# DECOMPOSITION PRODUCTS OF ENZYMATIC STARCH HYDROLYSIS

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## Introduction

In the Rabe process, starch is mechanically removed by a flotation process without leaving any residue in the juice.

The sugar industry now applies an enzymatic process to reduce the quantity of starch in raw sugar. This process is operated in such a way that the unaffinated raw sugar contains approximately 100 ppm of starch. The hydrolysis products, however, remain in the syrup and may be partly included in the crystal.

Various authors have published data on the hydrolysis of starch by B. subtilis *a*-amylase [6, 2, 3].

Robyt and French used various glucans, among others amylose and amylopectin. They added to these substrates (conc. 0.1—1%) 0.1 amylase unit * per mg of glucan.

The oligosaccharide distribution which they obtained in the digest is shown in Table 1.

**TABLE I**
**Hydrolysis with B subtilis α–Amylase**

| *Product of Hydrolysis* | *Amylose* | | *Amylopectin* | |
|---|---|---|---|---|
| | *60 min.* | *180 min* | *60 min* | *180 min* |
| | % | % | % | % |
| $G_1$ | 2.3 | 5.3 | 1.4 | 3.3 |
| $G_2$ | 10.1 | 12.3 | 5.5 | 8.3 |
| $G_3$ | 12.8 | 22.0 | 8.2 | 10.8 |
| $G_4$ | 6.0 | 10.5 | 0.9 | 2.5 |
| $G_5$ | 10.2 | 14.8 | 4.9 | 6.7 |
| $G_6$ | 20.6 | 30.1 | 14.0 | 26.8 |
| $G_7$ | 14.7 | 5.1 | 9.8 | 9.2 |
| Larger mol. than $G_7$ | 23.3 | 0.0 | 55.3 | 32.4 |

* One amylase unit as defined by French is that amount of enzyme which reduces the blue value of a 1% soluble starch solution at 40°C in one minute. At present one unit of enzyme activity (U) is that amount that will catalyse the transformation of 1μmol. of substrate (or 1μ equivalent of the group attacked) per minute under optimum conditions of pH and temperature[4].

Unfortunately it is rather difficult to analyse accurately for all the hydrolysis products of starch in syrup, juice or any other sugar-house product.

## Experimental

*Enzyme Digest*

No effort was made to treat amylose and amylopectin separately as the factory only treats the complete starch mixture.

The factory conditions were simulated by using a potato starch solution which was boiled for twenty minutes and autoclaved for five minutes at .3 bar. The concentration was adjusted to 400 mg/l and the pH to 6.5. Ten mg/l each of sodium chloride and calcium chloride were added. Bactamyl D.200 was added in the range 5-15 mg/l to the starch solution, which had been heated to 65°C. The hydrolysis was carried out in such a way that approximately 90-95% of the starch was decomposed.

Under these circumstances 5 mg/l of enzyme powder gave an increase in reducing power of 0.25 mg glucose equivalent per minute.

Little increase in hydrolysis was obtained between 30 and 120 minutes. Alcohol was added to the mixture (1.6 times the volume) to stop the reaction and the solution was evaporated on a rotary film evaporator. Final drying was carried out in a desiccator.

*Quantitative determination of Oligosaccharides*

The oligosaccharides were separated by paper chromatography at room temperature using butanol: ethanol: water (2:1:1) as eluant. The quantitative determination was a modification of the method of Whistler & Hickson[8].

Guide strips were developed in silver nitrate reagent.[7] After the centre of the paper had been cut in between the fractions, the individual oligosaccharides were extracted in water by digesting the papers for one hour. As it was found that filter paper fibres interfered with the determination, the extract was filtered through a .45μ millipore filter, washed and made up to a suitable volume. To an aliquot of the filtrate (normally 10 ml) 2 ml hydrochloric acid (10 N) was added and the mixture was heated for twenty-five minutes in a boiling water bath.

After careful neutralisation using Bromo Thymol Blue (0.005%) as an indicator, the oligosaccharides were subsequently determined as glucose, according to the method of Hagedorn and Jensen[1].

A chromatogram of the oligosaccharides is shown in Fig. 1—Determination of polysaccharides.

Oligosaccharides larger than $G_8$ (maltooctaose) are not easy to separate by paper chromatography, although it is possible. Thin layer chromatography will separate to $G_{20}$—$G_{25}$.

