PHOTOMETRIC DETERMINATION OF ALPHA- AND BETA-AMYLASE IN FLOUR

by

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INTRODUCTION

In establishing the baking quality of rye flour, the determination of the alpha-amylase content is one of the main factors. To the liquefying and dextrinizing action of this enzyme the existence of a so-called „sticky crumb" is attributed. In contrast with the wheat loaf the volume of the finished rye loaf is not an essential factor and most notice is taken of the crumb structure.

In order to be able to estimate the quality of rye flour by the alpha-amylase content, we need a method by which it is possible to determine variable quantities of alpha-amylase when beta-amylase is also present.

The action of alpha-amylase or dextrinogen amylase is characterized by its dextrinizing and liquefying power along with a less pronounced saccharogenic action, whereas the beta-amylase is chiefly of saccharogenic nature but also appears to have dextrinogenic properties.

These facts are repeatedly mentioned in the literature on the subject. The results obtained by different investigators are sometimes rather contradictory.

OHLSSON[1, 2] and HOLMBERGH[3] showed that beta-amylase affects the dextrinogenic activity of alpha-amylase in starch breakdown as shown in the changes in the colour produced by iodine, though OHLSSON supposed that beta-amylase did not influence the establishment of the colourless endpoint in alpha-amylase determinations by the method of WOHLGEMUTH[4].

OHLSSON and also FREEMAN and HOPKINS[5] demonstrated that starch-saccharification by a mixture of alpha- and beta-amylase was roughly an additive function.

BLOM, BAK, and BRAAE[6] showed the overlapping effects of alpha- and beta-amylase in starch dextrinization as well as in saccharification.

This was confirmed by HANES and CATTLE[7], who investigated the hydrolysis of starch by mixtures of alpha- and beta-amylases and found that the rate of destruction of the iodine colouring property is by no means defined by the amount of the alpha-component.

If these facts are ignored in cases where the alpha-amylase content is large compared with the beta-amylase, it is nevertheless still possible to get relatively good results. If on the contrary comparitively little alpha-amylase is present, it is impossible to differentiate the investigated samples by their respective alpha-amylase contents.

SANDSTEDT, KNEEN, and BLISH[8] described a method for the determination of alpha-amylase, in which the procedure of WOHLGEMUTH[4] is modified so as to eliminate the

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influence of beta-amylase. This is attained by the preliminary addition of a sufficient quantity of pure beta-amylase to the starch solution that is used as a substrate.

The objections to this procedure are:

1. One is obliged to work with a comparison solution and must therefore always continue the starch degradation until the adopted endpoint is reached.

2. For small quantities of alpha-amylase the time needed to reach the adopted endpoint can be very long.

3. The ability to reproduce the substrate is not sufficiently assured, chiefly with regard to the susceptibility to diastatic attack.

From the investigations by HANES and CATTLE[7] it appeared that the extinction of an iodine-starch solution is directly proportional to the starch concentration for all wave lengths. The same applies to the extinction of an iodine-dextrin solution. Thus it is possible to derive the concentration of a starch or dextrin solution from its extinction when coloured with iodine.

From the publication of SANDSTEDT et al.[8] it is evident that erythrodextrin, the endproduct of beta-amylolytic breakdown of starch, can be used as a specific substrate for the determination of alpha-amylase, whereas from the investigation from HANES et al. it appears that the concentration determination can be carried out in a simple way by means of the iodine reaction.

For these reasons we prepared erythrodextrin in the solid form. Thus it was possible to trace the influence of alpha- and beta-amylase on this substrate separately and to verify the assumption that the next possible diastatic attack on erythrodextrin is governed only by alpha-amylase, at least during the first part of the reaction. By taking a buffered solubilized starch solution instead of erythrodextrin solution the amount of starch that is broken down appeared to be a perfect additive function of the sum of alpha- and beta-amylase present. The amount of beta-amylase could then be found by a simple calculation.

