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Proteolytic Activity in the Genus Ficus[1]

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Abstract. **The latices of only 13 of a total of 46 species of *Ficus* examined contained appreciable proteolytic activity. Therefore, high proteolytic activity in the latex is not a distinguishing feature of the genus. The latex of *F. stenocarpa* had the highest specific activity followed closely by the latices of *F. carica* and *F. glabrata*. Latices of 6 species of *Ficus* were examined by chromatography on CM-cellulose and compared with the results obtained for 9 varieties of *F. carica*. All of the latices were found to contain multiple proteolytic enzymes. Chromatographically, the multiple enzyme components of the several varieties of *F. carica* were more similar than those of the several species examined. The latices of 16 varieties of *F. carica* were all different as determined by free boundary electrophoresis although the specific proteolytic activity of the latices was reasonably constant.**

The genus *Ficus* belongs to the family *Moraceae* and to the order *Urticales* (4). *Moraceae* is composed of trees and shrubs which characteristically have a milky juice. The milky juice of plants of at least 5 of the genera contain high proteolytic activity. These are *Maclura* [osage orange, *M. pomifera* Raf. (12)], *Morus* [mulberry, *M. nigra* L. (10)], *Brosimum* [bread nut, *B. alicastrum* (10)], *Broussonetia* [paper mulberry, *B. papyrifera* (10)] and *Ficus* [fig, several species (10)].

Ficus includes more than 1300 named species (3) which are widely scattered over the tropical and subtropical regions of the world. Such diverse forms as the common edible fig (*F. carica*), the sycamore fig (*F. sycomorus*), the banyan tree (*F. indica* and *F. benghalensis*), various strangling figs (several species which include *F. bonplandiana* and *F. padifolia*), the rubber tree (*F. elastica*) and the creeping fig (*F. repens*) belong to this genus. Perhaps the only unifying characteristics of this genus, which includes vines, shrubs, and trees and has been reported to differ in chromosome number (6), are the milky latex and the bearing of fruit at some period in the life of the plant. While the fruits differ markedly in size, shape, color, location on plant, and frequency of appearance among the species they are all syconia.

Perhaps another common feature of this genus is high proteolytic activity of the latex. Previously it has been shown that both *F. glabrata* and *F. carica* have very high proteolytic activity (5, 11). The present study includes a comparison of the proteolytic activity of the latices of 46 species of *Ficus*, a comparison of the proteolytic activity of 16 varieties of *F. carica*, and a comparison of the electrophoretic and chromatographic properties of the protein components of several of these species and varieties. A high proteolytic activity was found not to be a general characteristic of the genus *Ficus*.

Materials and Methods

Latices. *F. glabrata* latex from Peru was a gift of Enzyme Development Corporation (lot No. AP/1). *F. glabrata* latex powder, also from Peru, was a gift of Merck and Company, (lot No. 50567). All other samples were personally collected by the authors. The latices of varieties of *F. carica* were collected in August from the variety plot at Fresno, California. (The plot is maintained by the California Citrus Research Center, Riverside, in cooperation with the United States Department of Agriculture, Fresno.) Another sample of latex from *F. carica* variety Kadota was obtained from the variety plot at Davis, California. The remainder of the samples were collected in July from the botanical gardens at the University of California, Los Angeles, the arboretum at the University of California, Riverside, and from the botanical gardens of the Department of Arboreta and Botanic Gardens, Arcadia, California. All trees were labeled and the reliability of identification was assured by professional workers at those gardens and by Professor Ira Condit, Emeritus, of University of California, Riverside.

Collection was made from the severed ends of fruit (or leaves in a few cases). The droplets of latex were collected directly into polyethylene centrifuge tubes, frozen immediately in Dry-Ice and maintained in the frozen state at $-20°$ until the analyses were performed. In the case of samples from varieties of *F. carica* and *F. glabrata* (Enzyme Development Corporation), the gum was removed by centrifugation at 100,000 $\times$ g for 20 minutes at

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Table I. *Proteolytic Activity of Forty-six Species of* Ficus