Higher carbohydrate polymers are best separated by gel chromatography. This does not separate each individual sugar but separates groups of components according to their molecular weight.

The gel used was Sephadex G 50*, which separates

* Pharmacia Fine Chemicals Uppsala Sweden.

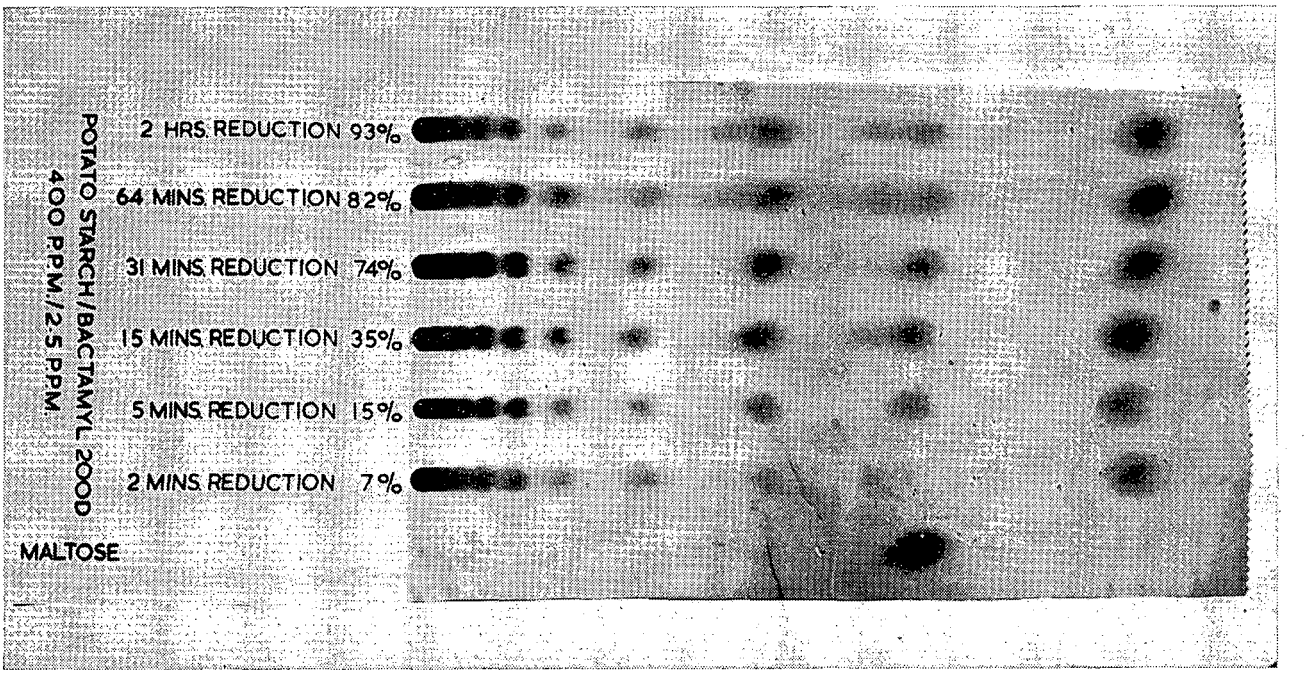


**FIGURE 1**

polysaccharides in the range Mw 500—10,000, excluding the higher Mw. The column used was 1.7 x 43 cm and 1 ml fractions were collected. The eluants used were either 0.15 M sodium chloride solution or distilled water. In these experiments no difference was observed between the two. One ml of eluant containing 1 mg of carbohydrate was placed in the column and washed into the gel with a few ml of eluant. Subsequently the reservoir with eluant was connected and the run started. The calibration of the column was carried out with Dextran T40* (Mw=40,000) Dextran $T_{10}$ (Mw=10,000) and glucose (Mw=180). The analysis of the fractions was carried out by a phenol-sulphuric acid method[5].

