INVESTIGATION OF THE RETROGRADATION OF AMYLOSE

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Amylose and starch solutions lose some of their original properties during standing; they are subjected to peculiar changes. Starch in cold water assumes its original insolubility. Maquenne named this phenomenon retrogradation or ageing [1, 2]; in the industry the term "set-back" is used [3].

Retrogradation is a colloidal phenomenon. According to some investigators [4] an enzyme called amylocoagulase exists, and this enzyme is supposed to be responsible for the retrogradation. Others, however, doubt the existence of this enzyme [5, 6].

Amylopectine also retrogrades during standing but much more slowly than amylose does, and to a considerably lesser extent [7]. The retrogradation of starch is almost entirely due to the properties of amylose, therefore this publication deals with the retrogradation of amylose only.

I. The aspects of retrogradation

A freshly prepared starch or amylose solution opalizes, according to its concentration and its origin. When standing the opalization increases; hence flocculation occurs. Below a concentration of 2,5% the viscosity decreases, at higher concentrations the solution forms a solid gel. The precipitating flocks undergo sedimentation and form a powderlike or fluffy sediment increasingly becoming more compact. The specific properties of the sediment are greatly influenced by the presence or the absence of amylopectine. The sediment, that can be considered as being the product of retrogradation, is not soluble in cold water. If heated from its original humid state to 100° C, the sediment slowly dissolves. In a somewhat alkaline medium this dissolution takes place quickly. If, however, the aged material had formerly been dried, the dissolution takes place only in a strongly alkaline medium under simultaneous degradation. Aged amylose resists enzymatic hydrolysis. One of the methods applied to determine the extent of retrogradation is based on this observation. The material also partly resists acidic hydrolysis. With the progress of the retrogradation the J_2 reaction moves from the original blue colour towards a purple-red. In the case of potato starch the conductivity of the paste increases [8]. This can be explained by the slow hydrolysis of the phosphate groups. X-ray investigation proves the formation of a semi-crystalline structure. SAMEC [9] while checking aged amylose by infrared spectrography, has found that in the band which is characteristic for hydrogen bonds, the absorption increases with the progressiveness of retrogradation. According to this view, H-bonds are formed.

II. The industrial importance of retrogradation

Starch is being employed mostly in gelatinized or dissolved form, *i. e.*, in a state in which it is likely to retrograde. Retrogradation has an undesirable influence on rheological properties of paste and strongly reduces its ability to enzymatic decomposition.

The rheological properties of paste are of utmost importance, especially in the textile industry. For the sake of the uniformity of mechanized processes, it is important that the various pastes or solutions should always be of identical quality and should never change their properties during the process. The same problems arise when using paste in the paper and the adhesive industry. In order to prevent the changes in the viscosity of paste and the forming of precipitate, it is considered as useful to store pastes at higher temperatures (60—70° C) or to use modified (soluble, oxidized or dextrinized) starches.

The retrogradation of starch has very unpleasant consequences in the canning industry, while manufacturing canned soups and vegetables. It constitutes a special problem, *e. g.* in the case of canned peas in glass jars. The starch of wrinkled-peas contains amylose up to 60—70%. During the sterilization a part of the amylose is being dissolved, and after some time the juice becomes turbid. This is particularly unpleasant in export shipments.

In the fermentation industry the ageing is harmful because it directly decreases the alcohol yield. The ageing might to a considerable amount preclude the way of the enzymes of starch during the saccharification.

The best known and most common instance of the ageing is the staleing of bread. Fresh bread is sticky, the soft texture becomes, however, hard, granulated and crumbly, its resistance to amylolytic enzymes increases and the amount of extractable starch decreases. The proportion of "bound water" changes while retrograding. The swelling ability of the paste decreases, too. Outwardly it gives the impression as if all these changes were caused by simple drying, although the bread also stales in a hermetically closed box. Bread is a complex mixture of starch, protein, hemicellulose, salt, water, etc., the predominant item being, however, starch. Therefore, bread might be considered as a highly concentrated paste. Recent experiments have proved, beyond any doubt, that starch is responsible for the retrogradation of bread. Some surface active agents inhibit the retrogradation of bread [10, 11]. However a large scale use of them has not taken place, as yet.

III. Methods for measuring the extent of retrogradation

Investigating retrogradation, quantitative measuring methods, to check the progress of the process, are of fundamental importance. Retrogradation can be investigated by the following methods :

1. The most widely used and the most sensitive method is based on the fact that the retrograded amylose becomes resistent to enzymatic decomposition. MAQUENNE [1, 12] has performed the saccharification with malt amylase, and SALLINGER with ptyaline [6].

