# DETERMINATION OF STARCH BY THE AMYLOGLUCOSIDASE METHOD

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In recent years amyloglucosidase has been taken into use in starch determination. Compared to other amylases, this enzyme has the advantage of splitting starch into glucose. The use of the method in routine work has only recently become possible, as is evident from the price development of the enzyme preparation: in the catalogue of the Koch-Light Laboratories Ltd. the price per 10 g was £ 150 in 1963—64 and in 1965 30 shillings.

The amyloglucosidase group of enzymes seems to be formed only by fungi, especially by the strains of *Aspergillus*, *Rhizopus* and *Endomyces*. Of these, *Aspergillus niger* is generally used in the commercial production of the enzyme. Amyloglucosidase acts by splitting glucose units from the non-reducing ends of starch chains. Studies by Pazur et al. (1959, 1960) with highly purified amyloglucosidase showed that it acts preferentially on longer chains and that it also hydrolyses $\alpha$-$(1 \rightarrow 6)$- and $\alpha$-$(1 \rightarrow 3)$ linkages, although more slowly than $\alpha$-$(1 \rightarrow 4)$ linkages. The enzyme also effects the hydrolysis of a variety of other glucosides, even if the process is very slow and there are large differences in the rates of hydrolysis of these compounds (Pazur and Kleppe 1962). According to Abdullah et al. (1963) the rate of hydrolysis of non-reducing $\alpha$-$(1 \rightarrow 4)$ bonds increases with the molecular weight of the substrate while the rate is dependent on the next bond in sequence.

Underkofler et al. (1965) studied the use of amylases in the starch industry, and compared characteristics of amyloglucosidase preparations from the strains of *Aspergillus*, *Rhizopus* and *Endomyces*. Investigations concerning the starch determination technique in connection with the use of amyloglucosidase seem to be rare, and the present authors have found only one investigation directly bearing on this subject (Thivend, Mercier and Guilbot 1965a, 1965b).

The purpose of the present study is to shed more light on the starch determina-

tion based on the use of amyloglucosidase, e.g. to examine whether the final determination can be carried out as reducing sugars.

*Method*

Reagents.

— 80 % (v/v) ethanol.
— 0.1 M acetic acid — Na-acetate buffer, pH 4.8, which contains 0.01 - % Na-merthiolate 0.5—1 ml/1 000 ml.
— Koch-Light amyloglucosidase preparation (950—1 200 U/gram).
— Reagents required in the determination of the reducing sugars (Somogyi 1945).
— Duolite A-$7^{CO_3^{--}}$ and Duolite C-$3^{H^+}$ (or Amberlite IR-$120^{H^+}$). Ionexchangers are required only for some materials poor in starch and even for these omitting the purification of the starch hydrolysate increases the result by only 0.1—0.3 per cent units.

Procedure. A level teaspoonful of quarz sand and 400—1000 mg of the sample are placed into a folded filter paper (Whatman no 541, Ø 18.5 cm) and mixed. For materials rich in sugars the sand prevents the sample from becoming compressed into a dense mass which would result in an inadequate extraction of sugars. The sample is extracted with 80 % ethanol in a Soxhlet apparatus for about 5 hours and left to dry in room temperature. Subsequently it is tranferred to a 100 ml Erlenmeyer flask (KPV 29) containing a few millilitres of water. The water prevents the starch from spreading along the sides of the flask. 25 ml of water is added in small portions and stirred with a glass rod to wet the sample. The mixture is boiled on an electrical stove for 5 minutes. The substance which has risen to the sides of the flask is rinsed with hot water (about 25 ml) and the boiling is continued for another 5 minutes. After cooling, 50 mg of amyloglucosidase and 25 ml of the buffer solution are added followed by water until the flask is almost filled. The flask is kept at 40°C for 20—22 hours shaking it once every hour during the first 3 — 4 hours. The solution is then filtered through a paper into a 500 ml volumetric flask and the residue is washed with water. The flask is filled up to the mark and the reducing sugars are determined by the method of Somogyi (1945).

An alternative method for the enzyme treatment: The flask is kept at 60°C for 4—5 hours and shaken every 30 minutes. The second alternative gives slightly more exact results for materials poor in starch (lucerne, swedes, soya bean meal), but the first alternative is more convenient and possibly even more reliable for materials rich in starch.

