Comparison of Nephelometric and Phadebas Methods of Determining Alpha-Amylase Activity in Wheat Flour Supplemented with Barley Malt[1]

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ABSTRACT

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α-Amylase activity in wheat flour supplemented with different levels of barley malt was determined by nephelometric and Phadebas methods. The methods were highly correlated with each other, indicating that one method can be used to predict the other. At low levels of malt supplementation (0.05–0.2%), the nephelometric method, which had greater variations among replicates, was more sensitive in detecting α-amylase activity than was the Phadebas method. However, the relationship between α-amylase activity and Amylase 1 readings was linear only to about 660 Amylase 1 units. This contrasted with the linearity shown by the Phadebas method that existed at absorbances as high as 1.6. A biochemical study showed that the β-limit dextrin substrate contained a high concentration of reducing sugars (2.5 mg/ml), which may have decreased α-amylase activity (via product inhibition) and accounted for the lack of linearity for Amylase 1 readings above 660. The average chain length of the high molecular weight fraction of the β-limit dextrin varied among bottles but ranged from 26 to 32 glucose units. The β-amylolysis limits for the 24-hr digestion of β-limit dextrin by sweet potato β-amylase averaged 22%; thus, the commercial substrate was not entirely specific for α-amylase.

A nephelometric method that uses β-limit dextrin as a substrate recently was applied toward determining α-amylase activity in wheat flours supplemented with fungal (Osborne et al 1981) and cereal (Kruger and Tipples 1981) α-amylase as well as activity in sprouted wheats (O'Connell et al 1980). High linear correlations were found between the nephelometric method and liquefaction numbers (O'Connell et al 1980), falling numbers (Campbell 1980b), and amylograph viscosities (Campbell 1979).

Barnes and Blakeney (1974) successfully used the Cibacron-labelled cross-linked potato starch (Phadebas tablets) developed by Ceska et al (1969) to colorimetrically determine α-amylase activity in wheat flours. The method is rapid and correlates well with other methods of measuring α-amylase (Barnes and Blakeney 1974). Mathewson and Pomeranz (1977,1979) and Mathewson et al (1978, 1981, 1982) recently modified the Barnes and Blakeney method to incorporate Cibacron blue-labelled potato starch with phosphate buffer, pH 6.2. That pH more closely approaches the optimum pH for cereal amylases than does the pH 7 of the Phadebas tablets. However, at the time of our study, the new tablets were not commercially available.

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Unlike the Phadebas method, which makes use of the simple laboratory colorimeter, the nephelometric method requires a special instrument that currently has no other proven application in cereal science, so its purchase would have to be justified on that use alone. Therefore, we compared the two methods to determine whether the nephelometric method showed any benefits over the Phadebas method in determining α-amylase activity in flours supplemented with barley malt.

MATERIALS AND METHODS

Materials

Kansas State hard wheat flour (protein 11.3%, ash 0.43%) having an amylograph viscosity of 1,830 Brabender units was used. This flour was supplemented with different levels (0.05–3.0%) of barley malt (120 SKB units/g).

Purifying of Alpha-Amylase

α-Amylase was purified from barley malt according to the procedure of Greenwood and Milne (1968) with the slight modifications shown in Fig. 1.

Alpha-Amylase Activity

Reducing Value Method. The activity of purified α-amylase was determined by the method of Robyt and Whelan (1968). Reducing sugars were determined by Nelson's colorimetric copper method (1944). Enzyme activity (units) is expressed as μmol of apparent maltose produced per milliliter of α-amylase solution per minute.

Nephelometric Method. The procedure for determining α-amylase with model 191 Grain Amylase Analyzer and the commercial lyophilized β-limit dextrin (Perkin-Elmer, Oak Brook, IL) was as described by Campbell (1980a). In our study α-amylase activity was reported in Amylase 1 units on the grain α-amylase analyzer.

Phadebas Method. α-Amylase activity was determined as the amount of soluble dye-labelled products produced during a 15-min reaction at 50°C (Barnes and Blakeney 1974). Results were expressed in mU/10 ml as defined by Robyt and Whelan (1968).