2. X-ray patterns : the primary amorphous Röntgenogram becomes semi-crystalline, yielding the so-called "B-type" Röntgenogram [13].

3. Viscosimetrically, the retrogradation of starch pastes can be checked only if the concentration is below 2,5%. Owing to the formation of gel, the formation of sediments gives these measurements, however, a doubtful value.

4. The decrease of light-transmittance ; due to turbidity, the amount of transmitted light decreases to a certain value, hence it starts to rise as soon as most of the amylose had settled. LANSKY and co-workers [14] have considered as characteristic for ageing, the time elapsing from the moment of the preparation of a 1% amylose solution till it becomes turbid.

5. ULMANN [15] studied the phenomenon of retrogradation by chromatography in a column with Al_2O_3 filling. His method can be particularly well applied for registering the presence of decomposition products.

6. SAMEC [9] investigated ageing with infrared spectrography, and ultraviolet absorption as well as with an electronic microscope.

7. WHISTLER and JOHNSON [16] determined the quantity of amylose, still in solution, by the chromic acid oxidation method of LAUNER [17].

8. On retrograding, the colour of the J_2 reaction turns from the original blue into red. The iodine reaction can be better evaluated if the amount of iodine suitable for bounding is potentiometrically or amperometrically established [18]. During the course of our experiments amperometric titration was employed. Retrogradation can be expressed in per cents with the aid of the iodine absorption value at the beginning of the experiment, and in the moment of the measurement, according to the following formula :

$$\text{Retrogradation } \% = \frac{(J_2\%)_0 - (J_2\%)_x}{(J_2\%)_0}$$

where

$(J_2\%)_0$ = the J_2 binding power of amylose in per cent at 0 time ;

$(J_2\%)_x$ = the J_2 binding power of amylose in per cent after x time had elapsed.

The experiments have been carried out in such a way that identical quantities of the same amylose solutions were titrated amperometrically [18, 19, 20] in various moments, and the consumption expressed in ml-s had been directly substituted into the above equation.

IV. Factors influencing retrogradation

The checking of factors influencing the phenomenon of retrogradation helps us to understand the mechanism of retrogradation, the knowledge of these factors are, however, important from the industrial standpoint, too. Mainly the following factors have bearing on retrogradation :

1. *The temperature.* The velocity of retrogradation decidedly increases with decreasing temperature. At a temperature of 0° C retrogradation is the quickest [12, 6], while at a temperature of between 60 and 70° C, retrogradation does not take place at all [21]. Melting a frozen starch paste the retrograded starch persists as an insoluble gel [22]. The properties of the gel also depend on the conditions of freezing [23].

In order to check the influence of temperature, the following experiments have been made : a potato amylose solution of a 249 mg/150 ml concentration has been prepared. The solution has been adjusted with a phosphate buffer to a pH of 6,5. 10 ml-s of the buffered solution have been introduced into test tubes, 1 drop of toluene added to each sample and the tubes closed with a rubber stopper were stored at 10, 30 and 40° C, resp. From time to time the contents of a tube have been amperometrically titrated, and from the quantity of the 0,01 n J_2 solution consumed the $J_2\%$ value has been figured, from which the per cent of retrogradation could be figured out with the above formula. The results are shown on Fig. 1.

The reasons of the interdependence of retrogradation and temperature will be discussed in our next publication.

2. *The influence of pH.* — According to the literature [24], the modifications of pH around the neutral point have no particular importance as regards retrogradation. According to SMIRNOV [25], the quickest retrogradation takes place at a pH 2, being four times as quick as at pH 6. At a higher pH retrogradation does not occur at all.

During the experiments hereafter described the role of pH was more thoroughly examined. A stock solution of constant concentration is prepared from potato and wheat starch amylose, resp. (recrystallized from aqueous butanol) so that butanol is completely removed from the solution by vacuum distillation. The solution is always subdivided into 5 portions. Each portion is adjusted to some pH value with a CLARK—LUBS [26] buffer solution. 10 ml-s of each solution have been introduced into test tubes to which one

