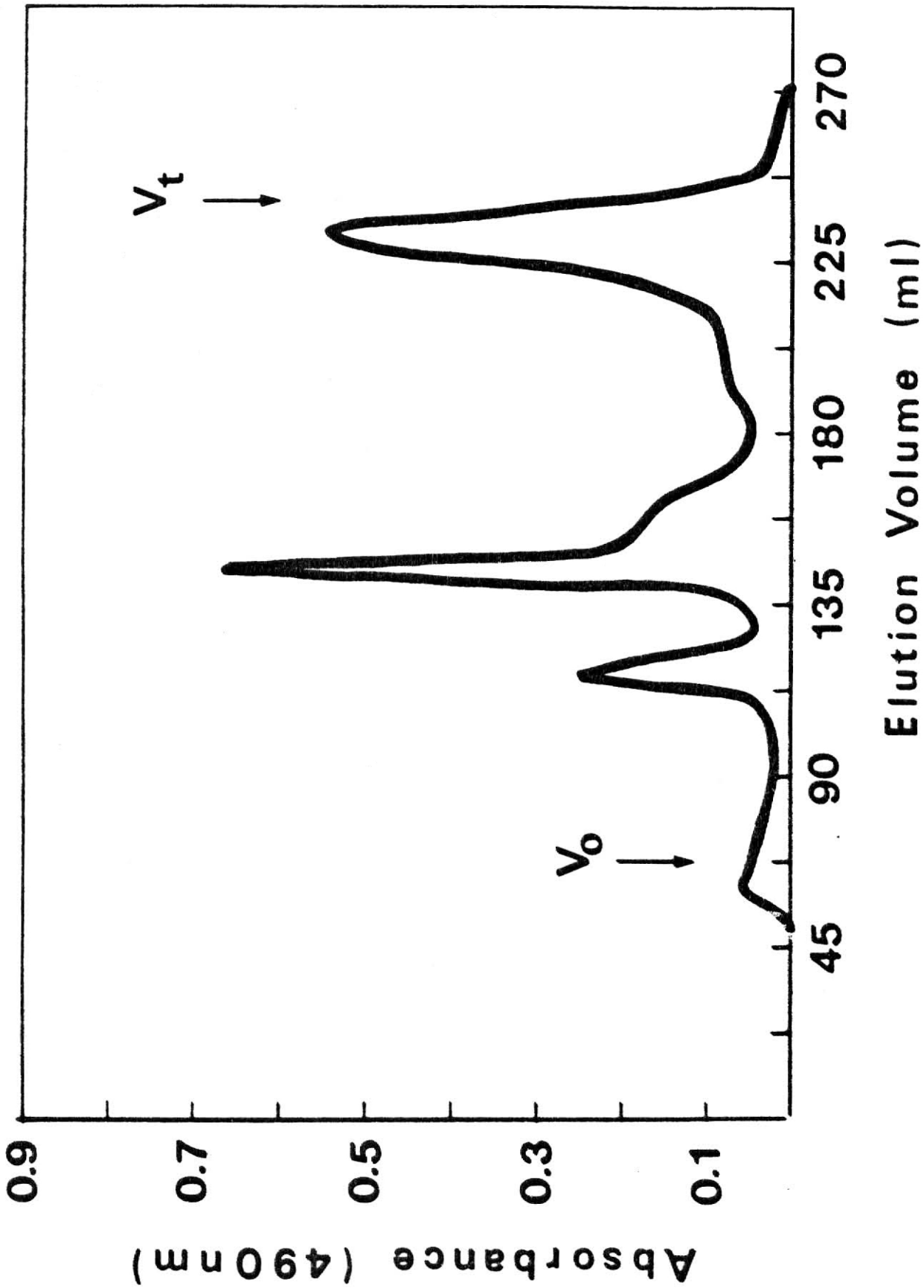

Absorbance (490nm)
0.9
0.7
0.5
0.3
0.1
V_o
V_t
45
90
135
180
225
270
Elution Volume (ml)

Table V. Reducing sugar content and average chain length ($\overline{C.L.}$) of the commercial β-limit dextrin.

Bottle No.	Reducing sugar[a] (maltose eq., mg/ml)	$\overline{C.L.}$[b]
1	2.42	26
2	2.49	30
3	2.50	32
4	2.41	28

[a] $s_{\bar{x}} = 0.05$

[b] $s_{\bar{x}} = 3$

Table VI. β-Amylolysis limits of commercial β-limit dextrin.