Ficus species[1]	Native locale	Activity	Protein[2]	Specific activity
		units/ml	*mg/ml*	*uints/mg protein*
1. Stenocarpa (A)	Australia	373	160	2.33
2. Carica (D)	Europe, Asia, Africa	320	156	2.05
3. Glabrata[3] (A)	Brazil	353	183	1.93
Glabrata[3] (R)	Brazil	259	126	2.06
Glabrata[3] (Enz. Develop. Corp.)	Brazil	201	119	1.69
Glabrata[3] (Merck and Co.)	Brazil	...	. .	1.38
4. Stephenocarpa (A)	Australia	636	420	1.52
5. Parcelli (L)	Pacific Isles	122	104	1.17
6. Benjamin (R)	Asia	82.2	68.6	1.20
7. Tueckheimeri (R)	...	60.5	72.6	0.834
8. Pumila (L)	Asia	240	396	0.606
9. Petiolaris (A)	Mexico	33.6	58.6	0.573
10. Jacquinaefolia (L)	Cuba	72.6	169.2	0.429
11. Glomerata (L)	Asia	128	386	0.332
12. Mysorensis (L)	Asia	51.3	159.6	0.322
13. Retusa (L)	Asia	103	336	0.307
14. Archeri (A)	Columbia	15.0	98.6	0.152
15. Racemosa (L)	Asia	39.3	312	0.126
16. Mallotocarpa (A)	Africa	23.5	194	0.121
17. Watkinsiana (L)	Australia	10.4	88	0.118
18. Elastica (L)	Asia	1.2	10.6	0.11
19. Murrayana[4] (A)	Brazil	16.5	147	0.112
20. Religiosa (A)	Asia	16.7	150	0.111
21. Capensis (L)	Australia	28.2	254	0.111
22. Wightiana (A)	Asia	15.2	141	0.108
23. Henneana (L)	Australia	14.7	145	0.101
24. Sycomorus (A)	Africa	52.4	534	0.0982
25. Thonningii[5] (A)	Africa	16.1	169	0.0953
26. Graphtholocarpa (L)	...	13.4	142	0.0945
27. Hispida (R)	Asia, Australia	5.62	61.6	0.0912
28. Infectoria (L)	Asia	4.6	50.6	0.091
29. Rumphii (R)	Asia	7.8	87.2	0.090
30. Nekbudu (R)	Africa	2.8	33.2	0.084
31. Rubiginosa (L)	Australia	4.1	49.2	0.084
32. Obliqua (L)	Pacific Isles	10.5	128	0.082
33. Padifolia (R)	Mexico	7.3	94.6	0.077
34. Aurea (A)	Florida	4.4	68.0	0.065
35. Nota (A)	...	9.1	167	0.054
36. Monckii (A)	Paraguay, Argentina	3.8	78.6	0.048
Monckii (R)	Paraguay, Argentina	4.5	113	0.040
37. Burkei (L)	Africa, Australia	1.2	29.2	0.041
38. Goldmanii (A)	Mexico	15.8	392	0.040
39. Bellengeri (L)	Australia	3.4	103	0.033
40. Benghalensis (L)	Asia	3.7	112	0.033
41. Bonplandiana (L)	Mexico	2.8	109	0.026
42. Macrophylla (L)	Australia	0.30	17.6	0.017
43. Lyrata (L)	Africa	0.60	40.6	0.015
44. Roxburghii (A)	Asia	0.10	6.8	0.015
45. Altissima (L)	Asia	0.40	36.0	0.011
46. Diversifolia (A)	Malaya	0.10	9.64	0.010

[1] The letter in parenthesis indicates the location of the specimen where A = Arcadia, D = Davis, L = Los Angeles and R = Riverside. See text for more details on location.

[2] The protein content was determined by the Lowry method (8).

[3] Anthelminthica preferred by Index Kewensis.

[4] Princeps preferred by Index Kewensis.

[5] Microcarpa preferred by Index Kewensis.

0°. Because of the limited amount of sample, the gum was not removed from the other latices.

Reagents. Carboxymethyl (CM−)-cellulose was prepared from Whatman cellulose powder (sieved to pass 100 mesh screen) by the method of Peterson and Sober (9). It contained 0.49 meq carboxyl groups per g resin. Casein, Hammarsten quality, was from Nutritional Biochemical Corporation. Cysteine hydrochloride was from Mann Research Laboratories, Inc., and Versene was from Eastman Kodak Company. All other materials were of reagent grade and deionized water was used throughout the work.

Protein Analysis. Protein content of the samples was determined either by the biuret method (2), the Lowry method (8) or by absorbance at 280 mμ. Bovine serum albumin (Armour Pharmaceutical Company, crystalline) was used as a standard protein for the first 2 methods.