EXPERIMENTAL

Erythrodextrin can be prepared by sufficiently long action of a pure beta-amylase preparation on a starch solution, followed by precipitation with ethanol.

To determine the alpha-amylase content, use is made of a buffered erythrodextrin solution to which a certain quantity of enzyme-containing solution is added. Known quantities of this substrate-enzyme mixture are added to a solution of iodine in KI. By measurement of the extinctions of the solution so obtained the erythrodextrin concentration at any moment can be found.

By plotting the erythrodextrin concentrations against time, straight lines are obtained in most cases, provided that not more than 50 % is converted. A measure for the quantity of enzyme present can be derived from the enzyme concentration-activity curves. If the decrease in erythrodextrin concentration after a specified time does not exceed more than 50 %, the quantity of alpha-amylase present in the reaction mixture is directly proportional to this decrease. Thus it is possible to express the activity of a certain sample of flour by the quantity of erythrodextrin converted in a certain time by an extract prepared from a fixed quantity of flour.

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A. REAGENTS

1) Erythrodextrin by the method of WEBER[9*].

200 g flour and 500 ml water are stirred into an even suspension, which after some hours is centrifuged and filtered. This flour is checked beforehand for the absence of alpha-amylase by the method of WIJSMAN[10].

To 1.5 l of a 3 % solubilized starch solution is added 50 ml of the flour extract. The mixture is placed in a thermostat at 40°, and after 24 hours another 50 ml flour-extract is added. By estimating the maltose content of the mixture the reaction can be followed. If after another 24 hours the limit of conversion, which lies at about 60 %[11], is reached, the reaction mixture is boiled to destroy the enzyme activity, after which it is cooled, Fuller's earth is added and the mixture is filtered. The erythrodextrin is precipitated by adding 96 % ethanol until the end concentration amounts to 80 %, and at the same time 15 ml 5 % NaCl solution is added. After decantation the precipitate is removed by suction and washed successively with 80 % ethanol, 96 % ethanol and acetone. The preparation is dried in a vacuum desiccator over concentrated H_2SO_4.

2) 0.625 % erythrodextrin solution:

3.125 g erythrodextrin (dry matter base) and 50 ml 0.2 M acetate buffer, p_H 5.3, are dissolved to a volume of 500 ml. Some toluene is added for preservation.

3) 0.2 % iodine solution (4 ml 1 N iodine solution plus 5 g KI are dissolved to 250 ml).

4) 1.25 % solubilized starch solution:

6.25 g solubilized starch (dry matter base) plus 50 ml 0.2 M acetate buffer are dissolved in boiling water and after cooling made up to 500 ml. Some toluene is added. This solution can be kept for about a week.

5) Purified alpha-amylase by the method of WEBER[9].

Finely ground malted barley is extracted with water at room temperature for some hours. The suspension is filtered and the filtrate is heated on a boiling water bath for 20 minutes to 75°. After cooling in ice water, the precipitate produced is sucked off. To the clear filtrate 96 % ethanol is added to an end concentration of 40 %. The precipitate is filtered off and to the filtrate ethanol is added to an end concentration of 80 %. Some NaCl is added. After 18 hours the precipitate is sucked off on a BÜCHNER funnel, washed with 80 % ethanol, then with 96 % ethanol, and dried over concentrated H_2SO_4 in vacuum.

6) Purified beta-amylase by the method of WEBER[9].

Finely ground pearl-barley, free from traces of alpha-amylase, is extracted with water at room temperature. After filtration ethanol is added to the clear filtrate to an end concentration of 50 %. The precipitate produced is filtered off and ethanol is added to the filtrate to an end concentration of 80 %. The rest of the procedure is the same as for alpha-amylase.

B. APPARATUS

To determine the extinction, use is made of a Moll extinctiometer (made by KIPP, Delft), provided on each side of the source of light with Schott glass filters OG_1 10 mm and BG_{17} 10 mm, a combination with maximum transmission at 5800 Å.