Bottle No.	Beta-Amylolysis (%)
1	22.5
2	22.1
3	22.9
4	22.3

found that extracts of the substrate without added $HgCl_2$ decreased the turbidity of the substrate faster than extracts containing $HgCl_2$ implying that beta-amylase digested the substrate during the alpha-amylase assay.

Using a flatbed polyacrylamide and a beta-limit dextrin plate technique, Kruger and Tipples (1981) reported that sweet potato beta-amylase contained traces of alpha-amylase. We tested our beta-amylase preparation for alpha-amylase using potato amylose cross-linked with Cibacron Blue (gift from Pharmacia, Piscataway, NJ). 40.0 mg of the substrate was incubated with 760 units of sweet potato beta-amylase in 5 ml of 0.02 M citrate buffer, pH 5.5, at 37°C. After a 24 hour digest, soluble dye products having an absorbance of 0.74 were detected. This corresponds to an alpha-amylase activity of 23 mU/10 ml. Although this is a low level of alpha-amylase compared to the high concentration of beta-amylase in the extract, it undoubtedly increased the values for beta-amylolysis that we obtained for the beta-limit dextrin substrate.

SUMMARY

Alpha-amylase activity in wheat flour supplemented with different levels of barley malt was determined by nephelometric, Phadebas, and falling number methods. The three methods were highly correlated with each other indicating that one method can be used to predict the others.

At low levels of malt supplementation (0.05 to 0.2%), the nephelometric method gave higher values for alpha-amylase activity than did the Phadebas method. However, the nephelometric method showed larger variations among replicates than did either the Phadebas or falling number methods. Furthermore, the relationship between alpha-amylase activity and Amylase 1 readings were linear only to about 660 Amylase 1 Units. This was in contrast to the Phadebas method where linearity existed at absorbances as high as 1.6.

A study of the biochemical characteristics of the substrate showed that there was a high concentration of reducing sugars (2.5 mg/ml) in the substrate which may decrease alpha-amylase activity (via product inhibition) and account for the lack of linearity observed for Amylase 1 readings above 660.

The average chain length ($\overline{C.L.}$) of the high molecular weight fraction of the β-limit dextrin varied from bottle to bottle but ranged from 26 to 32 glucose units. That high $\overline{C.L.}$ suggested that the substrate may be susceptible to β-amylase attack. The β-amylolysis limits for the 24 hr. digestion of β-limit dextrin by sweet potato β-amylase averaged 22% so the commercial substrate is not entirely specific for alpha-amylase. However, the affect of β-amylase on the nephelometric readings may not be significant under the normal test conditions since only a one minute reation time is used.

CONCLUSION

The nephelometric method is a satisfactory alternative to the falling number and Phadebas methods for measuring alpha-amylase activity in flours supplemented with barley malt. Unlike the falling number and the Phadebas methods, the nephelometric method is very sensitive to low levels of alpha-amylase. However, the problems associated with the substrate, i.e. high reducing sugar content and variability in $\overline{C.L.}$, should be resolved to increase the usefulness of the method particularly at high levels of alpha-amylase.

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EVALUATION OF A NEPHELOMETRIC METHOD TO DETERMINE ALPHA-AMYLASE ACTIVITY IN WHEAT FLOURS

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Alpha-amylase activity in wheat flour supplemented with different levels of barley malt was determined by nephelometric, Phadebas, and falling number methods. The three methods were highly correlated with each other indicating that one method can be used to predict the others.

At low levels of malt supplementation (0.05 to 0.2%), the nephelometric method gave higher values for alpha-amylase activity than did the Phadebas method. However, the nephelometric method showed larger variations among replicates than did either the Phadebas or falling number methods. Furthermore, the relationship between alpha-amylase activity and Amylase 1 readings were linear only to about 660 Amylase 1 Units. This was in contrast to the Phadebas method where linearity existed at absorbances as high as 1.6.

A study of the biochemical characteristics of the substrate showed that there was a high concentration of reducing sugars (2.5 mg/ml) in the substrate which may decrease alpha-amylase activity (via product inhibition) and account for the lack of linearity observed for Amylase 1 readings above 660.

The average chain length ($\overline{C.L.}$) of the high molecular weight fraction of the β-limit dextrin varied from bottle to bottle but ranged from 26 to 32 glucose units. That high $\overline{C.L.}$ suggested that the substrate may be susceptible to β-amylase attack. The β-amylolysis limits for the 24 hr. digestion of β-limit dextrin by sweet potato β-amylase averaged 22% so the commercial substrate is not entirely specific for alpha-amylase. However, the affect of β-amylase on the nephelometric readings may not be significant under the normal test conditions since only a one minute reation time is used.