ACTA BOTANICA NEERLANDICA, Vol. II, N. 1, 1953

PECTINASE INHIBITORS IN PEARS

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(*Received January 6th, 1953*)

INTRODUCTION

During the ripening of fruits, insoluble pectin is converted into the soluble form, which in its turn is broken down to galacturonic acid.

Although a pectin splitting enzyme with properties slightly different from pectinase was demonstrated in tomato fruit by McCOLLOCH and KERTESZ (8, 9) and evidence of the presence of similar enzymes in other fruits has recently been put forward by a number of authors, nothing as yet is known with certainty about the causes of the pectin conversions in ripening pears and apples.

In fact, as a result of the failure of many attempts to prove the presence of pectin splitting enzymes in these fruits, KERTESZ suggested that the conversions may well be non-enzymic (7), pointing out that in vitro long-chained pectin molecules could be split into smaller units by ascorbic acid and peroxides (6, 4). However, according to the results of the investigation presented below, the situation is shown to be more complex.

At least two pectinase inhibiting substances were found to be present in the sap of pears. One of these proved to be thermostable and was found only in the early stages of development of the fruit, whereas the other is thermolabile and appears when the fruit is about three months old. The amount then increases and reaches a maximum in the ripe fruit.

There is little evidence in the literature on naturally occurring inhibitors of pectinase. CHONA (2) found that malic acid in apples and potassium phosphate and magnesium sulphate in potatoes inhibited the activity of the enzyme. MEHLITZ and MAASS (10) suggested that tannin also had inhibiting properties, which however, was contradicted by WEBER and collab. (15).

This paper deals only with the thermolabile inhibitor found in *Doyenné Boussoch* and two other varieties of pears. Physiological data and other aspects of the pectin conversions in these fruits will be presented separately.

The older notations "pectinase" and "pectase" for the enzymes are

used instead of polygalacturonase and pectin-methyl esterase to indicate that none of the enzymes used in the experiments have been specially purified.

METHODS

One of the most sensitive methods to determine the activity of pectinase is to measure the decrease in viscosity of a pectin solution caused by the enzyme (7); this applies especially to the initial stages of the breakdown.

In our experiments commercial pectinase [1] and different kinds of commercial and self-prepared pectins were used. The viscosity was determined with a Hoeppler falling ball type viscosimeter at 25.00 ± 0.02° C.

The data obtained in the experiments are presented in the form of graphs. The change in the viscosity of the solutions is expressed as the decrease in the falling time of the ball in the viscosimeter tube and is plotted against the reaction time (Fig. 1).

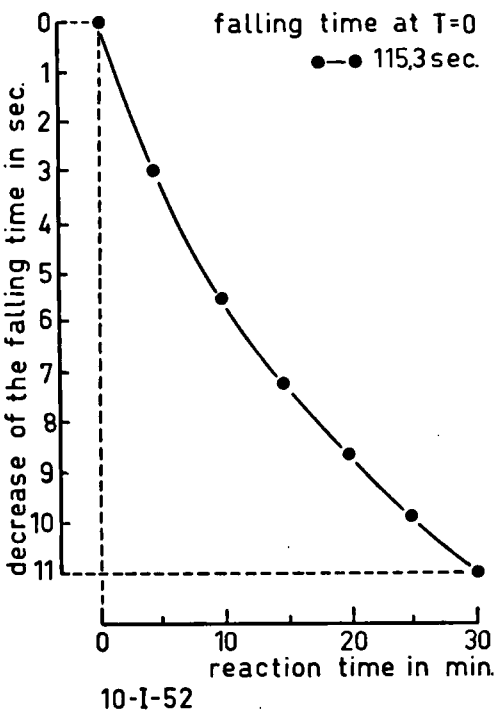

Fig. 1. The effect of pectinase on the viscosity of a pectin solution. 50 ml 0,22 % B.P.-pectin in 0.05 mol. succinic acid-borax buffer, pH 3,6; 1 ml 1 % Pectasin-A. Temp. 25° C.

[1] "Pectasin A" from Polak en Schwartz, N.V., Zaandam. Activity: 30 P.G.U./gr. (20° C); determ. accord. to KERTESZ (7). Containing: 3.4 P.M.U./gr. (20° C); determ. accord. to KERTESZ (7).

In all figures the falling time at the beginning of the experiment (fall. t. at T = 0) is given and hence the actual falling time during the experiment can be read from the graphs.

Experimental

The presence of a thermolabile pectinase inhibitor in the sap of *Doyenné Boussoch* pears can be demonstrated by comparing the rates of the viscosity drop on the addition of fresh and boiled sap to the reaction mixture respectively (Fig. 2).