C. EXTINCTION-CONCENTRATION STANDARD-CURVE

As the colour of the iodine-erythrodextrin solution or the iodine-starch solution is influenced by the temperature and there was not at our disposal a constant temperature room, it was necessary to make an extinction-concentration curve each day. For this purpose two points are sufficient. In fig. 1 the extinction-concentration curves for iodine-starch and iodine-erythrodextrin are given. From these curves it appears that up to a concentration of 0.45 ml 1.25 % starch solution (or 0.9 ml 0.625 % erythrodextrin solution) in 100 ml diluted iodine solution the extinction, corrected for the extinction of the blank, is directly proportional to the concentration. For higher concentrations the extinctions are not longer proportional to the concentration.

With a measuring pipette 0.4 and 0.8 ml erythrodextrin solution (or 0.16 and 0.32 ml starch solution) are transferred successively to two volumetric flasks of capacity 100 ml. Each of these flasks is provided beforehand with 3 ml 0.2 % iodine solution. After filling up to the mark with water the extinctions are measured. For the blank determination 3 ml 0.2 % iodine solution is diluted in a 100 ml flask.

D. PREPARATION OF ENZYME EXTRACT

An accurately weighed quantity of the flour sample is transferred to a mortar and some purified sand is added. This mixture is triturated for 3 minutes with enough water to obtain a suspension

* WEBER uses the denomination erythrogranulose.

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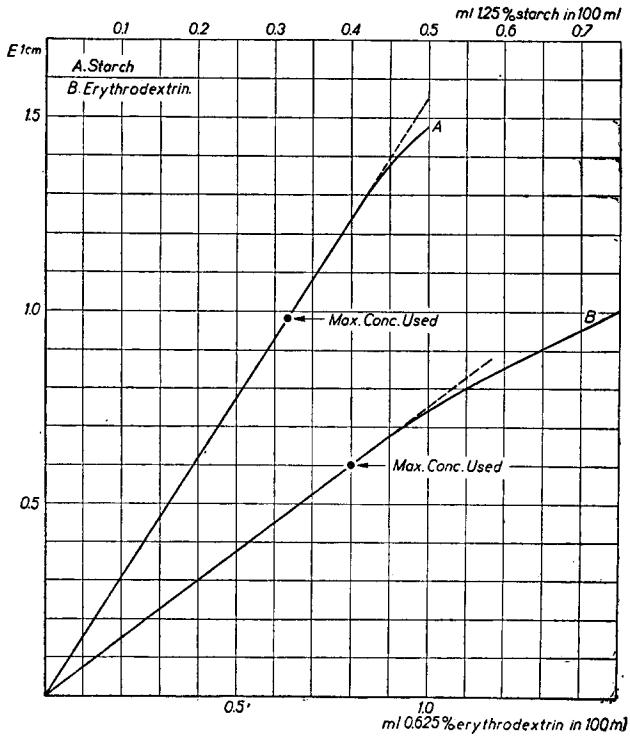

Fig. 1. Extinction concentration curves for iodine-starch and iodine-erythrodextrin

which is not too thick. The suspension is quantitatively transferred with water into a graduated shaking cylinder and diluted to 50 ml. After mixing, the cylinder is placed in a thermostat at 30° C and after one hour the suspension is filtered through a folded filter. If the first filtrate is turbid, it is filtered again. The maximum quantity of flour that can be used is 6 g in 50 ml; with greater quantities it is hardly possible to transfer the flour suspension with the available water, and moreover the filtration of the suspension would proceed with difficulty.

E. DETERMINATION

20 ml substrate solution are pipetted into a 100 ml flask. This solution and the flour extract are placed in a thermostat at 30° C, and after sufficient time to take up this temperature, 5 ml extract are added with a pipette to the substrate while shaking. The time is noted by means of a stopwatch. After x minutes 1 ml of enzyme-erythrodextrin solution (or 0.4 ml enzyme-starch solution) is transferred with a pipette into a 100 ml volumetric flask that already contains 3 ml 0.2 % iodine solution. This is repeated after 2x, 3x and 4x minutes. x depends on the velocity of the reaction and is on average 3–5 minutes. For very small amylase contents, one has to take x = 15 — 30 minutes to obtain a noticeable conversion. By varying the quantities of flour in preparing the extract, it is possible to keep x more or less constant.

