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THE ACTIVITY OF STARCH-HYDROLYSING ENZYMES IN PEARS DURING DEVELOPMENT AND COLD-STORAGE

BY

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INTRODUCTION

The keeping qualities of fresh vegetables and fruit can be prolonged by placing them in cold-storage. This counteracts the development of micro-organisms on one hand and slows down the life processes on the other. As the various physiological processes do not as a rule react identically to a reduction in temperature, disharmony may be created in the living object. This does not often prove to be a serious difficulty in the case of products having reached the final stage of their development prior to entering cold-storage, as long as the ensuing transformations are limited. This disharmony, however, is often the cause of storage being restricted to a relatively short period.

There is a further complication in the case of apples and pears, however, in that the fruit must complete its entire ripening after the period spent in cold storage before it is fit for consumption. This ripening is generally considered to be the functional breakdown of the tissue. Should excessive disharmony be created in the fruit during cold-storage, normal ripening is rendered impossible and the fruit can never become fit for consumption.

The behaviour of pears in cold-storage is partially governed by their stage of development, in other words by the activity of the enzyme systems at the moment the fruit was gathered.

The pears are invariably picked in an unripe condition; the subsequent ripening process being of longer or shorter duration, depending on the variety. Fruit picked too late actually ripen during cold-storage, whereas fruit picked too early will not ripen after leaving cold-storage. The interval of time between too early and too late picking is fairly short. This is not the case, however, with pears intended for direct consumption; the dates on which these pears can be picked may vary considerably, e.g. be spread over a period of 3-4 weeks.

We set out to study the activity of α- and β-amylase during the development of pears. The investigation also included the changes in

enzymic activity during the cold-storage of the fruit at 0.5° C. In addition, we endeavoured to ascertain the influence of the time of gathering on these changes and whether a number of varieties exhibited characteristic differences in this respect. Our inquiries also extended to the good ripening potentialities of each group after various periods in cold storage. The principal reason underlying this investigation was the phenomenon that starch is formed in pears during the course of their development, only to disappear again subsequently (see Fig. 4a). The first small amounts of starch can be found in the fruit, close under the peel, towards the end of June. The quantity of starch increases rapidly, spreading throughout the fruit and reaching its maximum about the beginning of August. From then onwards the quantity of starch decreases rapidly; towards the middle of September but little remains. As long as the pears are still on the tree, a small amount of starch is present in the fruit. This is generally the case, too, when normal ripening begins. As soon as the fruit has been gathered, the starch is broken down fairly quickly, disappearing completely within three days to a week. This degradation also continues, albeit more slowly, during cold-storage, so that no starch is present in cold-storage pears when ripening begins.

Even though numerous investigations have been made of the changes taking place both during the development as well as the cold-storage of fruit [BIALE (1950), NITSCH (1953), SMOCK (1944), SMOCK and NEUBERT (1950)], only a few research workers have paid attention to enzymic activities up to the present [EZELL and GERHARDT (1938, 1942), WEURMAN (1954a and b)].

MATERIALS AND METHODS

The pears used for the amylase determinations were mainly of the Doyenné Boussoch variety. Economically these pears are not of great importance as their flavour qualities are not rated highly This variety was selected, however, because it ripens well after cold-storage until March.

These pears were available in adequate quantities from one orchard, so that we were assured of a constant supply of pears of identical origin during a given growth and storage period, a factor of great importance when making series determinations. The orchard from which the Doyenné Boussoch pears were obtained is stocked with trees approximately 40 years old, so that they can supply a considerable quantity of fruit in good years. The orchard is at Langbroek near Doorn (Province of Utrecht). Soil type: heavy clay. Each sample consisted of 15 pears (more at the beginning of the season).

As the changes in amylase activity were characteristic, it appeared interesting to extend these investigations to two other varieties of pear, differing from the Doyenné Boussoch in the matter of their storage potentialities. Our choice fell on Conférence, one of our best dessert pears, and Comtesse de Paris; on the former because Conférence pears generally lend themselves well to storage over a lengthy period while preserving their ripening potentialities; on the Comtesse de

Paris, because difficulties frequently arise in connection with its ripening after cold-storage.

