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DEVELOPMENT OF PROTEOLYTIC ENZYMES IN GROWING PAPAYA FRUIT

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Abstract—Latex samples, collected from the same papaya fruit at fixed time intervals until the fruit ripened, were freeze-dried. The yield of latex, total nitrogen and protein composition, as well as proteolytic activities, were determined. The latex samples, tapped from fruit during its development, were chromatographed on hydroxylapatite: the resulting chromatograms show the change in proteinase content with fruit development. The overall proteolytic activity in the latex of young fruit is noticeably less than in more mature fruit.

INTRODUCTION

PAPAYA latex preparations have considerable industrial uses in such diverse fields as in the dehairing of hides,[1] in the ageing of raw rubber prior to vulcanization,[2] as meat tenderizers,[3] and in the prevention of turbidity when bottled beer is cooled.[4] Much academic interest is also manifested by numerous publications, reviewed by Hwang and Ivy[5] pertaining to the properties of papaya enzymes.

In this study, prompted by the availability of papaya (papaw) trees which grow readily here, latex was harvested from the same fruit, aged 5 days in the case of the first sample and thereafter at more or less regular intervals, the final sample having been collected when the fruit was ripening some 6 months later. No latex was obtained from fully ripe fruit. Analyses of the latex samples reveal changes in the nitrogen and proteinase contents of the sap, and the data giving yields of latex indicates when the maximum amount may be harvested.

RESULTS

Table 1 shows the yields of dried latex obtained in the nine latex samples that were collected from the same fruit; all were white powders, quite odourless except for sample No. 9, which had the pronounced fragrant aroma typical of ripe papaw fruit.

Analyses of the above samples by Kjeldahl procedure for total nitrogen and trichloroacetic acid-precipitable protein, and also the corresponding proteolytic activities, as determined by casein hydrolysis, of the unpurified samples, are recorded in Table 2.

Aqueous extracts of alternate latex samples (Nos. 1, 3, 5, 7 and 9 in Table 1) were chromatographed simultaneously on similar hydroxylapatite columns. The resulting elution patterns (absorbance at 280 nm for each fraction) are given in Fig. 1. The corresponding proteolytic activities, shown by black areas, are superimposed on the absorbtivity diagrams in each chromatogram.

[1] J. BALLAND, French Patent No. 548,606 (1921).
[2] G. E. VAN GILSL, *Rev. Gen. Caoutchouc* **29**, 117 (1952).
[3] G. Y. GOTTSCHALL and M. W. KIES, *Food Res.* **7**, 373 (1942).
[4] L. WALLERSTEIN, U.S. Patent No. 2,077,449 (1937).
[5] K. HWANG and A. C. IVY, *Ann. N.Y. Acad. Sci.* **54**, 161 (1951).

Peak A is non-protein material in all cases, and these fractions show no enzymic activity. When crystalline papain, prepared by the method of Kimmel and Smith,[6] is chromatographed hydroxylapatite, it is eluted by 0·09 M phosphate buffer, pH 7·0, so that peak B in the chromatograms of unpurified latex in Fig. 1 was identified as papain, and confirmed by physical criteria. (Attention is drawn to peak B in the chromatogram of sample No. 1; the fractions of this peak are inactive, possibly due to an inhibitor present at this stage of development of

TABLE 1. YIELDS OF FREEZE-DRIED LATEX FROM FRUIT AT DIFFERENT AGES

Sample No.	Age of fruit (days)	Yield (vacuum-dried latex) (g)
1	5	1·6
2	33	1·7
3	63	4·2
4	83	5·1
5	104	5·5
6	125	6·0
7	147	3·2
8	165	1·3
9	172	0·85

TABLE 2. KJELDAHL NITROGEN AND WATER-SOLUBLE PROTEINS IN LATEX SAMPLES

Sample No.	% Total nitrogen	% Protein	% Hydrolysis of casein	Specific activity
1	8·7	33·8	12·5	0·06
2	9·7	34·0	55·5	0·27
3	10·2	37·5	58·6	0·25
4	10·3	37·8	64·2	0·27
5	10·7	38·5	67·3	0·28
6	10·2	38·1	68·5	0·29
7	10·0	38·5	78·5	0·33
8	9·9	36·0	72·9	0·33
9	9·3	35·5	71·0	0·33

Proteolytic activities were determined by casein hydrolysis using glutathione as activator. Specific activities are expressed as the change in absorbance at 280 nm per min per mg protein nitrogen.

the fruit.) When chymopapain, prepared according to Ebata and Yasunobu,[7] was chromatographed on hydroxylapatite, it did not emerge until the phosphate buffer concentration was increased to 0·13 M, which pointed to peak C in Fig. 1 consisting of chymopapain. However, lysozyme activity was detected in the leading fractions of peak C, successive fractions having increasingly greater chymopapain activity. The enzyme which emerges as peak D is strongly