Enzymatic Activity. Proteolytic activity on casein was determined by a modification of the method of Kunitz (7) as described by Sgarbieri *et al.* (11). A unit of activity is defined as that amount which caused a change of 0.001 in absorbance in one minute at 35° and pH 7.0.

Electrophoresis. Preparation of the samples and electrophoresis in a Perkin-Elmer Tiselius apparatus were as described previously (11). All electrophoretic analyses were done at least in duplicate and the separation patterns were quite reproducible.

Chromatography. The equilibration of the CM-cellulose, preparation of the column and adsorption of the protein on CM-cellulose at pH 4.90 before addition to top of the column were as described previously (11). Chromatography was carried out either by a linear or a stepwise salt gradient elution scheme. The stepwise salt gradient elution has been described previously (11). In the linear gradient elution scheme the column was initially eluted with 72 ml of 0.01 M sodium phosphate buffer, pH 7.0. This was followed by the linear gradient elution. The mixing vessel contained 700 ml of 0.01 M sodium phosphate buffer, pH 7.0, while the second vessel contained 700 ml 0.01 M sodium phosphate—0.5 M sodium chloride buffer, pH 7.0. Finally, the column was eluted with 0.01 M sodium phosphate—1.0 M sodium chloride buffer, pH 7.0.

Results

Proteolytic Activity. In table I is given the proteolytic activity of latices from 46 species of *Ficus*. The proteolytic activity of the latices ranged from 0.01 unit per ml for *F. roxburghii* and *F. diversifolia* to 636 units per ml for *F. stephenocarpa*. There is not a correlation between the proteolytic activity and the protein content of the latex. For example, *F. nota* latex has 167 mg protein and 9.1 units activity per ml while *F. stenocarpa* latex has 160 mg protein and 373 units of activity per ml. In this connection it should be pointed out that over 90 % of the protein of *F. carica* latex contains proteolytic activity.

Because of variations in cultural practices and season, the more definitive characteristic of the latex of a given species is the activity per mg protein (specific activity). The specific activity ranged from a high of 2.33 for *F. stenocarpa* latex to a low of 0.01 for *F. diversifolia*. Latices from 30 of the 46 species had less than 5 % of the specific activity of *F. stenocarpa*. *F. glabrata* latices collected from 2 locations in California had nearly the same specific activity even though there was quite a marked difference in the activity and protein per ml of latex (table I). The specific activity of the latex from Peru is not strictly comparable because of uncontrolled storage conditions. This is further substantiated by the even lower specific activity of the *F. glabrata* powder. Other commercially available preparations of *F. glabrata* latex examined have ranged as low as 0.20 in specific activity.

The latices of *F. monckii* collected from 2 locations in California had essentially the same specific activity even though the activity and protein content of the 2 differed.

The best documentation for the constancy of the specific proteolytic activity within a species comes from data on a number of varieties of *F. carica* (table II). The average specific activity is 3.39 ± 0.26 and the specific activities range only from 3.90 for Blanquette to 2.95 for Calimyrna. We have examined the latices of *F. carica* variety Kadota

Table II. *The Proteolytic Activity of Sixteen Varieties of* F. carica[1]

F. carica variety	Activity	Protein[2]	Specific activity
	units/ml	*mg/ml*	*units/mg protein*
Adriatic	262	79.2	3.31
Beall	230	73.6	3.13
Black Mission	424	121	3.50
California Brown Turkey	167	52.0	3.22
Conadria	368	118	3.12
Earlimont	356	100	3.56
Fraga	260	77.0	3.38
Kadota	364	107	3.40
Martinique	238	66.0	3.61
Blanquette	554	142	3.90
Calimyrna	212	71.7	2.95
King	558	150	3.72
Marabout	304	82.5	3.68
Pied de Boeuf	310	95.5	3.25
Roeding-3 Caprifig	377	112	3.37
Stanford Caprifig	554	176	3.15

[1] All analyses were performed after dialysis of the latices.

[2] The protein content was determined by the biuret method (2).

from a number of locations in California and at various times during the year (unpublished data). While there was a marked variation in the amount of activity per ml depending on cultural practices and time of year the specific activity was found to be remarkably constant.

The difference in specific activity reported for *F. carica* latices in tables I and II is a result of the dialysis of all the samples in table II before analysis.