Both these varieties were obtained from a model orchard at Hoofddorp. Soil type: good sandy clay. The trees were much younger, viz. 15 years old. We used a row of 20 trees of identical age for the series determinations in connection with each variety. A sample consisted of 20 pears, one taken from each tree.

Activity determinations in individual pears showed that individual differences in amylase activity were so great that deviations up to 30 % in samples of 15-20 pears cannot be regarded as essential (MARIS MCARTHUR, 1955).

During the collection of these samples, care was taken to avoid gathering particularly small or large individuals. The pears were washed and dried; proportional parts by weight were taken by cutting a longitudinal section from each pear. These segments were then peeled, the core removed, rasped and mixed together.

The quantities of starch and the activity of the α- and β-amylase, inter alia, in the samples were determined. The α- and β-amylase activity was expressed as the number mg starch broken down by the α- and β-amylase respectively per 100 g pear pulp in one hour at 25° C. This activity is frequently expressed per pear in the following pages.

The starch content was determined by the method of LOOMIS and SHULL (1937). A few corrections had to be made for the material under examination (MARIS MCARTHUR, 1955).

For the determination of the α- and β-amylase we empolyed a method whereby the colour changes in starch-iodine and starch-erythrodextrin complexes respectively were measured. This method was developed by HOSKAM (1947) for determining amylase activity in various flour types. We have used this method successfully on pear material.

Erythrodextrin, which is broken down by α-amylase and not by β-amylase, was used as substrate in the determination of the α-amylase activity. Erythrodextrin is coloured red by iodine; this colorability disappears during hydrolysis by α-amylase. The activity of the α-amylase can be determined from this change, which can be followed colorimetrically. The activity of β-amylase cannot be measured separately as no substrate is known that is preferentially hydrolysed by β- and not by α-amylase. The combined activity of α- and β-amylase is therefore determined, soluble starch being added as substrate. Here too the colour change is measured colorimetrically. By subtracting the value obtained for the α-amylase alone from that for the α- and β-amylase together, the corresponding figure for the β-amylase can be calculated. As starch and erythrodextrin are not broken down at the same rate by α-amylase, it was necessary to ascertain the ratio between these rates under the test conditions. We found a value of 0.76 for this ratio, which also corresponds with that found by HOSKAM. For both determinations we employed a filter with a maximum transmission of about 572 mμ.

Further details of determinations in pear pulp are described elsewhere (MARIS MCARTHUR, 1955).

Tests of the amylase determinations in pears

HOSKAM's method only yields reliable results providing a number of conditions are satisfied:

Activity determinations must be made at the optimum pH. The pH of the reaction mixture at which pear amylases evidence optimum activity was therefore investigated. Fig. 1 shows that the pH range between 5.7 and 6 is most favourable for the activity of $(\alpha + \beta)$ amylase. The optimum breakdown of erythrodextrin by α-amylase occurs at a pH of about 6. All our amylase determinations in pear pulp were therefore carried out at a pH between 5.7 and 6.

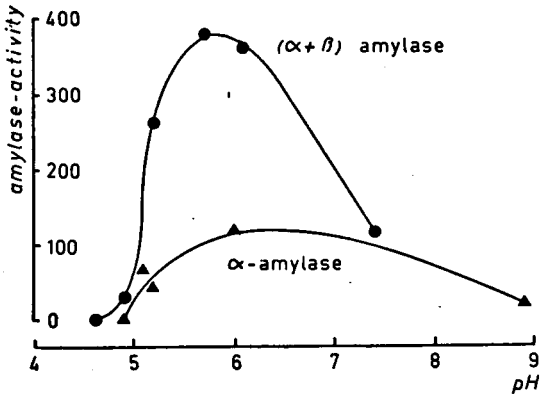

Fig. 1. *The influence of pH on the activity of* $(\alpha + \beta)$ *amylase* (*substrate: starch*) *and* α-*amylase* (*substrate: erythrodextrin*) *in pear pulp.*
Coarsely rasped pulp macerated with $NaHCO_3$; pH of pulp 6.2 — Samples modified to various pH with 0.5 N HCl or 5 % $NaHCO_3$ and made up to 15 ml with water — Reaction mixtures: 10 g pulp, 10 ml water and 20 ml starch solution 1.25 % or erythrodextrin solution 1.25 % — Duration of the reactions: 30-70 minutes — Temp. 25° C.