Gel Permeation Chromatography. Portions of the prepared commercial β-limit dextrin were applied to a Sepharose 2B column (2.6 × 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 V_o of the column were combined and concentrated, then applied to a Bio-Gel P-10 column (2.6 × 54 cm).

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

Beta-Amylolysis Limits. Susceptibility of the prepared β-limit dextrin substrate to β-amylolysis was determined according to Whelan (1964). Portions of the substrate were incubated with sweet potato β-amylase (crystalline, type I-B, Sigma Chemical Co., St. Louis, MO) at 37°C for 24 hr. The liberated maltose was determined by Nelson's colorimetric copper method (1944).

RESULTS AND DISCUSSION

Nephelometric Versus Phadebas Method

The nephelometric calibration curve shown in Fig. 2 was

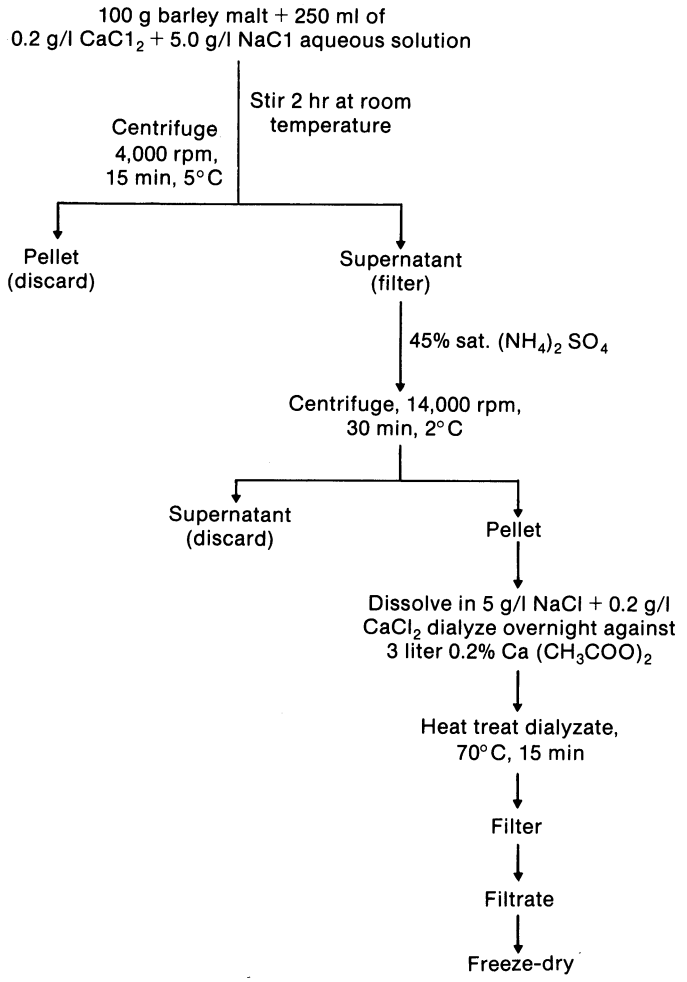

Fig. 1. Procedure for purifying α-amylase from barley malt.