For some materials poor in starch, an ionexchange purification before sugar determination reduces (corrects) the result by 0.1—0.3 per cent units, by eliminating, among others, cysteine from the solution. The purification is performed using the batch method in the following way: 0.5 g Duolite A-$7^{CO_3^{--}}$ + 0.5 g Amberlite IR—$120^{H^+}$ (or Duolite C—3) + 25 ml of the investigated solution are put into an Erlenmeyer flask, shaken for an hour and filtered through a paper. This purified solution can be used also for chromatographic examination.

*Studies concerning the method*

M a t e r i a l s. The wheat and potato starch were commercial products, the pea starch was a preparation from our own laboratory. The starches were analysed for their water, ash, crude protein and the 80-% ethanol extract contents. These impurities occurred in the following amounts:

| | | | |
|---|---|---|---|
| Wheat | starch | 1.48 % | of the dry matter |
| Potato | » | 0.43 » | » |
| Pea | » | 0.42 » | » |

The grains and vegetables were produced in Finland. The grains were examined as wholemeal and in the vegetables the edible parts were analysed. The rootstocks of the marsh horsetail (*Equisetum palustre*) formed a mixed sample collected from varying depths. The samples were ground with a Wiley mill using mesh No. 40 (0.42 mm).

E x t r a c t i o n w i t h e t h a n o l. Extraction with ethanol is indispensable for the elimination of the sugars, because the final determination is carried out as reducing sugars. This procedure has also other advantages: it effectively eliminates substances changing the pH of the solution (organic acids and their salts) so that the buffer concentration can be very low, even lower than the one used in this method. It also eliminates non-sugar reducing substances and makes the purification of the solution for the sugar determination practically unnecessary. In addition it dissolves substances clogging the filter so that the solution after the enzyme treatment usually is easily filterable. If this does not occur the mixture can be poured into a volumetric flask, filled up to the mark and an aliquot of it filtered.

T h e g e l a t i n i s i n g o f s t a r c h. Starch is gelatinised by boiling in water for 10 minutes. After this treatment the starch is hydrolysed completely into

Table 1. Results from starch preparations.

| | Buffer, pH and concentration of buffer in sugar determination | | | Hydrolysis Time | Temperature | Starch recovered, % |
|---|---|---|---|---|---|---|
| Potato starch | Citrate | pH 5.0 | 0,01 M | 22 hrs | 40° | 100.0 |
| » | Acetate | » 4.8 | » » | » » | » | 100.2 |
| » | » | » » | 0.005 » | » » | » | 99.4 |
| » | » | » » | » » | 5 » | 60° | 99.8 |
| Wheat starch | Citrate | » 5.0 | 0.01 » | 22 » | 40° | 99.6 |
| » | Acetate | » 4.8 | » » | » » | » | 99.0 |
| » | » | » » | 0.005 » | » » | » | 99.7 |
| » | » | » » | » » | 5 » | 60° | 99.6 |
| Pea starch | » | » » | » » | 22 » | 40° | 99.8 |
| » | » | » » | » » | » » | » | 99.9 |
| » | » | » » | » » | 5 » | 60° | 99.6 |

glucose by amyloglucosidase, as is seen from Table 1. The size of the samples in these experiments was 200.0 mg of air dry starch and the results are calculated for the ash-, protein- and lipid-free dry matter. Heating in autoclave at 130°C, which was considered indispensable by THIVEND et al. (1965), is not necessary, but does not cause erroneus results either. The boiling is performed in two stages, because at the start of the boiling many materials foam and there is a possibility that ungelatinised starch may stick to the sides of the flask if it is not washed down. The boiling time may possibly be cut down from 10 minutes.

The enzymatic hydrolysis. According to PAZUR and ANDO (1959), the optimum pH of highly purified amyloglucosidase prepared from *Aspergillus niger*, is 4.8 with approximately 50 per cent inactivation at pH values below 3.0 or above 6.5. THIVEND et al. (1956) give the optimum pH as 4.8, UNDERKOFLER et al. (1965) as 4.0. The two last mentioned groups of research workers consider the optimum temperature to be 60°C. According to the experiences of the present authors, the enzyme is not exacting with regard to either of its optima, at least not when the period of hydrolysis is round about 20 hours. Table 1 shows that with pure starch preparations a 100 % result was obtained when the pH was 4.8 and when it was 5.0, the temperature being 40°C. The same result was obtained with 5 hours of hydrolysis at 60°C, the pH being 4.8. According to preliminary trials the pH can be raised up to 5.2 if the treatment lasts overnight.