The determinations must be made in the presence of an excess of starch (or erythrodextrin) in order to ensure that the starch in pears does not influence the results. Our tests showed that the rate of starch breakdown increases as the concentration is raised, but that hydrolysis remains independent of the concentration for several hours in a reaction mixture containing 0.5 % starch. In this case the enzyme is saturated with substrate (see Table 1). All amylase determinations were, of course, executed in such a way as to ensure that this enzyme saturation was invariably achieved. This was certainly the case when the rate of hydrolysis, in a reaction mixture containing 0.6 % starch, did not exceed 0.8 mg per minute.

α-Amylase was saturated with erythrodextrin in a concentration of $\pm$ 0.4 % erythrodextrin (see Table 2).

For these determinations we used starch and erythrodextrin concentrations of 0.6 %, ensuring by dilution of the pulp that the reaction rate was lower than 0.5 mg per minute.

The calculation of the enzymic activity can be greatly simplified

TABLE 1

Influence of the starch concentration on the rate of hydrolysis by (α + β) amylase in pear pulp (high starch concentration)

Doyenné Boussoch pears — buffered starch solution 2.5 %, pH 5.6 — Reaction time: 100 minutes — Temp. 25° C — Constantly stirred

Pear pulp	10 g	10 g	10 g	10 g
Water	30 ml	25 ml	20 ml	15 ml
Starch solution	10 ml	15 ml	20 ml	25 ml
pH reaction mixture	5.82	5.79	5.75	5.71
Concentration starch in reaction mixture	0.50 %	0.75 %	1.0 %	1.25 %
Starch hydrolysed per minute	0.53 mg	0.56 mg	0.52 mg	0.52 mg
Activity (α + β) amylase	320	335	310	310

TABLE 2

Influence of the erythrodextrin concentration on the rate of hydrolysis by α-amylase in pear pulp

Doyenné Boussoch pears — buffered erythrodextrin solution 0.625 %, pH 5.6 — Reaction time: 90-120 minutes — Temp. 25° C — Constantly stirred — Determinations of α-amylase activity.

Pear pulp	10 g	10 g	10 g	10 g
Water	30 ml	20 ml	10 ml	—
Erythrodextrin solution	10 ml	20 ml	30 ml	40 ml
pH reaction mixture	5.87	5.80	5.80	5.80
Concentration erythrodextrin in reaction mixture	0.13 %	0.25 %	0.38 %	0.50 %
Erythrodextrin hydrolysed per minute	0.25 mg	0.35 mg	0.48 mg	0.44 mg
Activity α-amylase	—	—	220	200

by selecting the test conditions so that the rate of hydrolysis is proportionate to the time. It proved possible to realize this by permitting the enzymic process to take place at the optimum pH, adding adequate substrate to saturate the enzyme, maintaining the temperature constantly at, say, 25° C, and stirring the reaction mixture constantly and intensively. If the latter is neglected, the suspension precipitates rapidly, resulting in part of the enzyme present becoming less active.

It appeared that the rate of hydrolysis remains constant under the conditions referred to in the foregoing. This will be seen from the graphs at Fig. 2.

The rate of hydrolysis only decreases when the reaction has progressed to such an extent that there is no longer any excess substrate present. As this rate remains constant, even when the test is of longer duration, this value can be determined with sufficient accuracy.

In order to be able to calculate the enzymic activity in pear pulp, we had to ascertain whether the rate of hydrolysis was proportionate to the quantity of pulp. This is shown at Fig. 3.

RESULTS OF THE AMYLASE DETERMINATIONS IN DIFFERENT VARIETIES OF PEARS

α- and β-amylase determinations were made at regular intervals in several varieties of pears, during the growing season, the period in

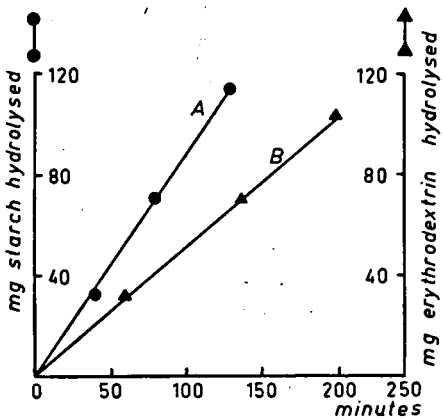

Fig. 2. *Hydrolysis under optimum conditions.*

A. *Starch as substrate.*
Doyenné Boussoch pears — Reaction mixture: 30 g pear pulp, 30 ml water and 60 ml starch solution 0.625 % — pH 5.62 — Temp. 25° C — Constantly stirred.