[6] J. R. KIMMEL and E. L. SMITH, *J. Biol. Chem.* **207**, 515 (1954).
[7] M. EBATA and K. T. YASUNOBU, *J. Biol. Chem.* **237**, 1086 (1962).

adsorbed by hydroxylapatite, and cannot be eluted unless buffer concentrations above 0·25 M are applied to the column.[8] This enzyme is less easily salted out of an aqueous solution than papain and chymopapain, and differs from these two proteinases in physical and enzyme properties. The enzyme in peak D was provisionally called proteinase D from its position in the hydroxylapatite chromatogram; it may possibly be identical with a chymopapain studied by Kunimitsu.[9]

DISCUSSION

The object of this study was mainly to determine whether any changes occurred in the proteinase composition of latex from developing papaya fruit. A secondary consideration was the yields of latex that are obtained. The absorbtivity chromatograms in Fig. 1 have much in common, while the superimposed casein activity patterns, the result of fraction by fraction assays, reveal differences in the proteinase content depending on the age of the fruit.

It is curious that only peak B fractions in the sample from fruit aged 5 days are not activated by cysteine or glutathione, the usual papain activators. If this was due to an inhibitor, it would be expected to be eliminated by elution in the non-protein peak A. Papain activity is present in all other samples of latex.

Chymopapain and proteinase D, judging from casein hydrolysis, seem to increase as the fruit matures. Analyses of crude latex samples show a maximum protein content at between 104 and 147 days' maturation of the fruit, while the specific activity towards casein by glutathione-activated enzyme extracts is maximum in fruit aged from 147 to 172 days.

The presence of lysozyme activity in the latex may be associated with defence by the plant since this enzyme is able to digest cell walls of bacteria and viruses. Papain and chymopapain have been shown to catalyse the synthesis *in vitro* of substances like small peptides[10] and anilides;[7] it would be interesting to ascertain whether any synthesis by these highly active proteinases occurs *in vivo*.

EXPERIMENTAL

Harvesting of the papaya latex was carried out by lightly pricking the skin of the fruit with a pointed glass rod, the sap being retained in a glass flask cooled in ice; the operation was effected in the early morning to avoid autolysis of the enzymes. Samples were immediately freeze-dried and kept at 0° until required.

Nitrogen Determinations

Samples of 100-mg dried latex were digested with conc. H_2SO_4 in the presence of a selenium catalyst. The ammonia was distilled into 2 % boric acid solution and titrated with standard N/10 HCl using Tashiro (screened methyl red) indicator.

Protein Determinations

Aqueous extracts of latex were made by mixing samples with 0·05 M sodium phosphate buffer, pH 7·0, for 2 hr at 20°; a second extraction was made using the same buffer by mixing for 1 hr. Solutions were filtered to remove the insoluble residue. Protein in the filtrate was precipitated with trichloroacetic acid, the precipitate, after centrifuging, was retained and nitrogen was determined as above. A nitrogen to protein conversion factor of 6·25 was used in the calculations.

Enzyme Determinations

(i) *Proteolytic.* The spectrophotometric method of Kunitz[11] was used, the conditions being as follows: to 0·2 ml of 0·5 % latex extract or chromatographic fraction, 0·1 ml of 0·05 M reduced glutathione was added

[8] G. S. SKELTON, *J. Chromatog.* **35**, 283 (1968).
[9] D. K. KUNIMITSU, Dissertation, Univ. Microfilms, Ann Arbor, Michigan (1964).
[10] M. BERGMANN and H. FRAENKEL-CONRAT, *J. Biol. Chem.* **119**, 707 (1937).
[11] M. KUNITZ, *J. Gen. Physiol.* **30**, 291 (1947).

as activator, and the mixture was incubated at 40° for 5 min; 3 ml of 1% casein solution in 0·1 M sodium citrate buffer, pH 6·0, were added; the time of reaction was 10 min at 40°. Hydrolysis was stopped by the addition of 2 ml 0·5 M trichloroacetic acid. After standing for 1 hr, the sample was filtered, and the absorbance of the filtrate was determined at 280 nm, hydrolysis being a function of the increase in absorbance. Substrate controls were run for each assay. Specific activity of the proteinases was defined as the increase in absorbance, during casein hydrolysis, at 280 nm per minute per mg protein nitrogen.

(ii) *Lysozyme*. This was determined qualitatively by the method of Dickman and Proctor.[12]

Column Chromatography on Hydroxylapatite

The procedure described by Skelton[8] was followed, except that no activator was used in the preparation of the aqueous extract of crude latex. It should be noted that a working temperature of 16° was used; changes in temperature influence the separations and modify the general aspect of the chromatograms.

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[12] S. R. DICKMAN and C. M. PROCTOR, *Arch. Biochem. Biophys.* **40**, 364 (1952).