Table 2 gives the obtained starch contents for different materials as well as the effect of the hydrolysis time and the temperature on the results. The pH was 4.8 in all trials. The results indicate that e.g. swedes, lucerne and red clover contain starch, even if the contents are very low. Carrot and celeriac are already rather

Table 2. The effect of the hydrolysis time and temperature.

| Material | Starch, % of dry matter | | | | |
|---|---|---|---|---|---|
| | 22 h 40 ° | 5 h 60 ° | 5 h 40 ° | 4 h 60 ° | 4 h 40 ° |
| Rye, Ensi | 57.0 | | | | |
| Wheat, Ring | 58.2 | 57.9 | 57.5 | | |
| Barley, Pirkka | 53.6 | 53.4 | 52.6 | 53.3 | 51.4 |
| Oats, Sisu | 48.7 | | | | |
| Wheat bran | 21.5 | | | | |
| Pea, Kalle | 40.2 | 40.1 | 37.8 | 39.1 | 37.3 |
| Garden bean, Fiskeby | 17.8 | | | | |
| Potato, Akvila | 59.1 | 59.3 | 56.7 | 58.3 | 56.3 |
| Celeriac, Aebleformet | 10.3 | | | | |
| Carrot, Nantes Typ Top | 3.8 | | | | |
| Swede, Göta | 1.4 | 1.3 | 1.3 | 1.3 | 1.3 |
| Ground-nut meal | 7.1 | | | | |
| Soya bean meal | 3.3 | 3.0 | 2.8 | 2.7 | 2.6 |
| Lucerne, leaf stage | 3.1 | 2.5 | 2.4 | 2.4 | 2.3 |
| Red clover, in bloom | 1.5 | | | | |
| Rootstocks of *Equisetum palustre* | 10.9 | 10.8 | 10.6 | | |

rich in starch. When comparing the results with those obtained by THIVEND et al. (1965a), relatively large differences are noticed for some cereals. These are, however, probably due to differences in climate and plant varieties.

The comparison concerning the hydrolysis time and the temperature (Table 2) shows that 5 hours at 60°C for most plant materials gives the same or almost the same result as 22 hours at 40°C, even 4 hours at 60°C seems in most cases to be reasonably adequate. A treatment of 4—5 hours at 40°C is less effective.

The paper chromatographic examinations (Fig. 1.) indicate that the starch hydrolysate of materials poor in starch contain besides glucose small amounts of arabinose and ribose, and certain materials also traces of galactose. In the 5 hour hydrolysates the amounts of these non-glucose sugars are less than one half of those in the 22 hour hydrolysates. The absolute amounts of the foreign sugars are, however, small, e. g. the 5 hour starch hydrolysate of lucerne contains arabinose about 0.2 % and ribose 0.05—0.1 % of the original dry matter or altogether 0.2—0.3 %, and the hydrolysate of swedes even less. For materials richer in starch, including bran and beans (about 20 % starch), a 90 microgram chromatogram did not show other sugars than glucose. It is possible, however, that also for these materials traces of non-glucose sugars could be demonstrated if the chromatograms were prepared from solutions concentrated enough so that amounts up to 0.05 % of the original dry matter could be noticed. In Figure 1 examples of the purity of starch hydrolysates are shown. The photograph of the chromatogram is, however, so much less distinct than the chromatogram paper itself that only arabinose, besides glucose, can clearly be seen. In routine work the error caused by non-glucose sugars is scarcely of importance. In order to reduce the error, the authors, however, recommend an enzyme hydrolysis of 4—5 hours at 60°C for materials poor in starch. For cereals, potatoes and peas, on the other hand, 20 hours at 40°C is a more convenient, and apparently also a more reliable procedure.

The occurrence of non-glucose sugars in the starch hydrolysate is due to the effect of the enzyme preparation on the plant material. Chromatograms from blank tests — from vegetables without enzyme as well as from enzyme preparation without vegetables — gave zero results as regards sugars. Either the enzyme preparation of the Koch-Light Laboratories contains some other carbohydrases besides amylases, or the amyloglucosidase or other amylases of the preparation are able to split also polysaccharides containing as components at least arabinose, ribose, galactose and mannose (in soya bean meal). Xylose could not be demonstrated in any of the hydrolysates although the plant materials are rich in xylose polymers (SALO 1965b). PAZUR and KLEPPE (1962) established that pure amyloglucosidase also hydrolyses a variety of other glucosides, even though the process is very slow. The glucosides subjected to their investigation do not, however, contain all the previously mentioned sugar units. On the other hand, PAZUR and ANDO (1959) mention that the »Diazyme» amyloglucosidase preparation (trademark of the enzyme preparation from the Miles Chemical company) contains α-amylase, maltase and two different kinds of amyloglucosidase. The preparation of the Koch-Light Laboratories is probably also a mixture of different enzymes.