Since sap from different varieties of pears or from fruit picked at different stages of development is compared, it should be borne in mind that it contains different amounts of pectin, thus changing the

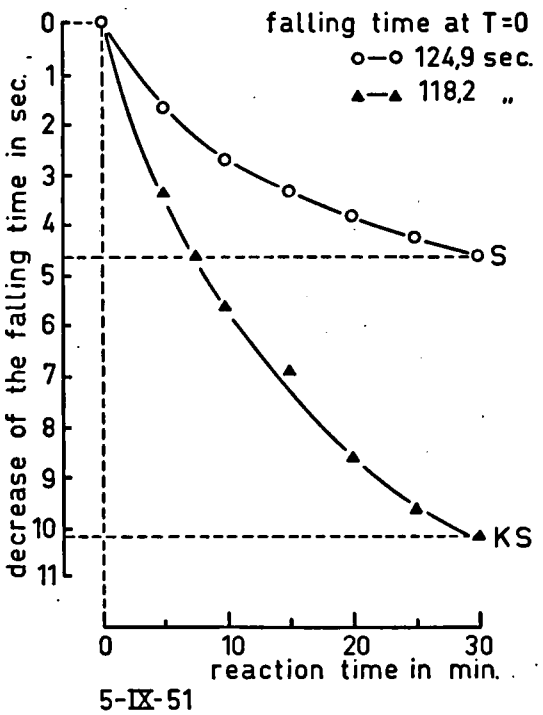

Fig. 2. The effect of the sap of pears on the decrease in viscosity of a pectin solution caused by pectinase.
50 ml 0,19 % B.P-pectin in 0,05 mol. succinic acid-borax buffer, pH 3,6; 5 ml sap (S) or 5 ml boiled sap (KS), 1 ml 1 % Pectasin-A. Temp. 25° C.

initial concentration of the reaction mixture. As a consequence the rate of decrease in viscosity by a certain amount of enzyme will be changed as well. The Figs. 3 and 4 give the extent of this influence in the range of the pectin concentrations used in the following experiments. From these graphs and the falling time at T = 0 given in

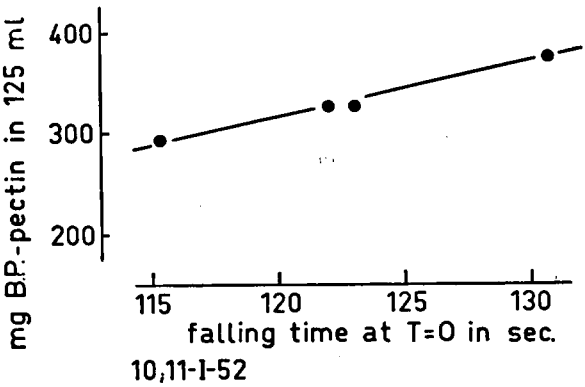

Fig. 3. The relation between the concentration of B.P.-pectin in succinic acid-borax buffer, pH 3,6, and the viscosity.

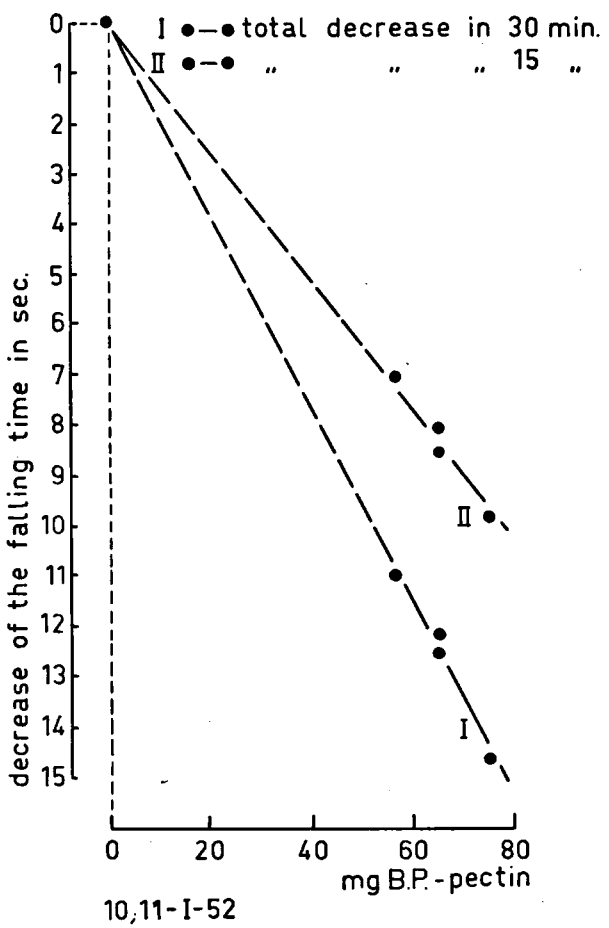

Fig. 4. The relation between the concentration of a pectin solution and the decrease in viscosity caused by pectinase in 15 and 30 min.