After filling up to the mark, the extinctions are measured. These extinctions corrected for the extinction of the blank are used to find the substrate concentration with the aid of the standard curve. The substrate concentrations are plotted against time, and provided that suitable flour quantities have been taken a straight conversion line is obtained.

The alpha-amylase activity is computed as the amount erythrodextrin in mg, converted per minute by the alpha-amylase present in 1 g flour.

DISCUSSION OF RESULTS

The change in erythrodextrin concentration with time under the influence of varying amounts of purified alpha- or beta-amylase and of mixtures of these preparations was followed.

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TABLE I

INFLUENCE OF PURIFIED ALPHA- AND BETA-AMYLASE ON ERYTHRODEXTRIN

mg amylase in 20 ml substrate		mg erythrodextrin converted per minute	alpha-activity · 10^2 for	
alpha-	beta-		alpha-amylase	beta-amylase
1.5	—	2.90	19.3	
2	—	3.91; 4.06	19.9	
2.5	—	4.84	19.4	
3	—	5.82	19.4	
4	—	7.56; 7.58	18.9	
—	50	0	—	0
—	100	0	—	0
1	1	2.03	20.3	—
3	3	5.78	19.3	—

The results are summarized in Table I, from which the following conclusions may be drawn:

1. Erythrodextrin is not converted by beta-amylase, even if large amounts of beta-amylase are used.

2. In a mixture containing alpha- and beta-amylase the conversion of erythrodextrin is determined by alpha-amylase only.

3. The decrease in concentration after 1 minute is directly proportional to the quantity of alpha-amylase in the substrate.

This is demonstrated by the activity which has, within the limits of error of the method, a constant value (mean: 19.5 × 10^2). With erythrodextrin solutions of smaller concentrations than used here the conversion curves are no longer rectilinear, while with larger concentrations the conversion rates are too small.

The influence of the p_H value of the substrate was traced over a p_H region 4.5 — 7.0, for two different acetate concentrations. The results obtained agree with those found in the literature. Between $p_H = 4.5$ and 5.6 the conversion is greatest and constant. A change in the acetate end concentration from 0.016 M to 0.008 M had no effect on the conversion rate of erythrodextrin. In accordance with these facts, $p_H = 5.3$ and an acetate end concentration of 0.016 M were chosen for the determination.

The alpha-activity was determined on about 30 samples of rye flour. In most cases more than one extract was prepared, using different amounts of flour. The conversion curves for some divergent samples are given in Fig. 2.

The activities found ranged

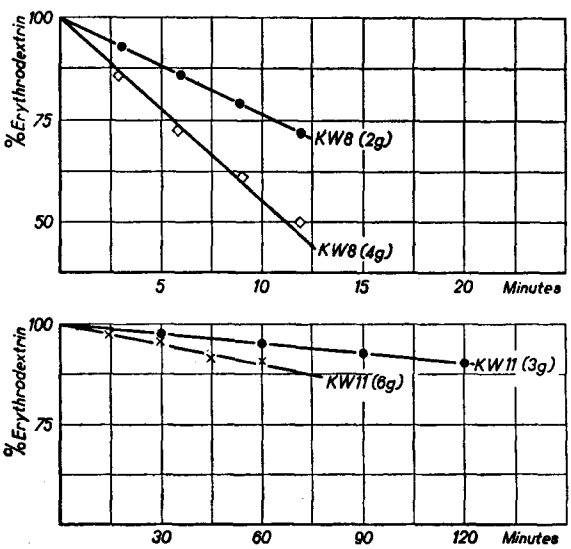

Fig. 2. Conversion of erythrodextrin by two different samples of rye flour. KW 8: High amylase content. KW 11: Low amylase content

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from 0.06 to 18. Six samples had an alpha-activity higher than 10, nine samples between 10 and 1, and the rest had an activity below 1.