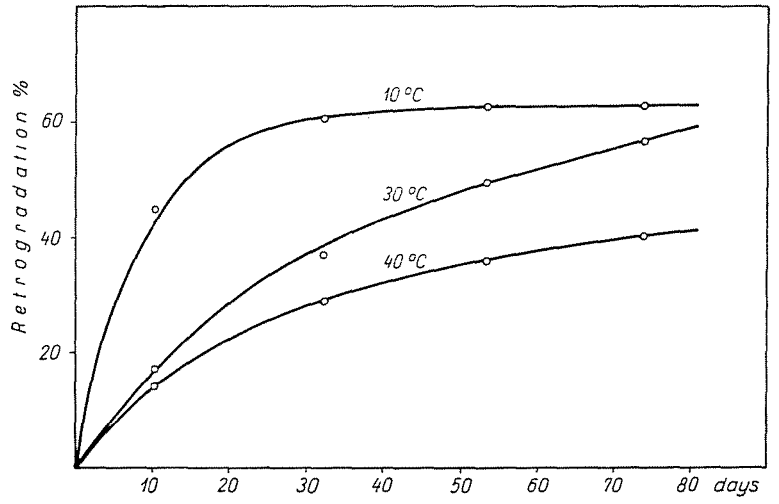

Fig. 1. The retrogradation of potato amylose in the function of temperature

drop of toluene has been added, the tube closed by a rubber stopper and thermostated at a temperature of 15° C. After a given time the content of one tube was amperometrically titrated. From the so obtained data the per cent of retrogradation has been figured out and represented on Fig. 2.

The curves show the following:

a) Retrogradation occurs in different ways in solutions having different pH values. Amylose retrogrades the most quickly at a pH of around 7. Fig. 3

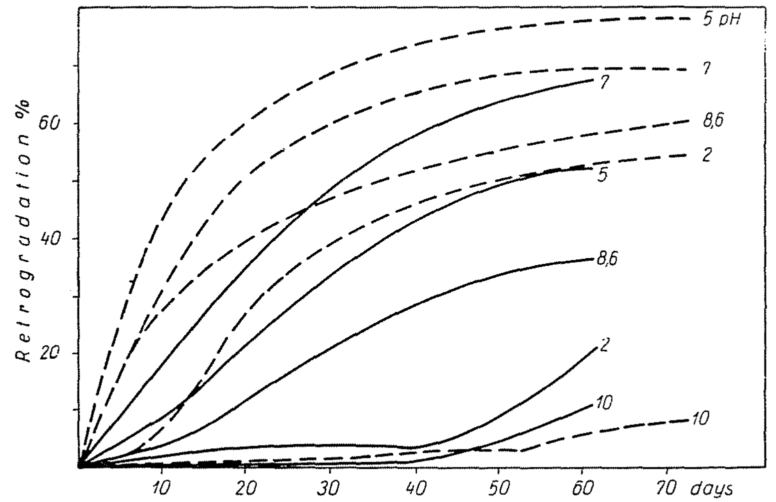

Fig. 2. Retrogradation of wheat and potato amylose, resp. in the function of time, in solutions of different pH-s. Individual curves connect data measured in solutions of identical pH-s but at different times. ———— = potato amylose — — — — — = wheat amylose

represents a kind of "cross-section" of the retrogradation curves of potato amylose. This Figure represents the per cent of ageing in the function of pH, measured on the same days [27]. This renders the role of pH particularly evident.

As will be later seen, the role of pH consists in the influence on the stability of the hydrate water hull.

b) Wheat amylose generally retrogrades quicker than potato amylose. Corn amylose has been investigated, too. The results obtained were analogous. Potato amylose always retrogrades slower. The potato amylose solution starts

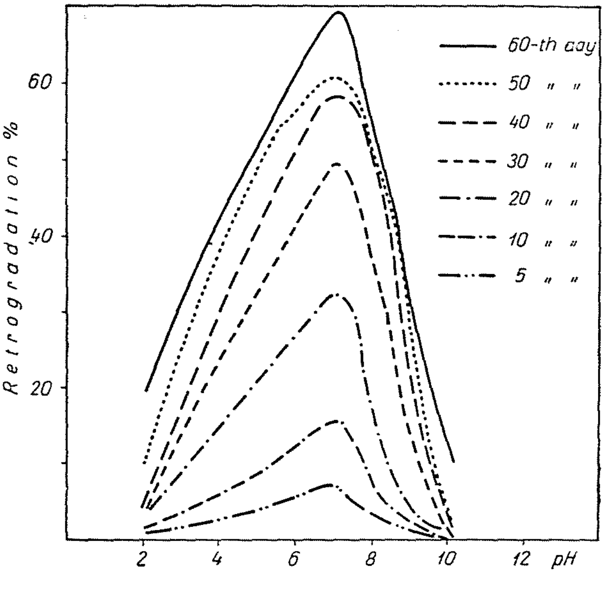

Fig. 3. Retrogradation of potato amylose in the function of pH. Individual curves connect data taken in the same moment but in solutions of different pH-s

to opalize later at the same concentration, and precipititation also occurs later. This will be explained in connection with the next point.