50 ml B.P.-pectin in 0,05 mol. succinic acid-borax buffer, pH 3,6, containing the amount of pectin indicated in 25 ml; 1 ml 1 % Pectasin-A Temp. 25° C.

all figures it is clear that the interpretation of the results is not altered by this factor.

However, as a result of the method used, the fact that the decrease in viscosity is lessened by the sap should not be attributed to an inhibitor of the enzyme without considering other possible explanations.

Since the demethoxylating enzyme pectase can increase the viscosity of pectin solutions, the addition of sap containing this enzyme to the reaction mixture might produce effects, which cannot be distinguished from a true inhibition of the pectinase (Fig. 5).

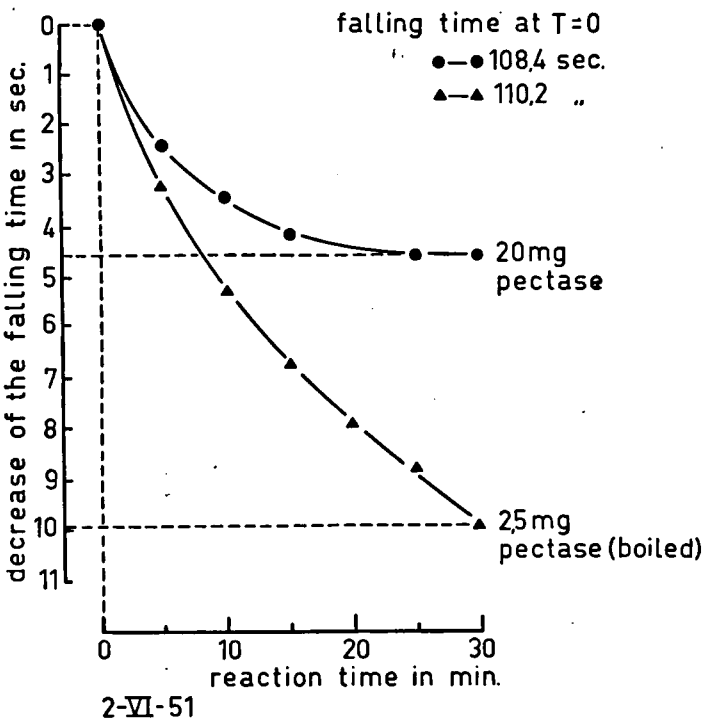

Fig. 5. The effect of pectase on the decrease in viscosity of a pectin solution caused by pectinase.
50 ml 0,19 % B.P.-pectin in 0,05 mol. succinic acid-borax buffer, pH 3,6; 5 ml 0,4 % citrus-pectase; 1 ml 1 % Pectasin-A. Temp. 25° C.

On the other hand, small amounts of pectase present in the pectinase preparation used in the experiments strengthen the activity of the latter enzyme (5, 7), so an inhibition of the pectase might result in a decrease of the pectinase activity.

Other possible explanations for the phenomena observed will be dealt with below.

All the experiments presented below were performed with the inhibitor, RI, isolated from the sap by precipitation with three times the volume of acetone. The precipitate, washed free of water with

acetone and dried by air, showed in aqueous solution the same pectinase inhibiting properties as the sap itself; boiled solutions, having no influence on the pectinase, were used as controls.

In the Figures 6, a–f, experiments are presented which strengthen the likelihood that the substance isolated from the sap is indeed an inhibitor of pectinase. In these experiments pectic acid was used as a substrate for the enzyme instead of pectin. Separate tests showed that the viscosity of the pectic acid solutions was not changed by citrus pectase. By comparing the graphs a and b it is seen that the total decrease in the falling time, caused by the pectinase, is 9.2 and 4.6 sec./30 min. when the inactivated and the active RI solutions are added respectively.

Since the activity of the pectinase is lessened when either pectic acid or pectin is used as a substrate, explanations in which any influence is attributed to pectase or pectase inhibitors are untenable.