Proteolytic Enzyme Composition. The electrophoretic properties of the proteins of *F. glabrata* and of several varieties of *F. carica* are shown in figure 1. It is apparent that there are a number of proteins in each of the latices and that the protein components of each latex differ both qualitatively (different electrophoretic mobility) and quantitatively from those in other latices. We have also examined several of the latices of different species of *Ficus* by polyacrylamide disk gel electrophoresis. The latices examined by this technique were all different in their protein composition.

The electrophoretic techniques for comparing the latices suffered from the disadvantage of not being able to adequately quantitate the protein and enzyme activity. Column chromatography, while requiring much longer for the separations, does permit such a quantitative description. The results of some typical analyses of latices of 4 species of *Ficus* are shown in figure 2.

In figure 3 data for several species of *Ficus* are shown on a comparative basis. This is compared with that obtained for several varieties of *F. carica* (fig 4, ref 11). Location of the bar diagram along the X-axis indicates the elution volume for the peak of a component while the height of the bar indicates the percent of the total activity or protein, as recovered from the column, contributed by that component. The relative specific activity of the components can be determined by comparing heights of the black (activity) and white (protein) bars of each component. Those components with identical letters within each figure are identical chromatog-

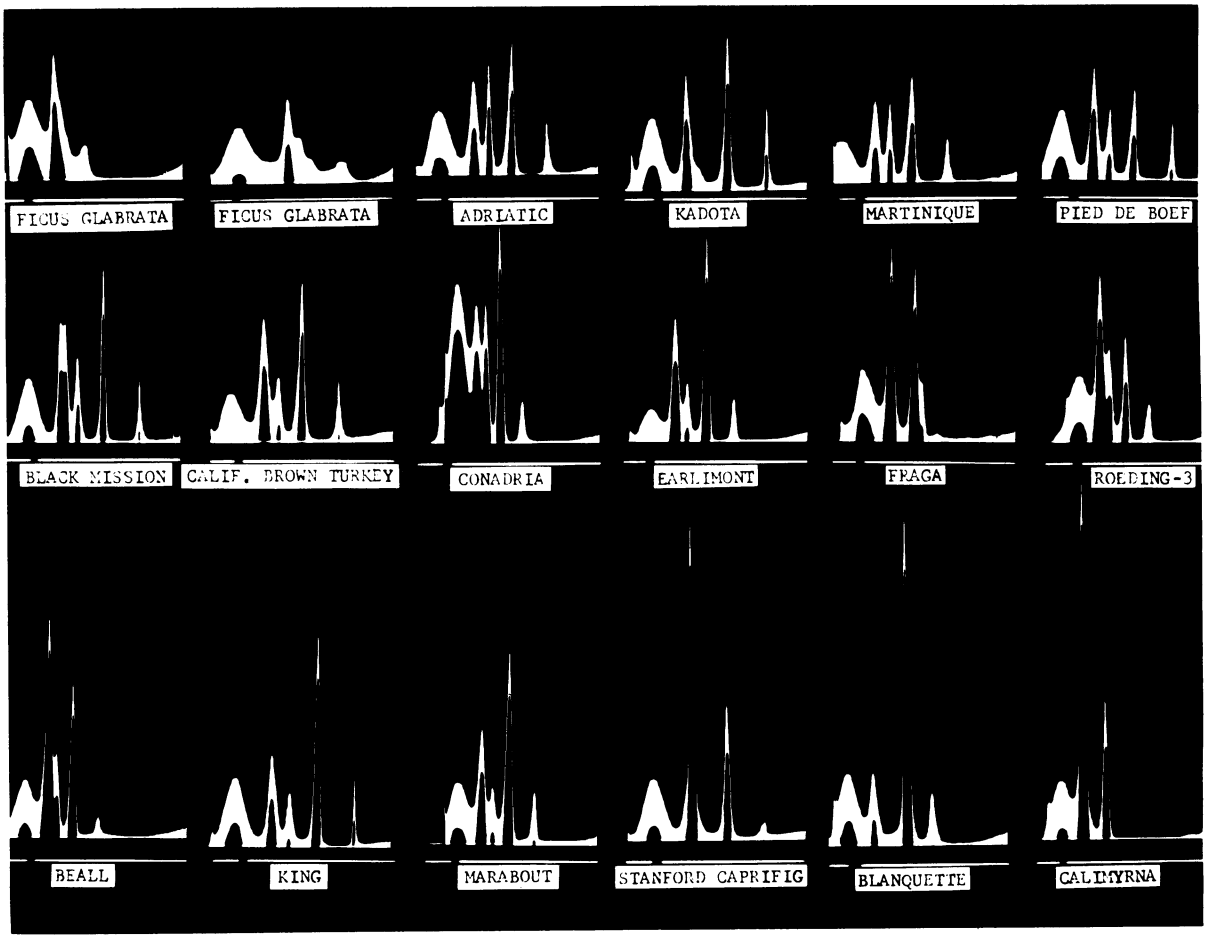

FIG. 1. (top left) Ascending free boundary electrophoretic patterns of latices from 16 varieties of *F. carica* and from *F. glabrata*. The ascending boundaries were photographed after 4000 seconds except the second one of *F. glabrata* was at 7200 seconds. The initial starting point is at the break in the base line at bottom of each pattern. The cathode was on right of the pattern. Specific conductivity of the solutions was 113 ohms per cm. The patterns for *F. glabrata* (4000 sec), Kadota, Black Mission, and Calimyrna have been reported previously (11).

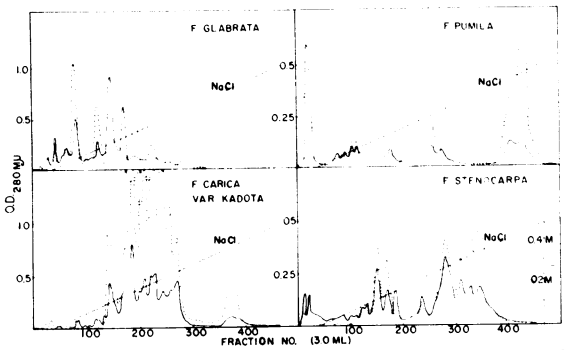

FIG. 2. Chromatographic separation of proteins of latices from four species of *Ficus* on a CM-cellulose column (1.2 × 45 cm) at pH 7.0. The initial starting buffer was 0.01 M phosphate buffer, pH 7.0. Elution was with a linear NaCl gradient as indicated by the diagonal line. Absorbance at 280 mμ is indicated by the solid line; activity per ml per min is indicated by the dashed line. *F. glabrata latex* was from Peru.