To keep the pH relatively unchanged, 25 ml of a 0.1 M buffer solution per 1 g

of plant material extracted with ethanol is sufficient. The buffering capacity of the acetate buffer is somewhat greater than that of the citrate buffer, though the latter is efficient enough for the purpose. If required, the amount of the sample for non-homogenous materials poor in starch can be raised up to a few grams without increasing the volume of the buffer solution.

The sugar determination. The starch hydrolysed into glucose is determined as reducing sugars (SOMOGYI 1945). The necessity of purifying the solution was examined by carrying out the sugar determinations of the hydrolysates as such, and after ionexchange purification. The comparison indicated that for all cereal products, and for peas, potatoes, celeriac and ground-nut meal the purification was unnecessary. In the other samples purification reduced the results by 0.1—0.3

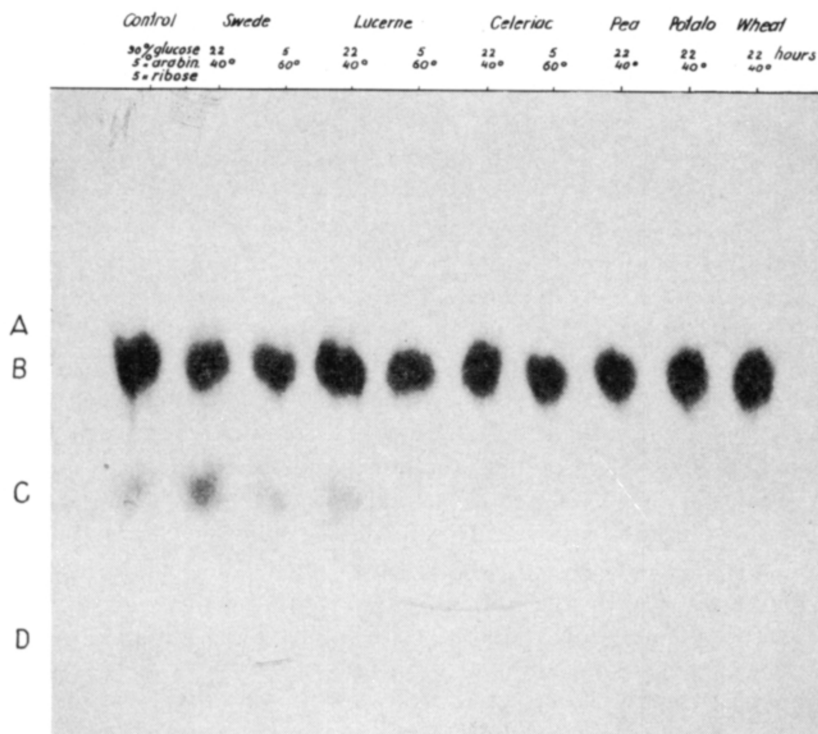


Fig. 1. Paper chromatogram of sugars in the starch hydrolysates of some plant materials. A galactose, B glucose, C arabinose, D ribose.

per cent units. The difference was greatest in some green plant materials, in which the hydrolysate contained cysteine (reduces copper reagent into green). The values in Table 2 are for the purified solutions. The purity of all hyrolysates as regards the non-glucose sugars was examined by paper chromatography (Fig 1). As solvent n-butanol — acetic acid — water (4 : 1 : 5) was used, the running time was about 90 hours and the colour reagent was aniline phthalate (SALO 1965a, p. 32).

The buffer concentration of the hydrolysate should be kept within certain

limits because of the sugar determination. It was observed that if a concentrated buffer solution was examined for reducing sugars as such, a zero result was obtained, but when a mixture of the buffer solution and glucose was analysed, the results obtained for glucose were too low. A 0.01 M solution seems to indicate the upper limit. A solution half as concentrated as the above (dilution 0.005 M) was, however, used in the final method, since the pH remained unchanged. The citric acid — Na-citrate buffer causes errors in the sugar determination at slightly lower concentrations than does the acetic acid — Na-acetate buffer. Perhaps the »approximate» results (92—98 %) observed by PAZUR and ANDO (1959) were due to too high a concentration of the citrate buffer.