Changes in the pH of pectin solutions may change their viscosity

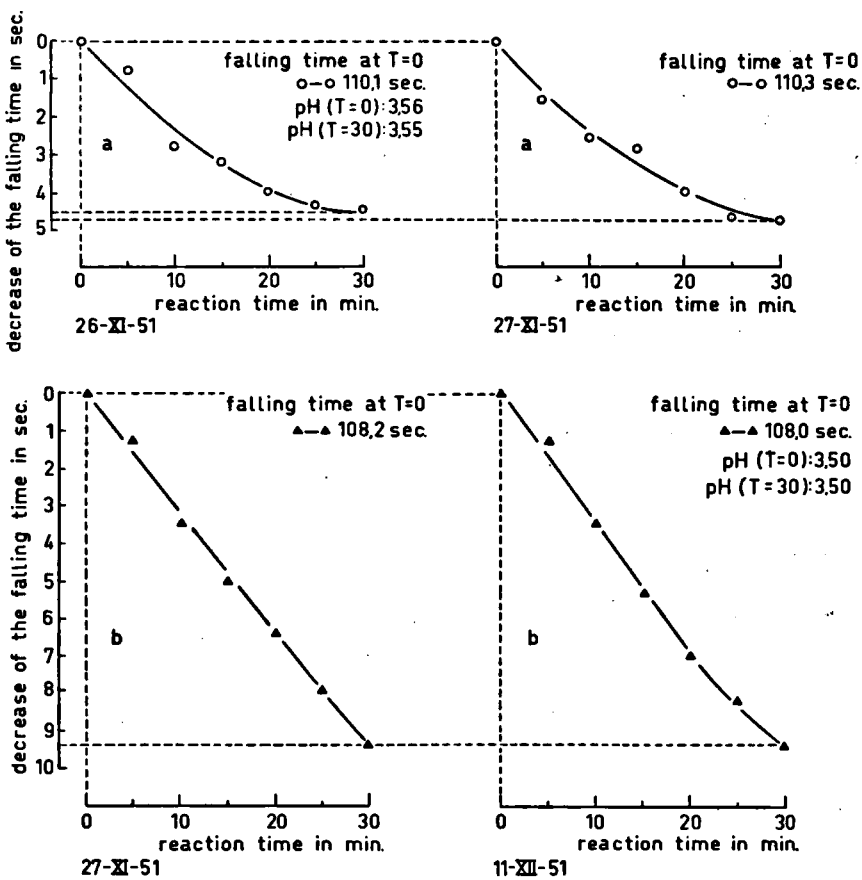

Fig. 6, a and b; for explanation see p. 113

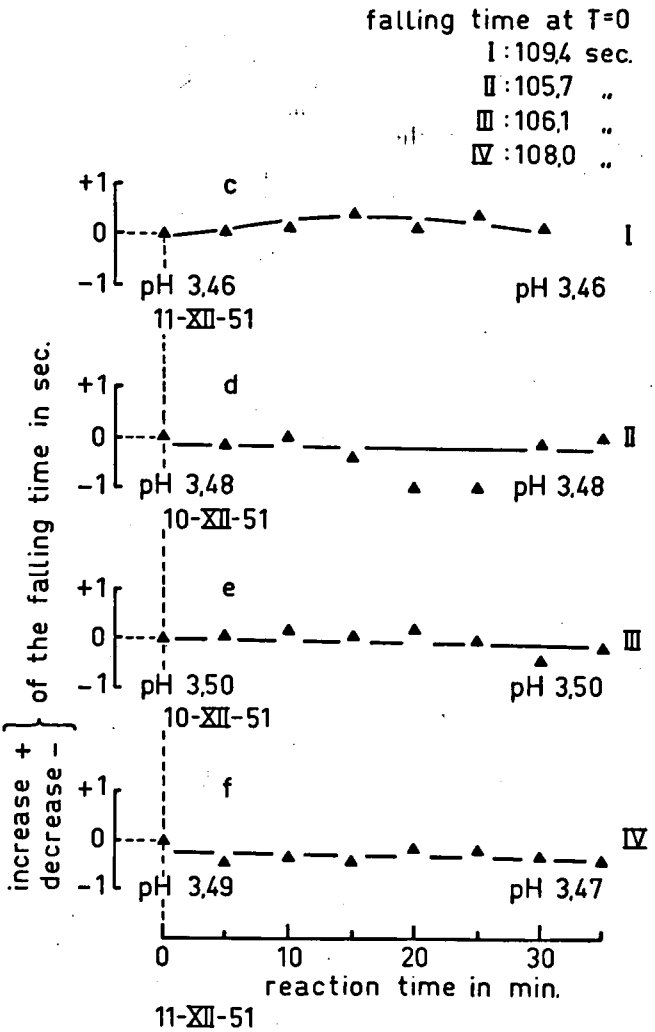

Fig. 6. The effect of different reagents on the viscosity of a pectic acid solution uninfluenced by pectase.

50 ml 0,14 % M.P.Z.-pectic acid in 0,05 mol. succinic acid-borax buffer, pH 3,6. Temp. 25° C.

a. 5 ml inhibitor solution containing 25 mg RI; 1 ml 1 % Pectasin-A.
b. 5 ml inactivated inhibitor solution containing 25 mg RI; 1 ml 1 % Pectasin-A
c. 5 ml inhibitor solution containing 25 mg RI; 1 ml water.
d. 5 ml water; 1 ml 1 % inactivated Pectasin-A.
e. 5 ml inactivated inhibitor solution containing 25 mg RI; 1 ml water.
f. 6 ml water.

(11, 13) but the pH values at the beginning and at the end of the experiments given in the figures, indicate that no explanation for the phenomenon can be derived in this way. The same holds true for viscosity changes which might have arisen from mixing the solutions or from accidental salt additions with the RI solutions as is shown by the graphs c, d, e and f.