The aim of the investigation was, in the first place, to find a reliable method for the determination of alpha-amylase. From preliminary tests it appeared that the breakdown of starch by the combined attack of alpha- and beta-amylase (in the circumstances investigated) was a perfect additive function. With a view to obtaining a complete idea of both amylases, determinations were performed also with solubilized starch as a substrate. Table II clearly demonstrates the additivity of the alpha- and beta-amylase action.

TABLE II

INFLUENCE OF PURIFIED ALPHA- AND BETA-AMYLASE ON STARCH

enzyme in 20 ml substrate solution	% starch converted in 10 minutes	
	found	calculated
2 mg α	11.9	—
2 mg β	25	—
2 mg α + 2 mg β	36.9	36.9
0.67 mg α + 1.33 mg β	20.6	20.6

The observed and calculated values agree satisfactorily. Moreover, it is seen that a certain amount of beta-amylase causes a larger conversion than the same amount of alpha-amylase.

For the determination of the combined alpha- and beta-amylase activity 500 mg flour is sufficient.

The combined activity is expressed as mg starch converted per minute by 1 g of the sample. The combined (alpha + beta)-activities for the rye flour samples ranged from 28 to 137. They were most frequently situated between 50 and 100. Only two values below 50 were obtained.

For the alpha-amylase preparation the "starch"-activity (A^s_α) is 14.9×10^2, and for the beta-preparation used 31.3×10^2 (A^s_β).

By a simple calculation one can find the beta-amylase activity, expressed in mg starch converted per minute by 1 g flour. In the preceding pages the alpha-amylase activity (A^e_α) of the purified alpha-amylase sample, expressed as mg erythrodextrin converted by 1 g sample per minute, is found to be 19.5×10^2. A^s_α for the same sample is 14.9×10^2.

$$\frac{\text{alpha-activity (starch)}}{\text{alpha-act. (erythrodextrin)}} = \frac{14.9 \cdot 10^2}{19.5 \cdot 10^2} = 0.76.$$

The factor 0.76 depends on the susceptibilities of both substrates, and it has to be re-determined when new stock substrates are taken in use.

On multiplying the observed alpha-amylase activities (A^e_α) by this factor 0.76, the alpha-amylase activity for starch (A^s_α) is found. A^s_β can then be found by subtraction: $A^s_{(\alpha+\beta)} - A^s_\alpha = A^s_\beta$.

The alpha-amylase values found were used to test a viscosimetric alpha-amylase determination worked out in our laboratory by one of our collaborators. By this method the alpha-amylase activity is determined from the early changes in viscosity of a solu-

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bilized starch solution prepared by special method. This investigation will be published later. From the scatter diagram (Fig. 3). it may be concluded that a fair correlation exists between the alpha-amylase activity as found by the photometric method and the liquefying power of the same samples.