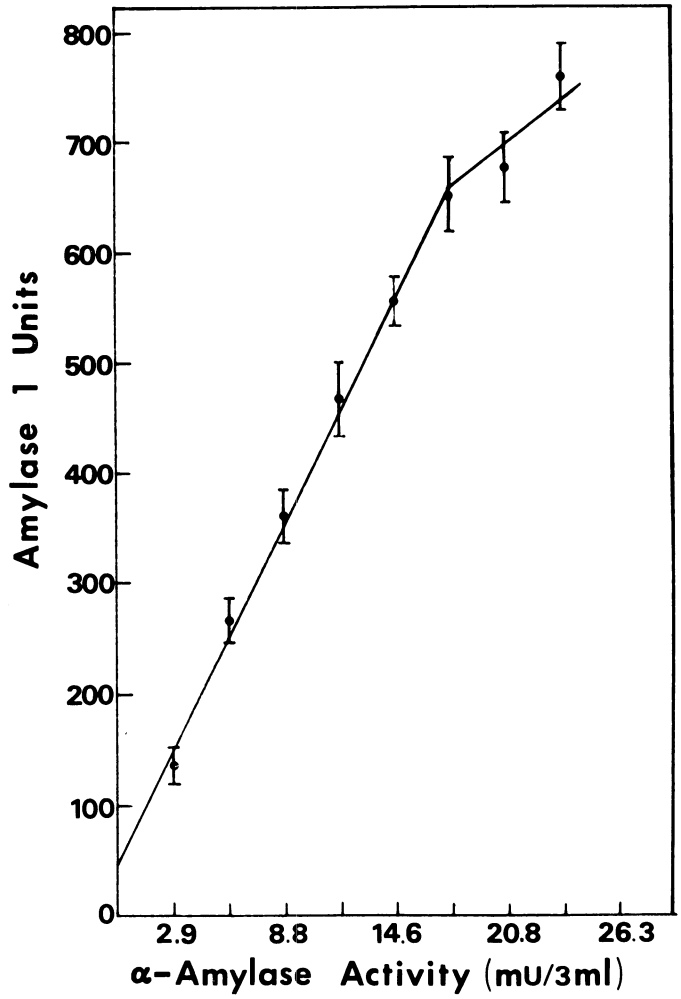

Fig. 2. Nephelometric calibration curve using purified α-amylase from malted barley.

prepared by using known concentrations of purified α-amylase from malted barley and the commercial β-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 α-amylase concentration and Amylase 1 units up to 660. Beyond 660 Amylase 1 units, the readings deviate from linearity so that, if the activity of a solution is above the linear region of the scale, the extract must be diluted and the test repeated. Campbell (1980a) and O'Connell et al (1980) reported linearity up to 700 and 720 Amylase 1 units for the grain α-amylase calibrator (Perkin-Elmer, Oak Brook, IL) and sprouted soft white wheats, respectively. Undoubtedly, standard curves for different enzyme sources will be different, and that 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 is due to substrate depletion or product inhibition.

The Phadebas calibration curve prepared by using purified α-amylase from malted barley is shown in Fig. 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 barley malt supplementation of flour on α-amylase activity as determined by the nephelometric and Phadebas methods is shown in Table I. The data are expressed in standard enzyme units per gram of flour. The nephelometric method yielded higher values than did the Phadebas method at the same level of barley malt addition. However, the random error in the nephelometric method ranged from 6 to 21%, whereas for the Phadebas method the range was only 4 to 14%. There was a high linear correlation between the nephelometric and Phadebas methods (0.993, $P = 0.05$). The nephelometric method showed a higher correlation with the percent of barley malt added than did the Phadebas method (0.999 vs 0.994, respectively). 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 samples. No correlations between the nephelometric method and the Phadebas method were reported previously.

In practice, malted barley supplementation to flour is at levels less than 1%. A comparison of α-amylase activity per gram of flour

TABLE I
Alph-Amylase Activity per Gram of Flour as Determined by Nephelometric and Phadebas Methods[a]

Percent Barley Malt	Nephelometer (mU/g)[b]	Phadebas (mU/g)[b]
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	1,545 ± 93	870 ± 49
2.00	3,603 ± 435	1,726 ± 196
3.00	5,221 ± 653	3,096 ± 444

[a] Values represent the mean ± the standard deviation from the mean.
[b] mU = Milli-enzyme units.