One more possibility has to be considered. It could be argued that the commercial pectin preparations used in the experiments do contain substances which, apart from the pectins, show in solution a rather high viscosity of their own. The pectinase on the other hand might contain enzymes which break down these unknown compounds

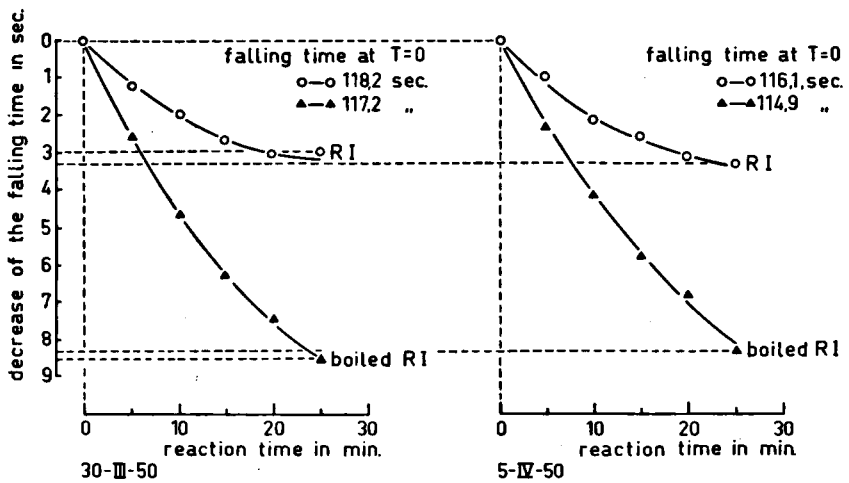

Fig. 7. The effect of the degree of purity of the pectin on the inhibition of pectinase by RI.
50 ml 0,20 % B.P.-pectin (pure pectin 60,9 %) and 0,17 % Z.B.P.-pectin (pure pectin 86,1 %) resp. in 0,05 mol. succinic acid-borax buffer, pH 3,6; 5 ml RI solution containing 1,55 mg RI; 1 ml 1 % Pectasin-A. Temp. 25° C.

and so produce a decrease in viscosity parallel to the one caused by the pectinase. The sap or the RI solution might inhibit these last mentioned enzymes, thus lessening the decrease in viscosity.

An experiment in which the same pectin is used in two different degrees of purity shows that this supposition is not correct since the inhibition of the pectinase is the same whether the purity of the pectin amounts to 61 or 86 % (Fig. 7).

Thus it has been proved beyond doubt that some substance present in the sap of pears is an inhibitor for the enzyme pectinase.

Some properties of the pectinase inhibitor

When increasing amounts of the inhibitor are added to replicate mixtures of pectin and pectinase, the inhibition of the enzyme is found to increase almost linear with the lower concentrations of the inhibitor.

Above a certain amount, however, no further increase in inhibition

is met with (Fig. 8, a and b). If these experiments are repeated with pectic acid as a substrate the same results are obtained. Whereas the maximum inhibition with pectin amounts to 82 %, for pectic acid the inhibition is only 50 %. So the substrate seems to be an important factor in the inhibition.