3. *The influence of the origin of amylose on retrogradation.* The above Fig. 2 shows that there is a decided difference between the velocity of retrogradation of wheat and potato amylose. This circumstance has already been pointed out by other investigators [16], and KERR [28] believes that potato starch retrogrades more slowly because the molecules are of a "V" shape.

KERR and CLEVELAND [29] concluded, on the basis of enzymatic experiments performed in 1952 that potato amylose contains 1—2 ramifications per molecule. Therefore, in our opinion, the molecules are rather "T" shaped.

This idea seems to be supported by the reduced readiness of retrogradation of potato amylose. The amylose helices have to form while ageing crystalline micells oriented one along the other in a stretched state. This orientation is greatly hindered by side chains of various length.

4. *The influence of salts.* It is a well-known fact that some salts, especially the salts of the earth alkaline metals, either accelerate or retard ageing.

Our experiments are principally aimed at examining the influence of $MgSO_4$ as the BUS—HIEMSTRA—MUETGEERT amylose producing method [30] employs a medium containing $MgSO_4$ in a concentration of 13% to fractionate starch. It seemed, therefore, rather interesting to find out how $MgSO_4$ acts, whether a simple salting out takes place only, or if ageing begins immediately.

A 0,1% solution of potato amylose was prepared and subsequently as much $MgSO_4$ was added as to reach a 13% concentration. This solution was adjusted to various pH values and a sample was taken from time to time after thorough shaking and titration with 0,05 n J_2.

In order to establish whether the ions of the buffer do not have any bearing on retrogradation, two series of tests were performed. In one of these series adjustment of the pH was performed with the aid of a CLARK—LUBS buffer solution [26]. In the other case the adjustment was done with H_2SO_4 and KOH, resp. The results were identical in both cases. Thus it can be concluded that the ions of the buffer have no bearing on the ageing process.

The retrogradation of solutions of different pH-s adjusted by acid or alkali plotted against time is shown on Fig. 4.

From Fig. 4 the following may be concluded :

a) The action of $MgSO_4$ is not based primarily on the formation of complexes. About five minutes after the addition of $MgSO_4$, the total amount of amylose is precipitated from the solution, while the J_2% value decreases only by 10—12%. The curve of the amperometric titration has a normal shape, just the horizontal section becoming somewhat shorter. Namely, the addition of complex-forming agents (*e. g.* butanol) somewhat steepens the horizontal section of the amperogram [31]. In the present case this did not happen.

b) The pH value by no means affects the process of retrogradation in a $MgSO_4$-containing medium. The retrogradation curves are nearly identical in solutions of different pH-values. This is of particular interest as in solutions without $MgSO_4$ the pH has a remarkable influence on retrogradation.

The pH above all affects the stability of the hydrate water hull (see below).

In the present case the pH is likely to lose its importance because $MgSO_4$ eliminates through simple salting out the hydrate water hull, independently of the pH.

c) The salting out of amylose is a primary process (considering that it takes place within about 10 minutes), while retrogradation itself is a secondary one.

Precipitated amylose molecules being near to each other, and deprived of their hydrate water hull, relatively quickly retrograde. The process takes, however, some hours at least (see Fig. 4).

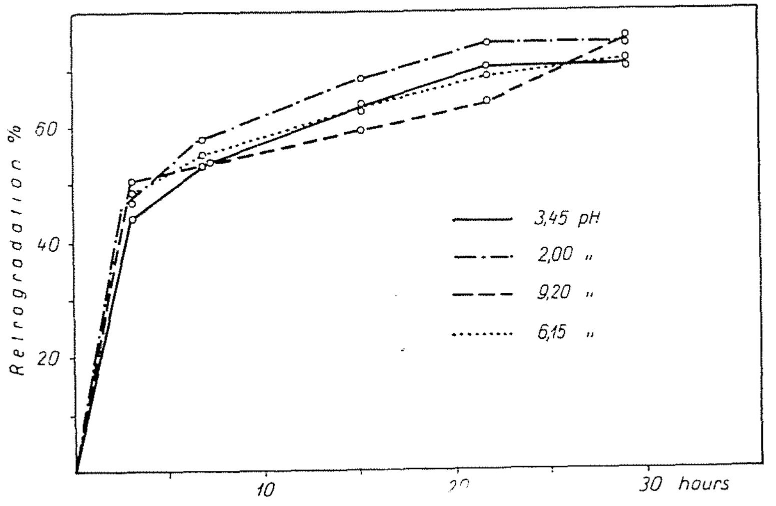

Fig. 4. Retrogradation of potato amylose in solutions containing $MgSO_4$ in 13% and of different pH-s