The repeatability of the method is good, 1—1½%. For materials rich in starch the repeatability of the results after a 4—5 hour hydrolysis is somewhat lower than after about 20 hours of hydrolysis.

The method is suitable for serial laboratory work. The analysis of a series of 8—9 samples takes about 12 hours, spread over 3—4 days. The sugar determinations are then carried out as duplicates.

The method as compared to that of Thivend et al. The authors consider that the method has the following advantages compared to the method of THIVEND et al.:

1) The performance of the analysis requires less work, for instance because heating in autoclave has been omitted as unnecessary. The extraction with ethanol and the sugar determination are obviously also less time-consuming than those of Thivend et al.

2) The analysis can be carried out within 6—6½ hour working-days, which is important from the practical point of view. For several materials the enzyme hydrolysis can be accomplished during the night. For materials poor in starch for which a 4—5 hour hydrolysis period is more suitable, the hydrolysis procedure can also be carried out within 6—6½ hour periods, since the pre-boiling takes only 10 minutes. The sugar determination can be made the following day.

3) The apparatus needed is simple and cheap, since a spectrophotometer, an autoclave or a water bath with a shaking apparatus are not required. The enzyme hydrolysis can be performed in a laboratory oven. Thus the method is suitable also for laboratories that have only limited facilities.

## *Summary*

A method of determining starch based on the use of amyloglucosidase is presented. In the method the sugars are extracted from the sample with 80 % ethanol, the starch is gelatinised by boiling with water for 10 minutes, after which it is hydrolysed into glucose by amyloglucosidase at pH 4.8 at 40°C (20—22 hours) or at 60°C (4—5 hours). The reducing sugars are determined. Purification of the solution for the sugar determination is not necessary for cereal products, peas and potatoes. For vegetables and legumes ionexchange purification reduces the results by 0—0.3 per cent units. The buffer concentration in the sugar determination should be less than 0.01 M.

The method was found to give a 100 per cent result for pure starch preparations. The purity of the hydrolysates was examined by paper chromatography and it was observed that, at least for some materials poor in starch, the starch hydrolysate contains small amounts of other sugars besides glucose. However, with a short hydrolysing time these amount to only 0.2—0.3 % of the original dry matter.

The repeatability of the method is good, 1—1 ½ %. The analysis of a series of 8—9 samples takes about 12 hours.

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## SELOSTUS:

## TÄRKKELYKSEN MÄÄRITTÄMINEN AMYLOGLUKOSIDAASIMENETELMÄLLÄ

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Kirjoituksessa esitellään amylglukosidaasin käyttöön perustuva tärkkelyksen määritysmenetelmä. Näytteestä uutetaan 80 % etanolilla sokerit, tärkkelys liisteröidään keittämällä vedessä 10 min., minkä jälkeen se hydrolysoidaan amyloglukosidaasin avulla glukoosiksi pH:n ollessa 4.8 ja lämpötilan 40 ° (20—22 t) tai 60 ° (4—5 t). Määritetään pelkistävät sokerit. Liuosta ei tarvitse puhdistaa sokerimääritystä varten, jos tutkimuksen kohteena ovat viljatuotteet, herneet tai perunat. Kasviksilla ja apiloillakin ioninvaihtajapuhdistus alentaa tulosta vain 0—0.3 prosenttiyksikköä. Puskurikonsentraation pitää sokerimäärityksessä olla alle 0.01 M.

Menetelmällä on todettu puhtaille tärkkelyspreparaateille saatavan 100-prosenttinen tulos. Hydrolysaattien puhtautta on tarkistettu paperikromatografialla ja todettu, että ainakin eräillä tärkkelysköyhillä materiaaleilla tärkkelyshydrolysaatti sisältää glukoosin ohella pienen määrän muitakin sokereita, mutta lyhyttä hydrolyysiaikaa käytettäessä niiden määrä on vain 0.2—0.3 % alkuperäisestä kuiva-aineesta.

Tulosten toistettavuus menetelmässä on hyvä, 1—1.5 %. 8—9 näytteen sarjan analysoimiseen kuluu noin 12 tuntia.