B. *Erythrodextrin as substrate.*
Doyenné Boussoch pears — Reaction mixture: 15 g pear pulp, 15 ml water and 30 ml erythrodextrin solution 0.625 % — pH 5.7 — Temp. 25° C — Constantly stirred.

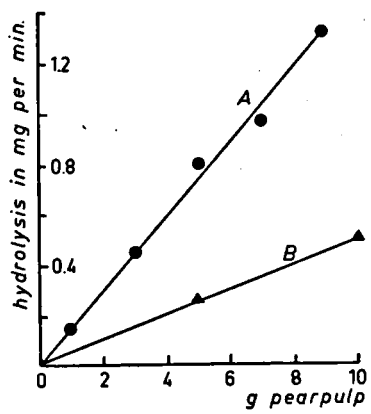

Fig. 3. *Influence of the quantity of pear pulp (enzyme) on the rate of hydrolysis.*

A. *Starch as substrate.*
Doyenné Boussoch pears — Buffered starch solution 1.25 %, pH 5.8 — Reaction time: 1-2 hours — Temp. 25° C — Constantly stirred.

B. *Erythrodextrin as substrate.*
Doyenné Boussoch pears — Buffered erythrodextrin solution 0.625 %, pH 5.8 — Reaction time: 2 hours — Temp. 25° C — Permanently stirred.