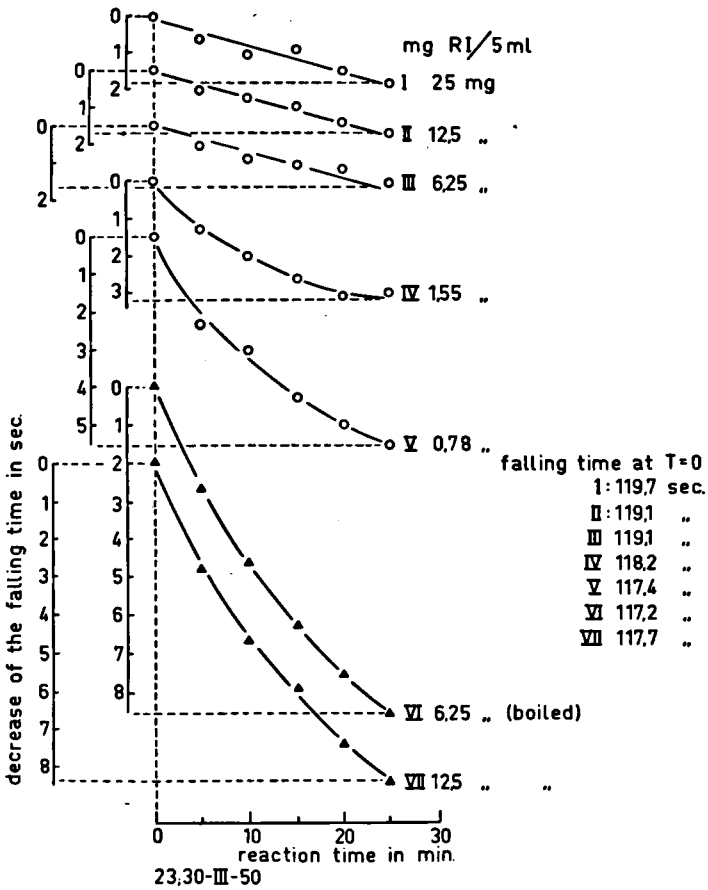

Fig. 8a. The effect of different amounts of inhibitor on the decrease in viscosity of a pectin solution caused by pectinase.
50 ml 0,20 % B.P.-pectin in 0,05 mol. succinic acid-borax buffer, pH 3,6; 5 ml inhibitor solution containing different amounts of RI; 1 ml 1 % Pectasin-A. Temp. 25° C.

This is supported by the results of experiments presented in the Figs. 9, 10 and 11 where pectins from different sources are used. With the pectin prepared from pears, as with the apple pectin used in the experiments described above, a marked inhibition of the enzyme was found. The experiments with pectins from oranges and lemons show a very weak or no inhibition. Data about the properties of the pectins,

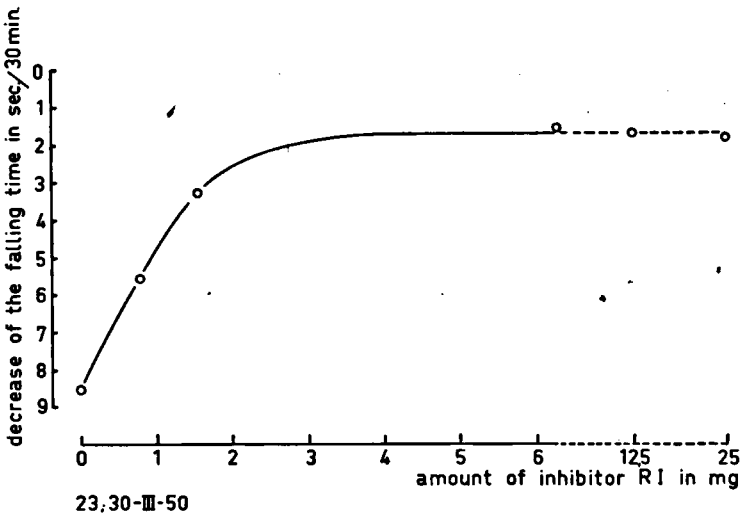

Fig. 8b. The relation between the decrease in viscosity of a pectin solution caused by pectinase and the amount of inhibitor.
Consolidation of the results given in Fig. 8a.

TABLE I

Notation	Type of pectic material	Ash in %	Free carboxyl	Carbox. esterified	Equivalent weight	Degree of esterification in %	pure pection in %
B.P.	Apple pectin commercial	4,3	0,097	0,236	628	70,4	60,9
Z.B.P.	B.P. purified.	0,4	0,147	0,317	586	68,3	86,1
M.P.	Apple pectin commercial	6,6	0,162	0,237	454	59,8	73,5
Z.M.P.	M.P. purified	0,5	0,212	0,261	410	55,5	86,9
M.P.Z.	Pectic acid prep. from M.P.	2,2	0,472	0,005	178	1,2	84,0
C.P.	Citrus pectin commercial	4,3	0,097	0,134	450	58,5	43,5
S.P.	Orange pectin prep. from orange albedo	1,1	0,090	0,316	847	77,9	75,8
P.P.	Pear pectin prep. from pears	0,5	0,072	0,247	830	77,0	59,6

determined according to DEUEL (3) are presented in Table 1; they offer no explanation for the differences in inhibition encountered.

The phenomenon of a maximum inhibition, by which a certain amount of breakdown of the substrate is uninfluenced by further

additions of RI, cannot be explained by the experiments given. It is possible that the commercial pectinase used may be a mixture of several enzymes, all of which lower the viscosity of pectin solutions but only some of which are affected by the inhibitor. Apart from McCOLLOCH and KERTESZ, other investigators have pointed out the possible existence of more then one pectinase (1, 12, 14, 17). As a matter of fact a preliminary experiment with the pectinase ("depolymerase") prepared from tomatoes according to McCOLLOCH and