*Results and Discussion*

The distribution of oligosaccharides formed during enzymatic hydrolysis is given in Table II below:

**TABLE II**

**Oligosaccharide composition of a starch hydrolysate after treatment with B. subtilis α-amylase Potato Starch conc: 400 mg/l**

| | *5 ppm enzyme* % | *10 ppm enzyme* % | *25 ppm enzyme* % |
|---|---|---|---|
| $G_1$ | 1.8 | 4.0 | 8.0 |
| $G_2$ | 4.8 | 7.8 | 8.7 |
| $G_3$ | 8.8 | 10.2 | 10.0 |
| $G_4$ | 3.2 | 4.5 | 3.8 |
| $G_5$ | 4.5 | 7.8 | 12.8 |
| $G_6$ | 18.5 | 21.4 | 26.8 |
| $G_7$ | 15.0 | 11.3 | 4.8 |
| $G_8$ | 3.3 | — | — |
| $>G_8$ | 40.1 | $>G_7$ 32.0 | $>G_7$ 26.1 |
| Total recovered carbohydrate in % | 99 | 97 | 101 |

This distribution of oligosaccharides compares well with those published by Robyt and French. The main oligosaccharides formed are $G_3$, $G_6$, $G_7$. With higher enzyme concentrations the amount of $G_1$ (glucose) increases and $G_7$ decreases.

The results of the gel chromatography of the starch hydrolysate are shown in Fig. 2. The calibration of the column is shown in Fig. 3.

The fractions 56—90 were collected in a separate analysis and combined. After evaporation of the eluant the residue was separated by paper-chromatography and proved to contain the oligosaccharides $G_1$—$G_7$ in the same proportions as found before. The first peak, which was 30% of the total amount of carbohydrate present in the enzymatic hydrolysate has its maximum at fraction 32. The Dextran $T_{10}$ shows a maximum at fraction 28. The average Mw of the polysaccharides in the enzymatic hydrolysate is for this reason <10,000.

## Conclusion

The hydrolysis of starch B. subtilis *a*-amylase results in two groups of carbohydrates. One contains a mixture of oligosaccharides (70%) in which $G_3$, $G_6$ and $G_7$ are predominant. With higher quantities of enzyme the amount of $G_7$ decreases. The second group are polysaccharides (30%) with an average Mw <10,000.

## Summary

The decomposition products in a starch hydrolysate obtained by B. subtilis *a*-amylace were investigated.

The starch concentration used was 400 mg/l and the concentration of enzyme powder (a commercial product) between 5 and 25 mg/l.

The oligosaccharides in the hydrolysate were analysed by quantitative paper chromatography. The predominant oligosaccharides were $G_3$, $G_6$ and $G_7$.

The polysaccharides formed were analysed by gel chromatography using Sephadex G 50. The average Mw of these polysaccharides was found to be below 10,000 and represented approximately 30% of the total quantity of carbohydrate in the hydrolysate.