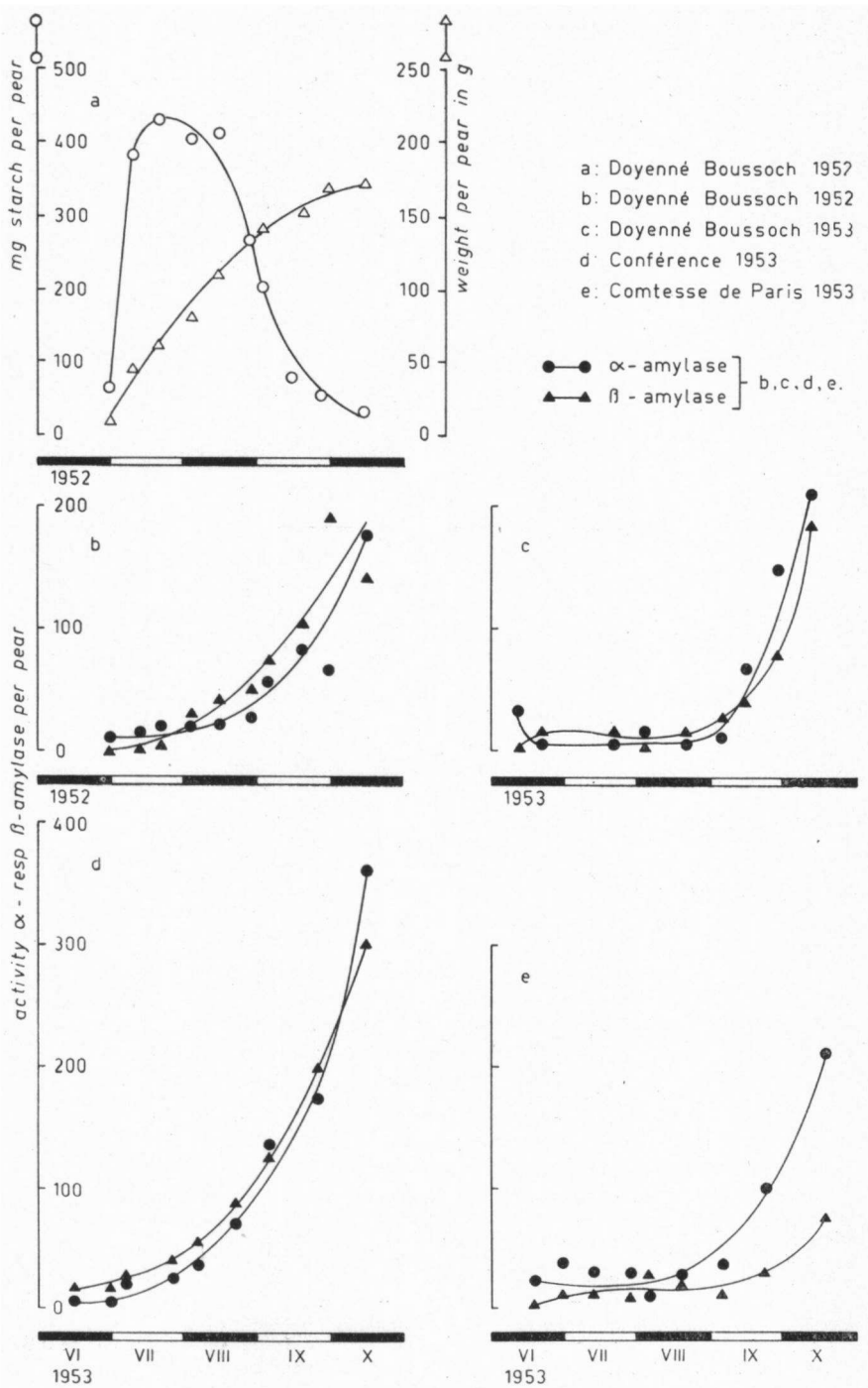

Fig. 4.

a. Average weights and quantities of starch per pear of Doyenné Boussoch in 1952.
b-e. Quantities of α- and β-amylase per pear during development on the tree.

cold-storage and ripening. In addition, samples of cold-storage pears gathered on different dates were also compared with one another.

The growth period

The amounts of amylase present during growth on the tree are summarized at Fig. 4. In each case the amount was calculated separately per pear. In addition, the average weights of the pears and the quantities of starch per pear are shown at Fig. 4a. The starch content reaches its maximum towards the end of July.

The general impression is that the amounts of both α- and β-amylase increase only slightly during the first half of the growth period, but fairly sharply during the second half. In general, the trends are identical for all three varieties, differing only in minor details.

A striking fact is that the major increase in the amount of amylase begins after the largest quantity of starch has already disappeared.

The storage period

Fig. 5a shows the amounts of α-amylase per pear in the three varieties during cold-storage. The earliest gathering dates were selected in this case (for more detailed figures regarding all gathering dates, see MARIS MCARTHUR, 1955).

The graph shows clearly the period up to which ripening is possible.

It will be noted that the increase in the amounts of α-amylase, which actually began on the tree (see Fig. 4) still continues during cold-storage to such an extent, that the quantities of α-amylase achieve values never reached on the tree.

The increase in the amount of α-amylase presents an entirely different picture in each of the three varieties. The increase is only slight in the case of Conférence pears, enormous in Comtesse de Paris, while it lies between the two in the Doyenné Boussoch variety.

Fig. 5b shows the amounts of β-amylase during the storage period. It appears that no important changes in the quantities of β-amylase occur in any of the three varieties.

The ripening

Our investigations also extended to ascertaining whether any changes in amylase activity took place during ripening. To determine this, cold-storage pears of the Doyenné Boussoch variety were stored for some time at room temperature during various stages of the investigation. The results are incorporated in Table 3.

This table shows that changes do actually take place during ripening. There is an obvious decrease in the amount of α-amylase, the differences being much greater than the anomalies which could possibly be attributed to the sampling methods; the amount of β-amylase varies irregularly; it is possible that it undergoes practically no change.