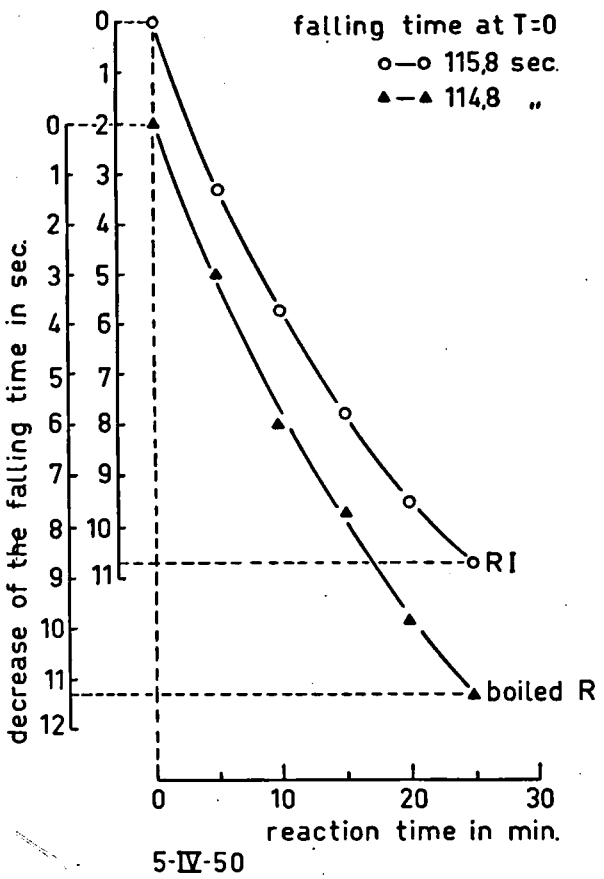

Fig. 9. The effect of the inhibitor on the decrease in viscosity of a citrus-pectin solution caused by pectinase.
50 ml 0,34 % C.P.-pectin in 0,05 mol. succinic acid-borax buffer, pH 3,6; 5 ml inhibitor solution containing 1,55 mg RI; 1 ml 1 % Pectasin-A. Temp. 25° C.

KERTESZ showed no inhibition of the enzyme by the RI when pectic acid was used as a substrate (Fig. 12).

By comparing the influence of cystein-activated papainase and the inhibitor on the activity of pectinase, it can be demonstrated that the

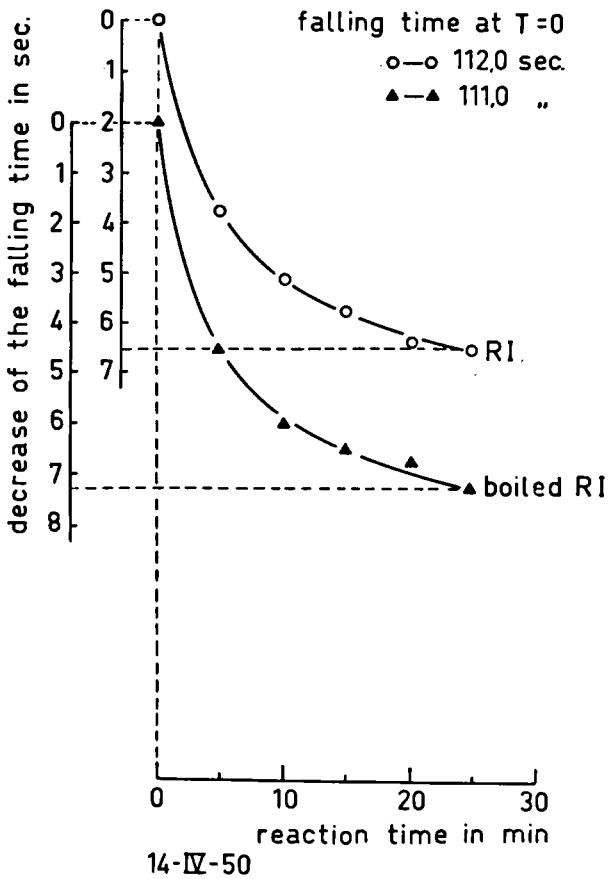

Fig. 10. The effect of the inhibitor on the decrease in viscosity of an orange-pectin solution caused by pectinase.
50 ml 0,08 % S.P.-pectin in 0,05 mol. succinic acid-borax buffer, pH 3,6; 5 ml inhibitor solution containing 1,55 mg RI; 1 ml 1 % Pectasin-A. Temp. 25° C.

inhibition is not caused by an enzymic breakdown of the protein body of the pectinase. The experiments are presented in detail elsewhere (16).

It was also shown that when a solution of malic acid was added to a mixture of pectin and pectinase no change in the activity of the enzyme was found when concentrations of the acid were applied comparable with those of the inhibitor (CHONA (2)).

The sap of two other pear varieties (*Comtesse de Paris*, *Fondante de Charneu*) which were tested in this respect were also found to contain the inhibitor.