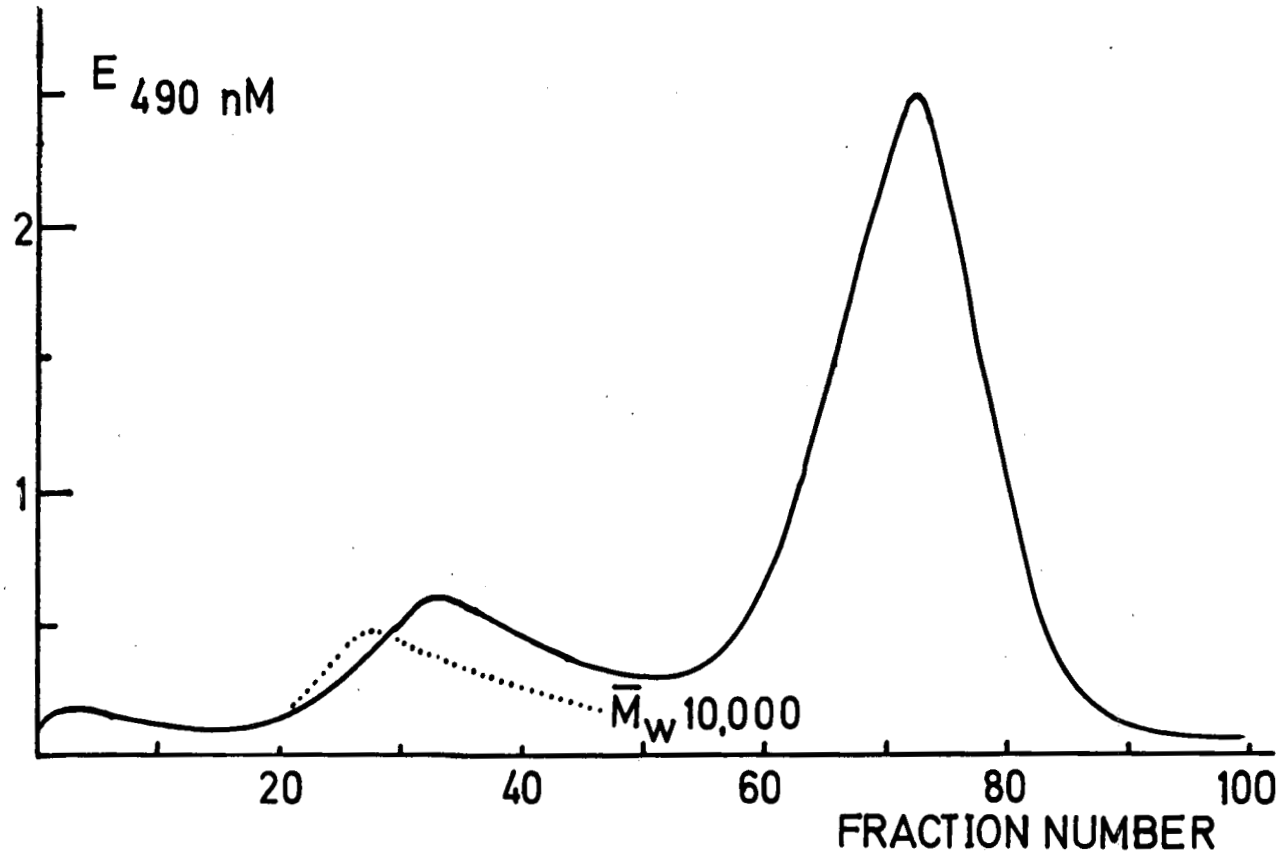


**FIGURE 2:** Gel chromatography of a starch hydrolysate using Sephadex G50.

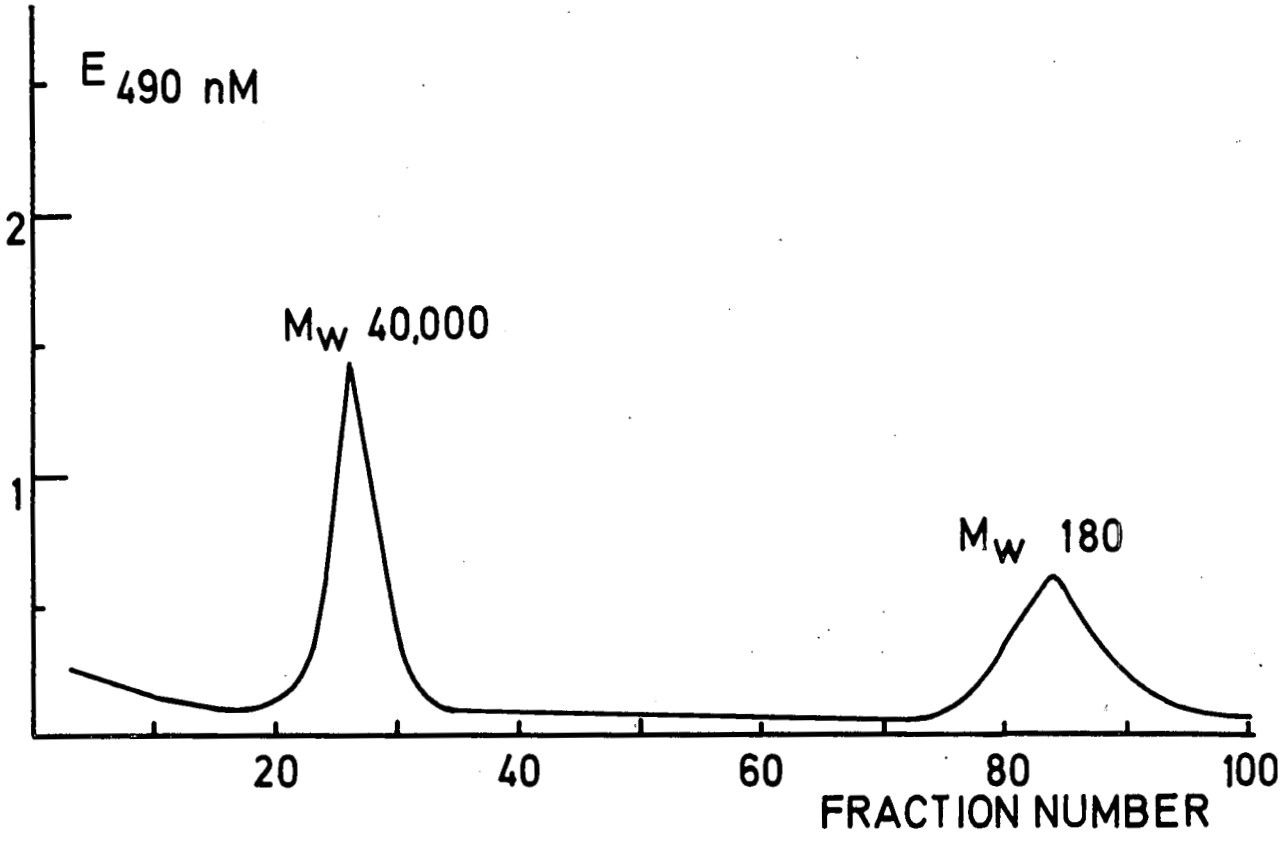


**FIGURE 3:** Calibration of a Sephadex G50 column with Dextran T40 and glucose.

**Acknowledgement**

Thanks are due to Mrs. M. Roberts for the experimental work.

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**Discussion**

**Mr. Alexander** (in the chair): Do any of the oligosaccharides in the hydrolysate co-crystallise with sucrose?

**Mr. Bruijn:** I have not done any work on this. But a subject under investigation at the moment is to try and find out why some factories produce elongated crystals, which, according to an Australian publication, are due to dextran.

We have analysed massecuites, which gave elongated crystals, for their total polysaccharide content but found no difference in the total quantity compared with other massecuites.

At present I am isolating the total quantity of polysaccharides and putting them through columns to try and find any difference in molecular weight.

**Mr. Comrie:** Mr. Bruijn mentions that the oligosaccharides $G_{20}$ to $G_{25}$ were separated by thin layer chromatography, using silica gel. We have had no success with this method.

Also, did he use streaking techniques for his paper chromatography, or only the spots as shown on the slides?

I must congratulate him on Table II, where his total carbohydrate recovery was near 100%.

**Mr. Bruijn:** I did carry out thin layer chromatography, but not very successfully, hence I used paper chromatography.

**Dr. Murray:** I would like to know more about the kinetics of starch hydrolysis. The kinetics shown by Mr. Smith appear to be zero order under his experimental conditions.

However, the kinetics shown by Mr. Bruijn's experiments under factory conditions are not of zero order. Now in a normal chemical reaction, if the order changes then the products of the reaction may change and, of course, if the products change this means that the subsequent chemical and physical behaviour of the system will change. If then one produces different products by changing hydrolysis conditions then might this not have an effect on the crystallisation of sucrose and impurities intercalated at a later stage?