raphically. The precision of the chromatographic analyses, as determined by duplicate analyses and from previous experience particularly with chromatography of *F. carica* variety Kadota latex, was used in assigning letters to the individual components. Where a sequence of components is found within any 1 latex (for example K through R in *F. carica* variety Kadota, fig 3) assignment of letters is easy. In a very few cases, the assignment of letters may be somewhat arbitrary although statistically significant on the basis of data available.

In the case of the latices of the 6 species of *Ficus* examined the data indicate there are a minimum of 26 chromatographically distinct protein components with proteolytic activity (fig 3). There is no single component which occurs in all the species examined. As might be expected, the proteolytic enzyme composition of the latices of the 9 varieties of *F. carica* examined was more uniform than that of the different species (fig 4). In the latices of the 9 varieties there appears to be a minimum of 16 chromatographically distinct components. It should be noted that the stepwise elution scheme used in the chromatography of the latices of the several varieties of *F. carica* was more discriminating than the linear gradient elution scheme used for the latices of the various species. Eight of the 9 latices were found to contain components J and P while components A, B, H, and L were found in 6 of the 9 varieties. On the other hand, the latex from the variety Kadota had 10 proteolytic enzyme components while latices of varieties Blanquette and Calimyrna had only 4 proteolytic enzyme components each. In the latex of Calimyrna component H was predominant while in Blanquette latex the predominant component was J.

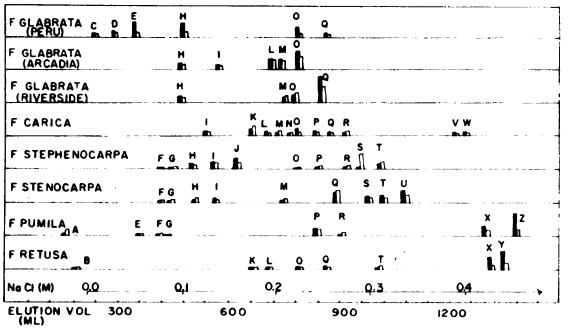

FIG. 3. Diagrammatic representation of the separation of proteolytically active proteins of latices from 6 species of *Ficus*. The experimental conditions are described in figure 2 and the text. The heights of the black (activity) and white (protein) bars indicate the relative contribution of each component to the overall activity or protein as recovered from the column. The location along the x-axis indicates the elution volume of the peak of the component. Components designated by the same letter are chromatographically identical.