Meanwhile, two other control determinations had to be made before it could be concluded with certainty that there is a marked biosynthesis of α-amylase during the cold-storage of pears. After all,

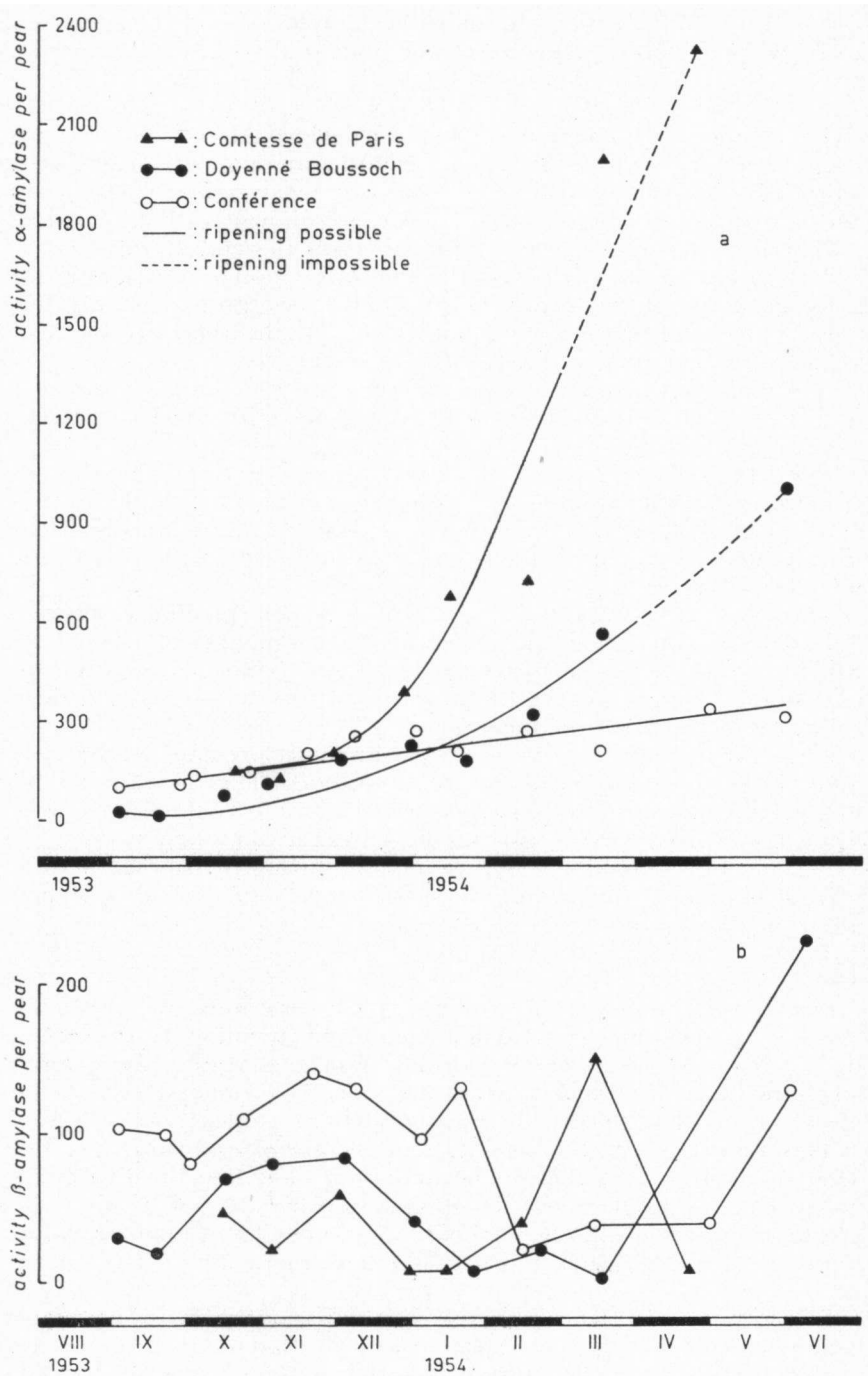

Fig. 5.
a. Quantities of α-amylase per pear during cold-storage.
b Quantities of β-amylase per pear during cold-storage.