Does the mechanism of starch hydrolysis vary with reaction conditions?

**Mr. Bruijn:** A lot has been published about the mechanism of the amylase attack on starch but I would have to study this to answer your question.

**Mr. Cox:** Starch hydrolysis has the characteristics of a first order reaction. One of the characteristics of this type of reaction is that it has a fixed and definite half life; therefore the percentage of starch hydrolysed in mixtures will remain constant, since until the point is reached at which all the actual sites on the enzyme are saturated, the speed of reaction will increase in proportion to the concentration of starch present. When all the active sites are saturated the kinetics change to zero order. This does not entail however that the molecularity of the reaction has changed, only that the availability of active sites, which under constant enzyme concentration will remain constant, has become the limiting factor. Under these conditions the mechanism of the reaction should remain exactly the same as when there still remained some active sites available, that is, as when the kinetics were still of the first order.

**Dr. Matic:** Over ninety percent of the original material was hydrolysed as determined by the starch-iodine colour determination. However, thirty percent of the hydrolysate according to gel-chromatography had an average molecular weight of 10.000 and therefore it would be expected to give blue colour. These two results seem to be contradictory.

**Mr. Bruijn:** It has been published that malto oligosaccharides with a molecular weight greater than 10 give a coloured iodine complex.

There seems to be a discrepancy in the results. It is however likely that the malto oligosaccharide has to be unbranched to give this colour. In the hydrolysate obtained most of the larger oligosaccharides obtained will be branched.