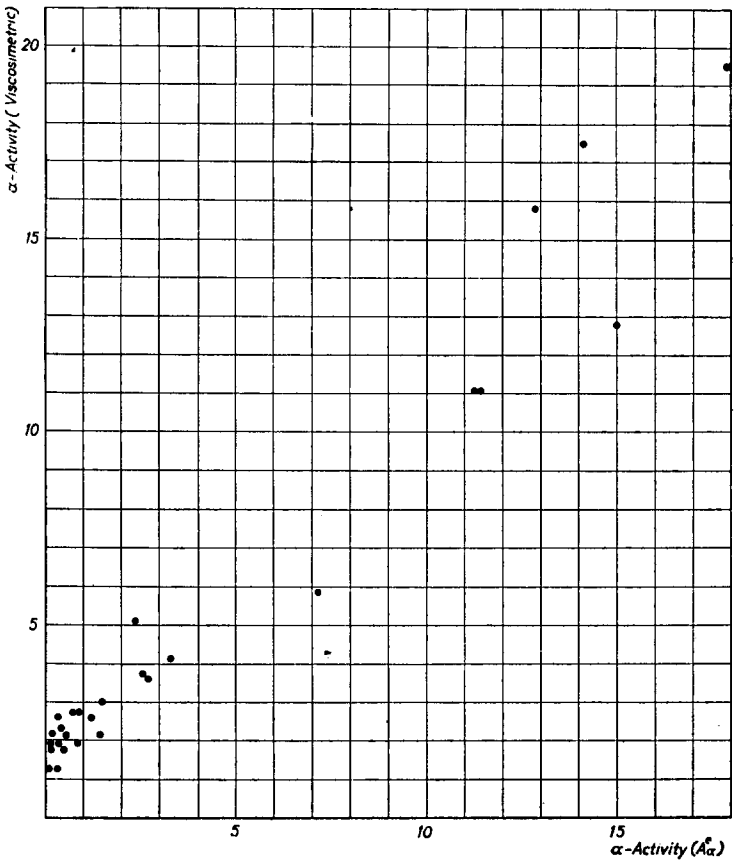

Fig. 3. Scatter diagram illustrating the correlation between alpha-amylase activities determined by two independent methods

In Fig. 4 the calculated values for beta-amylase-activity (A^s_β) are plotted against the saccharogenic values, obtained by determining the free amylase content by the method of MYRBÄCK and ÖRTENBLAD[12].

The correlation found here is also satisfactory. In most cases the conversion by combined alpha- and beta-amylase action was practically the same as by beta-amylase alone, suggesting that in normal flour samples the starch conversion as measured by the colour changes of the iodine starch reaction during the first period is governed by beta-amylolysis only; in some cases, however, the subtraction allowance for alpha-amylase amounted to 20–50 % of the beta-amylase activity, and then a departure from the correlation is to be expected. In Fig. 4 four points are marked by means of a circle for which the subtraction is more than 10 % of the calculated beta-value. Three out of the four points show deviations of the direction that might be expected.

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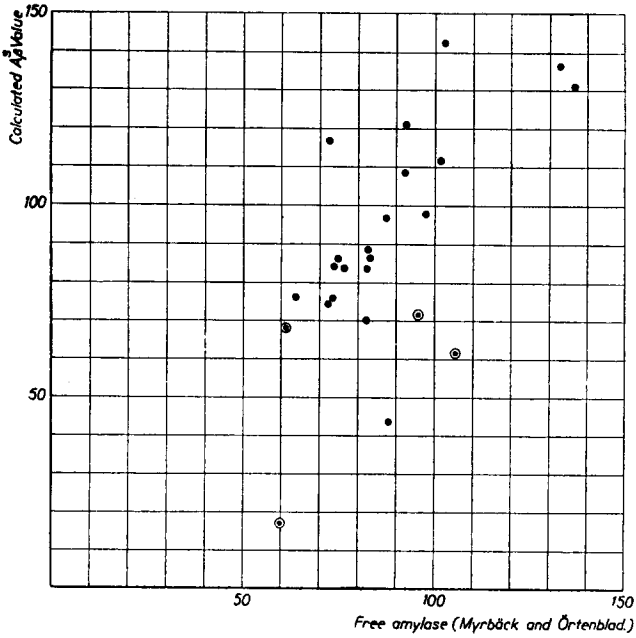

Fig. 4. Scatter diagram illustrating the correlation between calculated beta-activity and free amylase content by the method of MYRBÄCK and ÖRTENBLAD

CONCLUSION

The method for alpha-amylase determination described above, using erythrodextrin as a substrate, gives good results, as has been checked with purified alpha- and beta-amylase samples.