TABLE 3

Changes in α- and β-amylase activity during the ripening of Doyenné Boussoch pears

Pears gathered unripe on 10/9/52 — Kept in cold-storage at 0.5° C — Ripening at 23° C

	Date test	Details	Activity α-amylase	Activity β-amylase
A.	8/1/53	Unripe pears from cold-storage ...	90	55
	16/1/53	9 days ripening, ripe for consumption.	55	35
B.	23/1/53	Unripe pears from cold-storage	115	55
	28/1/53	7 days ripening, ripe for consumption.	85	90
	3/2/53	12 days ripening overripe and mealy .	35	70
C.	11/2/53	Unripe pears from cold-storage	245	95
	17/2/53	7 days ripening, ripe for consumption.	180	75
	25/2/53	15 days ripening, over-ripe and mealy .	45	80

it is feasible that the observed changes in activity could be attributed either to the presence of amylase-inhibiting substances during the evolution of the pears, or to the formation of amylase-stimulating compounds during the cold-storage of the fruit. The following experiments were conducted in an endeavour to elucidate this question.

Tests were made at various times to ascertain whether macerated pear pulp exerted any influence on the activity of α- and β-amylase preparations, prepared from malt and pearl barley respectively. This involved determining the amylase activity of the macerated pear pulp and that of the amylase preparation on one hand, and that of both together on the other. The results of a few of these experiments are presented in Table 4.

This table shows that between August and January pear pulp exercised a slight inhibitive action on the activity of the α- and β-amylase preparations. This inhibition, however, amounts at the most to one-third of the total activity. If inhibiting substances are actually present, then their action must be so weak that it can have no practical significance in explaining the observed changes in the activity of the amylases.

It follows from these observations too that no substances occur in the pulp of pears kept in cold-storage, which stimulate the amylase activity. If this was the case, the breakdown of the starch by an amylase preparation plus pear pulp would have been greater than the sum of the breakdown by both components individually. This was not the case however.

Consideration also had to be given to the possibility of combined amylases occurring in pears. It is known that α-amylase occurs in

TABLE 4

Influence of macerated pear pulp on the activity of α- and β-amylase preparations

Doyenné Boussoch pears, finely ground with $NaHCO_3$, pH pulp 5.7 — α- and β-amylase solutions in 0.2 M acetic acid-acetate buffer, pH 5.7. Buffered starch solution of 1.25 %, pH 5.7 — Reaction mixtures consisting of: 10 ml enzyme solution/10 g pear pulp, 10 ml water and 20 ml starch solution — or — 10 ml enzyme solution, 10 g pear pulp and 20 ml starch solution.

Date	Enzyme preparation	Starch breakdown in mg/hour		
		Amylase	pulp	Amylase + pulp
12/ 8/53	β-amylase	18	3	13
13/ 8/53	α-amylase	24	1	19
1/ 9/53	α-amylsae	26	4	20
6/10/53	α-amylase	29	1	22
26/11/53	β-amylase	60	16	70
27/11/53	α-amylase	34	24	46
19/ 1/54	α-amylase	20	0	12
20/ 1/54	β-amylase	52	5	51
26/ 4/54	α-amylase	26	44	71
27/ 4/54	β-amylase	36	21	58

combined form with protein in barley grain; the activity of this enzyme, however, is then weak. This linkage can be broken by the addition of a proteolytic enzyme — papain — resulting in the α-amylase activity increasing considerably, as has been demonstrated by experiments of FORD and GUTHRIE (1908).

β-amylase, too, can be liberated in this way from oat grain.

This increase in the amylase activity of germinating seed such as barley, is probably due to the action of a protease.

If the amylases occurred in combination (probably) with proteins in the pears, it would be possible for the increase in activity, which we found during the cold-storage of pears, to be ascribed not to a change in the amount of amylase, but to the breakdown of this inactive complex. To establish this, an investigation was made to ascertain whether there was any increase in the activity of the amylases consequent upon the interaction of papain. Doyenné Boussoch pears, kept in cold-storage to the end of January or the beginning of February, were selected as test material. The greatest increase in activity took place thereafter, so that it could be assumed that — if this hypothesis was correct — the interaction of papain would have the greatest effect.

A number of experiments were conducted, during which papain — to which cysteine had been added as an activator of the papain — was allowed to interact on macerated pulp at pH 7.5-8, a little toluol also being added. The pH of the reaction mixture was subsequently modified to 5.7 and the amylase activity determined.

It appeared that there was absolutely no question of any increased amylase activity consequent upon the interaction of papain.

The results of these experiments may be summarized by stating that the increase in α- and β-amylase activity during the last stage of

growth of pears and the increased α-amylase activity during cold-storage can only be explained by a powerful biosynthesis of both enzymes.