5. *Hydrolysis* increases the velocity of retrogradation till a certain value after which it quickly decreases. Big molecules arrange themselves for steric reasons, especially in a thin solution, with difficulty. The too small molecules, however, are slow in retrograding, due to their high solubility. This question has been thoroughly investigated by WHISTLER and JOHNSON [16].

Adding enzymes to a starch paste, the process of retrogradation, stagnating till then starts. According to some investigators [4], a supposed enzyme, named amylocoagulase accompanying amylase causes this phenomenon. It seems much more likely, however, that the increased retrogradation during the enzymatic hydrolysis is due to two separate reasons :

a) The viscosity of the paste decreases [5],

b) The small molecules playing the role of a protecting colloid [6] are being consumed before the sizes of the big molecules have been reduced to the size necessary for retrogradation.

6. *Increasing the concentration* the velocity of retrogradation of course ncreases, too. If the concentration of starch paste exceeds the 2,5%, the whole ageing paste rigidifies to a gel. In the case of amylose this depends, to

a great extent, on the origin of the amylose, its degree of polymerization and on its homogeneity as to the degree of polymerization [31, 32].

7. *The influence of inoculating centres.* — It has been investigated, how the retrogradation of a freshly prepared amylose solution goes on, if some centres of an already aged amylose are present. The solution of a freshly prepared wheat amylose has been divided into two portions, and one of the portions has been shaken with an amount of 10% of wheat amylose of the same concentration as exposed for one week to retrogradation, *i. e.* "inoculated" with aged centres. From time to time samples have been taken from the inoculated and from the non-inoculated solutions. The J_2 absorption has amperometrically been checked in order to figure out the per cent of retrogradation. The results are shown on Fig. 5. It can be concluded that the inoculated centres decidedly accelerate the process of retrogradation on the initial stage. The limit values of ageing have of course the same trend as in the non-inoculated solutions.

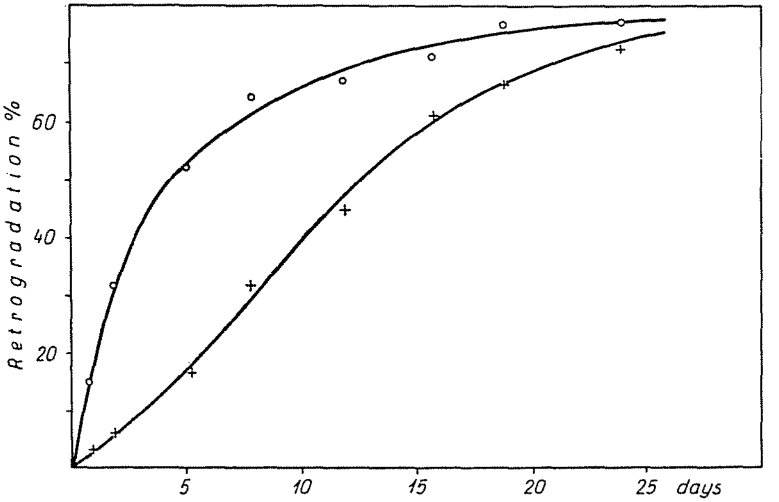

Fig. 5. Ageing of potato amylose in the presence of inoculating centres (0) and in their absence, resp. (+) The pH of the solutions was 6,46

The conclusions which may be reached from the above experiments will be discussed in our next publication.

Summary

1. During the investigation of retrogradation of amylose the decrease of iodine binding capacity is being used. The iodine binding capacity has been amperometrically measured.
2. The velocity of retrogradation decreases with increasing temperature.
3. Amylose retrogrades the quickest around the neutral point.
4. Potato amylose containing 1—2 ramifications per molecule, ages less rapidly than wheat amylose containing no ramification.

5. Under the influence of salts amylose first loses its hydrate water hull, and only later precipitates and retrogrades.

6. Inoculating fresh amylose solution with aged centres the retrogradation can be accelerated.

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