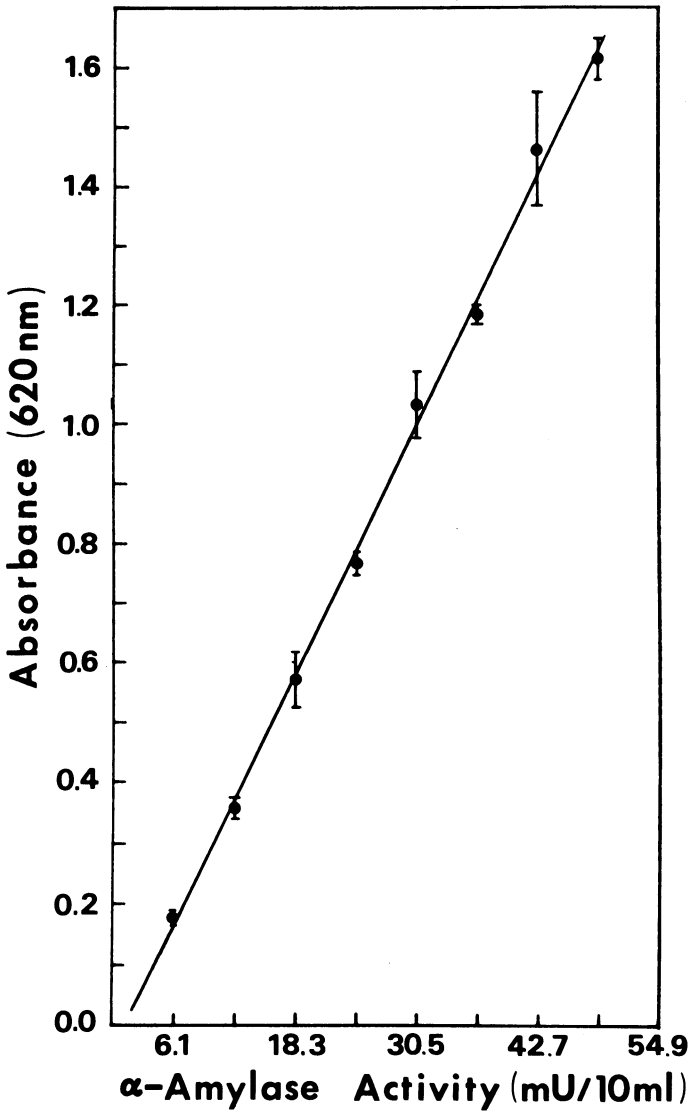

Fig. 3. Phadebas calibration curve using purified α-amylase from malted barley.

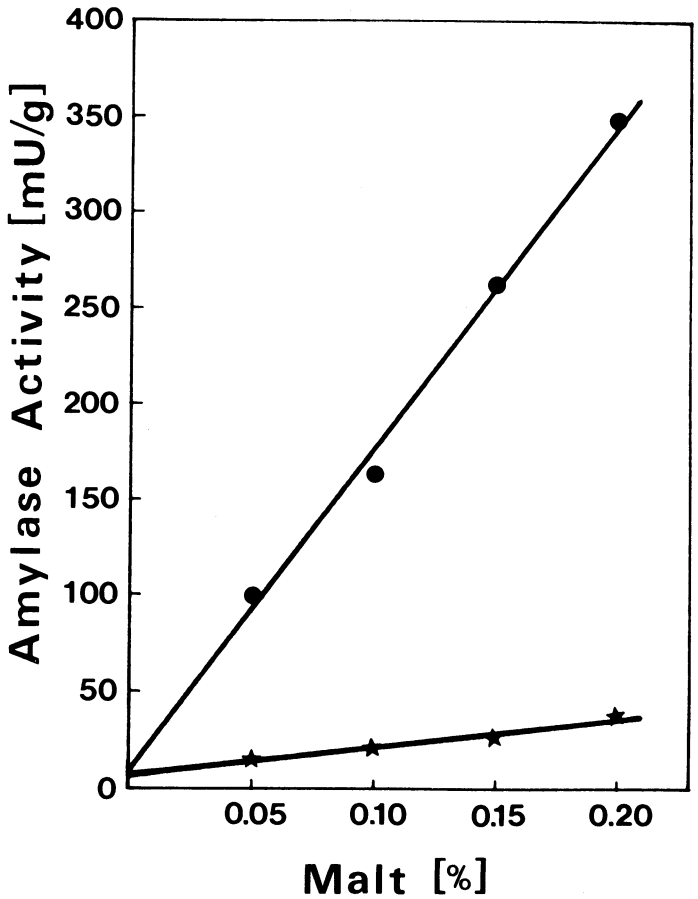

Fig. 4. Comparison of nephelometric (•——•) and Phadebas methods (*——*) at low levels of malt addition.