Discussion of the results

The extremely intensive biosynthesis of α-amylase in cold-storage pears is a remarkable phenomenon, especially when it is remembered that no substrate whatsoever for the enzyme is present in cold-storage pears. This becomes even more interesting when we compare these observations with the ripening potentialities of the pears (see Fig. 5a): Conférence, the variety exhibiting only a slight increase, continued ripening until the bitter end (stocks were exhausted by the end of June). Comtesse de Paris, in which the increase was very marked, continued ripening until the end of February. Doyenné Boussoch, intermediate between the two other varieties in this respect, continues ripening until April.

There is thus a parallel between the amount of α-amylase at a given moment and the good ripening potentialities. This potentiality holds good for pears providing the increase in the amount of α-amylase is not excessive. If it is excessive, on the other hand, the possibility of ripening disappears.

The parallel between the increase in α-amylase and the ripening potentialities is also apparent from a comparison of the first and second gatherings of Comtesse de Paris. The pears of the first gathering revealed a more rapid increase in the amount of α-amylase than those of the second. Corresponding with these facts the pears of the first gathering were able to ripen for a shorter period (up to the end of February) than those of the second (up to the end of April).

If we regard an abnormal increase in the amount of α-amylase as a symptom of a disturbed harmony in the cold-storage fruit, it is quite feasible that these disturbances might well reach such proportions at a given moment that ripening becomes impossible.

An approximate calculation (see Maris McArthur, 1955) shows that the following holds good for the Doyenné Boussoch and Comtesse de Paris varieties: if the α-amylase activity, expressed per 100 g pulp (A_a), reaches a value 6-8 times higher than what it was about the time the fruit was gathered, ripening is no longer possible. The A_a in respect of the Conférence pears never reached this value; these pears can therefore ripen well for a very long time (until June).

We thus have an indication as to whether cold-storage pears can subsequently ripen well or not in the ratio between the value for the activity of the α-amylase (A_a) in these pears at a given moment and that for A_a around about September and October. Each variety possibly has its own critical ratio value.

The fact that there is a correlation between the changes in the A_a and the ripening potentialities does not imply that there must be a direct causative link between the two phenomena. The modified α-amylase activity should be regarded more as an indication of a disturbance in the harmonic equilibrium.

These investigations show that not all processes are necessarily delayed by cooling. The influence of a low temperature may be such that a process can continue at the same rate or even accelerate. This applies to the biosynthesis of α-amylase in pears. Certain materials may consequently be accumulated. In the case under investigation this was due to an enzyme unable to find a substrate. This could well be the case, too with enzymes that actually find a substrate; the result might be that conversions occur that would not take place under natural conditions, and if they did, to a far lesser extent.

The changed composition of the fruit — especially as far as the enzymes are concerned — may lead to such disharmony that normal ripening is no longer possible.

The accumulation of amylase is a phenomenon which probably has no repercussions for the fruit. Quite accidentally, the α-amylase activity curve proved to be an indication for the ripening potentialities at a given moment.

SUMMARY

Some varieties of pears can no longer ripen after spending some time in cold-storage.

An investigation has been made of the changes in the activity of a small number of enzymes in this fruit during growth and cold storage. The changes in the activity of α- and β-amylase in three varieties of pears, gathered at different dates, were examined; these varieties differ considerably from the point of view of their post cold-storage ripening potentialities.

During the pear's development on the tree, the quantities of α- and β-amylase increase to a certain extent. During cold-storage, the quantity of α-amylase continues to increase, reaching a value which is never achieved under natural conditions. The rise in α-amylase activity during cold storage proved to be entirely different for the three varieties of pears.

There is a certain parallel between the rise in α-amylase activity and the possibility of ripening after cold storage, which was also found when a comparison was made of the first and second picking of the variety Comtesse de Paris. If the increase is not too excessive, ripening remains possible. If the rise in α-amylase activity is too great, at a rough estimate six to eight times the value at commercial picking-time, ripening is rendered impossible.

In the ratio between the value for α-amylase activity in pears during cold-storage at a given moment and that about commercial picking time, we found an indication as to whether pears can still ripen or not. It is possible that a specific critical value exists for each variety.

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