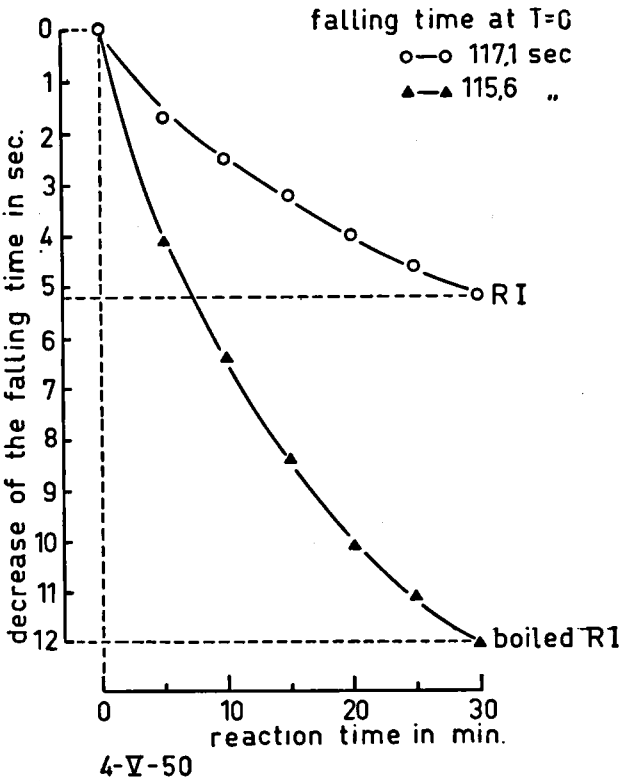

Fig. 11. The effect of the inhibitor on the decrease in viscosity of a pear-pectin solution caused by pectinase.
50 ml 0,06 % P.P.-pectin in 0,05 mol. succinic acid-borax buffer, pH 3,6; 5 m inhibitor solution containing 1,55 mg RI; 1 ml 1 % Pectasin-A. Temp. 25° Cl

Summary

In the sap of several varieties of pears a thermolabile inhibitor for the enzyme pectinase is found. The inhibitor can be isolated by precipitation with acetone. The inhibition of the enzyme increases with increasing amounts of the inhibitor up to a maximum, above which no further increase in inhibition is found. The rate of the residual breakdown of the pectin is influenced by the properties of the substrate.

Evidence from the experiments points to the existence of different kinds of pectinase.

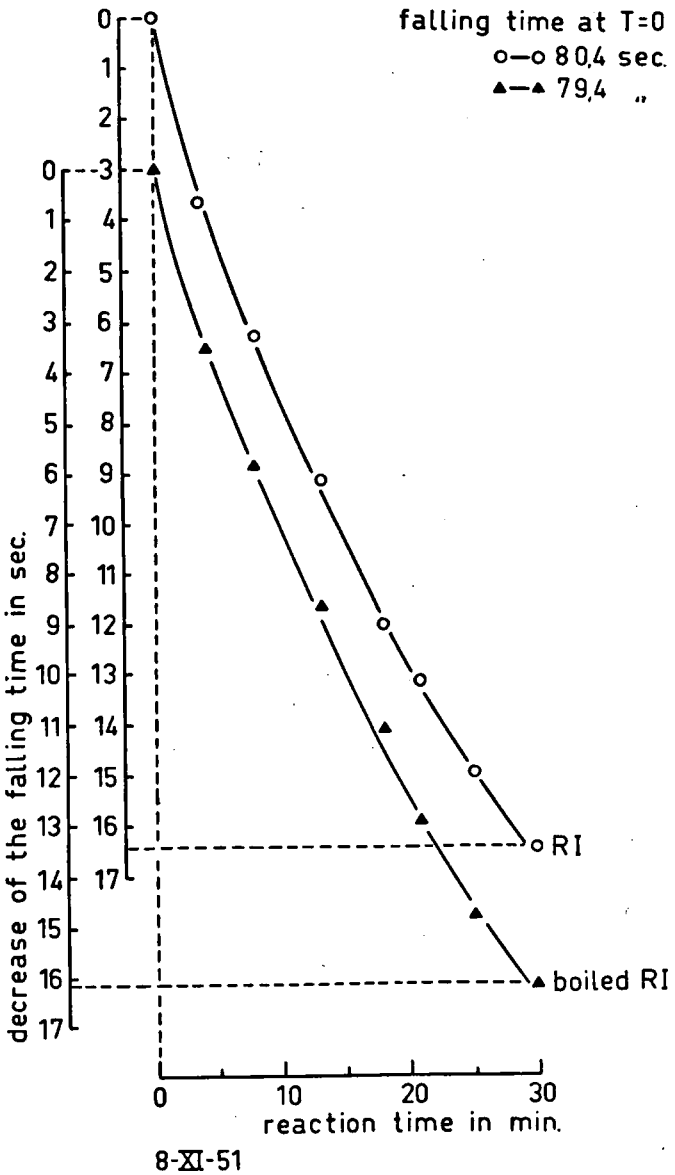

Fig. 12. The effect of the inhibitor on the decrease in viscosity of a pectic-acid solution caused by tomato-pectinase.
50 ml 2 % M.P.Z.-pectic-acid in water, pH 4,0; 5 ml inhibitor solution containing 50 mg RI; 5 ml "depolymerase" extract. Temp. 25° C.

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