TABLE II
Alpha-Amylolysis of Cibacron Blue-Labelled Potato Starch as Affected by pH

Alpha-Amylase Source[a]	Method Using Phadebas Tablets[b] ($A_{620\ nm}$)	Cibacron Blue Substrate[c] in Phosphate Buffer, pH 6 ($A_{620\ nm}$)
Malted barley, 12 mU[d]/10 ml	0.38 ± 0.02	0.32 ± 0.01
Pearl millet, 43 mU[d]/10 ml	0.11 ± 0.02	0.59 ± 0.02

[a] Total activity of purified α-amylase in the reaction solution as determined by Robyt and Whelan's (1968) method.
[b] Barnes and Blakeney (1972).
[c] Cibacron blue-labelled potato starch (Pharmacia) was used in combination with 0.02 *M* phosphate buffer (pH 6) containing 0.01 *M* $CaCl_2$. Incubation temperature and time were the same as the Barnes and Blakeney method.
[d] mU = Milli-enzyme units.

TABLE III
Reducing Sugar Content and Average Chain Length ($\overline{C.L.}$) of the Commercial Beta-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] $S_{\bar{x}} = 3$.

as determined by nephelometric and Phadebas methods at levels of 0–0.2% is shown in Fig. 4. The much greater slope of the α-amylase curve with the nephelometric than with the Phadebas method suggests that, at equal malt additions, the former method is more sensitive to α-amylolysis. The nephelometer yielded higher readings for α-amylase activity than the Phadebas method, probably because of differences in reaction conditions, eg, pH and temperature. The optimum temperature for α-amylase from barley malt is 35° C, and the optimum pH is between 4.0 and 5.8 (Robyt and Whelan 1968). The conditions for the nephelometric method (37° C, pH 5.5) more closely follow those optima than do the reaction conditions for the Phadebas method (50° C, pH 7). Furthermore, the β-limit dextrin is likely to be more susceptible to hydrolysis than is the Phadebas substrate. Lineback and Ponpipon (1977) reported that, based on data by scanning electron microscopy, millet α-amylase appeared to be very active; high activity was not reflected in the reading obtained with the Phadebas substrate. The authors suggested that the Phadebas substrate may not be applicable for assaying α-amylase from that grain. Such problems with this synthetic substrate may also apply to malted barley α-amylase. Mathewson et al (1978) showed that the pH of the Phadebas tablets limits its usefulness in measuring cereal α-amylase activity, so they combined Cibacron blue-potato starch substrate with phosphate buffer (pH 6.2) salts and found an improvement in activity. We studied the α-amylolysis of the synthetic substrate as affected by pH (Table II) and found that decreasing the pH to 6.0 greatly improved the activity of pearl millet α-amylase but not that of malted barley α-amylase. Therefore, a factor other than pH, eg, steric hindrance due to the covalently bound Cibacron dye, limits the hydrolysis of that substrate by malted barley α-amylase.

One factor that might have contributed to the lessened sensitivity of the Phadebas method compared with that of the nephelometer is that the extracted sample used in the test contained soluble starch, which acts as a competitive substrate for α-amylase. For the nephelometric method only 200 μl of the extract per 3 ml of substrate is used (compared to 5 ml/10 ml of reaction mixture for the Phadebas method); such a small aliquot does not add significant levels of soluble starch to the reaction mixture.

Evaluating Commercial Beta-Limit Dextrin

We believed that problems with the commercial β-limit dextrin might have caused the large standard deviation obtained with the nephelometric method, so we studied the chemical characteristics of the substrate. O'Connell et al (1980) found that amylase I 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 III. The high concentration of reducing sugars in the substrate might have caused product inhibition, which explains 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 β-limit dextrin is shown in Fig. 5. The first peak, eluting at the void volume, consisted of particles having a molecular weight greater than 20×10^6; the second peak, eluting at the V_t of the column, consisted of particles with molecular weights 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_o 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 and obtained the elution pattern shown in Fig. 6. A major portion of the molecules had a molecular weight of about 10,000, as determined by chromatography of molecular weight standards on the same column, but some chains had