SANDSTEDT, KNEEN and BLISH, without putting it into words, also make use of the fact, that erythrodextrin is not further broken down by beta-amylase. Since, however, new substrate is prepared for each series of determinations, there is a greater possibility of different susceptibilities appearing than when solid erythrodextrin is prepared. The observed additivity of alpha- and beta-amylase acting on starch in the circumstances described was used only as a means of determining alpha- and beta-amylase activity separately. No hypothesis is made as to the underlying reaction mechanism. We are fully aware of the possibility that if the conditions are different the additivity of alpha- and beta-amylase action on starch may disappear.

RÉSUMÉ

Une solution d'érythrodextrine solide, tamponnée à p_H 5.3 a été utilisée comme substrat pour le dosage de l'alpha-amylase dans la farine, au moyen de la coloration produite par l'iode. La beta-amylase n'attaque pas l'érythrodextrine et n'a pas d'influence sur le comportement de l'alpha-amylase vis à vis de l'érythrodextrine. L'activité de l'alpha-amylase de la farine est exprimée en mg d'érythrodextrine transformée par minute, pour 1 g de farine.

La dégradation de l'amidon soluble dans des conditions déterminées est une fonction additive des quantités d'alpha- et de beta-amylase présentes. Il en résulte que l'activité de la beta-amylase peut être déduite de l'action combinée de l'alpha- et de la beta-amylase sur l'amidon, et de l'action de l'alpha-amylase sur l'érythrodextrine; cette activité est exprimée en mg d'amidon transformé par minute par 1 g de farine.

Les activités alpha- et beta- d'environ 30 échantillons de farine de seigle ont été ainsi déterminées. Les valeurs trouvées ont été comparées avec les résultats obtenus respectivement par une méthode viscosimétrique pour l'alpha-amylase, et une méthode saccharogène pour la beta-amylase.

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SUMMARY

A buffered solution of solid erythrodextrin at p_H 5.3 has been used as a substrate for the alpha-amylase determination in flour by means of the colour produced with iodine.

Beta-amylase does not attack erythrodextrin and it has no influence on the behaviour of alpha-amylase with regard to erythrodextrin. The alpha-amylase activity of flour was expressed as mg erythrodextrin converted per minute by 1 g flour.

Breakdown of solubilized starch under specified conditions appeared to be an additive function of the amounts of alpha- and beta-amylase present. Consequently the beta-amylase activity could be computed from the combined action of alpha- and beta-amylase on starch and the action of alpha-amylase on erythrodextrin; it was expressed as mg starch converted per minute by 1 g flour.

From about 30 samples of rye flour the alpha- and beta-activities were determined. The observed activities were compared with the results of a viscosimetric method for alpha-amylase and a saccharogenic method for beta-amylase.

ZUSAMMENFASSUNG

Eine gepufferte Lösung festen Erythrodextrins von p_H 5.3 wurde als Substrat für die alpha-Amylasebestimmung in Mehl mit Hilfe der mit Jod entstehenden Farbe benutzt.

Beta-amylase greift Erythrodextrin nicht an und hat keinen Einfluss auf die Einwirkung von alpha-Amylase auf Erythrodextrin. Die alpha-Amylaseaktivität von Mehl wurde in per Minute von einem Gramm Mehl umgesetzten mg Erythrodextrin ausgedrückt.

Abbau von in Lösung gebrachter Stärke unter bestimmten Bedingungen war, wie sich herausstellte, eine additive Funktion der vorhandenen alpha- und beta-Amylasemengen. Als Folge davon konnte die beta-Amylaseaktivität aus der kombinierten Wirkung von alpha- und beta-Amylase auf Stärke und der Wirkung von alpha-Amylase auf Erythrodextrin berechnet werden; sie wurde in mg Stärke, die pro Minute von einem Gramm Mehl umgesetzt werden, ausgedrückt.

Von ungefähr 30 Proben Roggenmehl wurden die alpha- und beta-Aktivität bestimmt. Die gefundenen Aktivitäten wurden mit den Ergebnissen einer viscosimetrischen Methode für alpha-Amylase bzw. einer saccharogenen Methode für beta-Amylase verglichen.

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