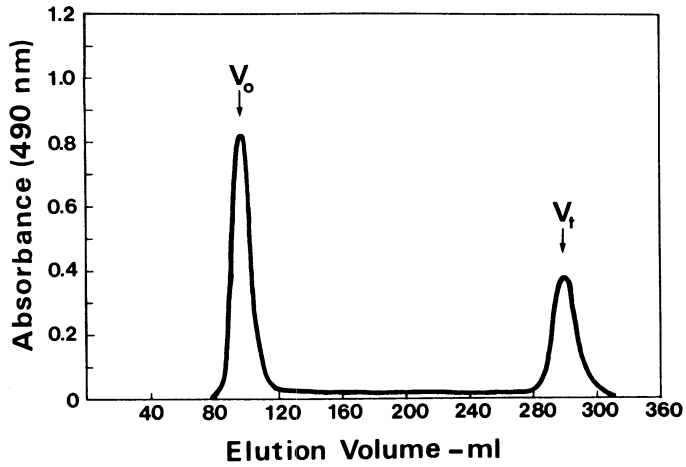

Fig. 5. Elution diagram of β-limit dextrin on Sepharose 2B Cl column.

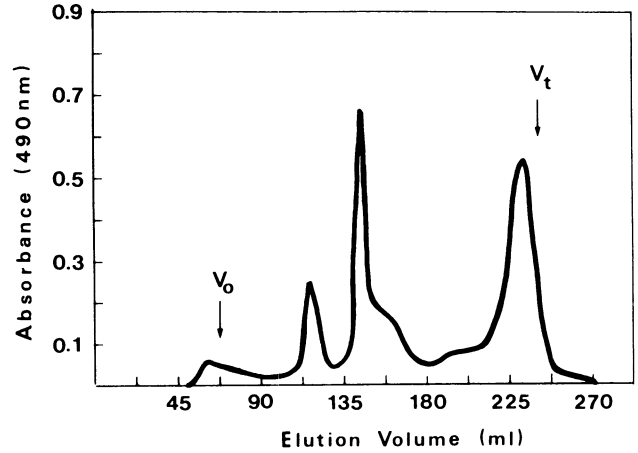

Fig. 6. Elution pattern of debranched β-limit dextrin on Bio-Gel P10.

TABLE IV
Beta-Amylolysis Limits of Commercial Beta-Limit Dextrin

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

molecular weights of 5,000 or less. The variability among bottles in the C.L. of the debranched β-limit dextrin is shown in Table III. The C.L. ranged from 26 to 32 glucose units. Although the standard deviation (±3) suggested significant variability in C.L. from bottle to bottle, it is difficult to determine whether those differences would greatly affect the turbidity of the solution during amylolysis.

If the substrate is to be specific for α-amylase, then it should not be susceptible to attack by β-amylase. If the C.L. of the β-limit dextrin is 26 to 32 glucose residues, then one might expect that the substrate is susceptible to β-amylolysis. To determine the susceptibility of the commercial β-limit dextrin to β-amylase, β-amylolysis limits were measured using sweet potato β-amylase as described by Whelan (1964). β-Amylolysis limits, found to be consistent from bottle to bottle, averaged 22% (Table IV) and indicated that the substrate was susceptible to sweet potato β-amylase. O'Connell et al (1980) compared the rates of amylolysis of β-limit dextrin by sprouted wheat extracts using $HgCl_2$ as a β-amylase inhibitor. They found that extracts of the substrate without added $HgCl_2$ decreased the turbidity of the substrate faster than did extracts containing $HgCl_2$, implying that β-amylase digested the substrate during the α-amylase assay.

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

CONCLUSION

The nephelometric method is a good alternative to the Phadebas method for measuring α-amylase activity in flours supplemented with barley malt. Unlike the Phadebas method, the nephelometric method is very sensitive to low levels of α-amylase. However, the problems associated with the substrate (ie, high reducing sugar content and variability in C.L.) should be resolved to increase the usefulness of the method, particularly at high levels of α-amylase.

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