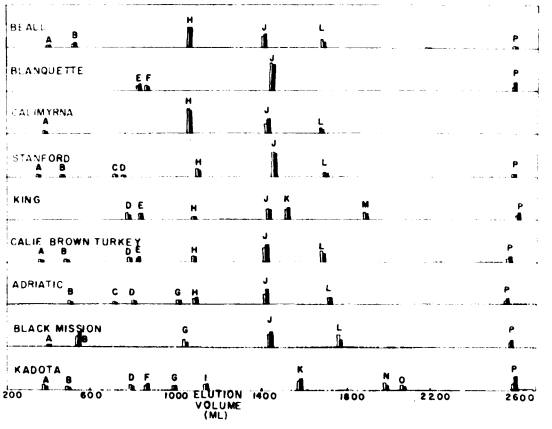

FIG. 4. Diagrammatic representation of the separation of proteolytically active proteins of latices of 9 varieties of *F. carica* (11). The bar diagrams have the meaning indicated in figure 3.

There were marked chromatographic differences among the *F. glabrata* latices from 3 different sources (fig 3). In the latex from Peru there are 3 components (C, D, and E) which do not occur in the latices from Riverside and Arcadia. Only 50 % (4) of the combined components of the latices of *F. glabrata* from Riverside and Arcadia were chromatographically identical.

Discussion

High proteolytic activity in the latex is not a distinguishing feature of the genus *Ficus*. Of the 46 species of *Ficus* examined only 13 had what might be classed as high proteolytic activity. This observation confirms the much earlier report of Robbins and Lamson (10) who reported that the latices of 7 of 16 species of *Ficus* had no measurable proteolytic activity. Their results, in which gelatin

was used as substrate, were somewhat qualitative as the activity was given only in terms of the volume of latex used in the assay. As pointed out above, the protein content of the latex can vary tremendously depending on the cultural conditions and the season.

The proteolytic activity was found to be distributed among several chromatographically distinct proteins in all latices examined. These multiple components are not artifacts of the storage and separation conditions. Latices of *F. carica* varieties Kadota and Black Mission have been examined from a number of locations in California and at various times of the year. While the total activity (units per ml latex) was found to be markedly different, the proteolytic enzyme components were constant and entirely characteristic of the variety. Latices have been collected directly into sodium *p*-chloromercuribenzoate (or into sodium tetrathionate) which reacts with the essential sulfhydryl group of the enzyme to eliminate all proteolytic activity. Chromatographic separation of this completely inhibited material has yielded the same number of components with proteolytic activity (after reactivation with cysteine). In deliberate attempts to cause artifacts latices have been incubated at 35° for 3 to 4 days. Except for a lowering in specific activity, there was no formation of new components with proteolytic activity. In our extensive investigation on *F. glabrata* and *F. carica* variety Kadota latices we have shown that isolated components rechromatograph as unique components (5, Williams and Whitaker, unpublished data). In only 1 case so far have we been successful in observing the formation of one component from another. If component F of *F. carica* variety Kadota latex (fig 5) is stored at 4° in phosphate buffer, pH 7.0, for several weeks it is slowly converted to component D (Kramer and Whitaker, unpublished data).

From the data presented and other data available to us we must conclude that the proteolytic activity of all latices examined is contained in multiple protein components and that this distribution is characteristic of species and varieties from which the latex is derived. We do not believe the variability of components in the 3 samples of *F. glabrata* latex invalidates this conclusion. While the 2 trees at Riverside and Arcadia are identical by classical taxonomic characteristics, they do not appear to be quite identical based on the chromatographic properties of the proteolytic enzymes. Undoubtedly, there are varieties of *F. glabrata* just as there are at least 700 named varieties of *F. carica* (1). The *F. glabrata* latex from Peru may be from still another variety. We have examined the proteolytic enzyme composition of latices and powders obtained from all major suppliers of ficin (the proteolytic enzymes of fig latex) in the United States and have found them to be chromatographically identical on a qualitative basis. The quantity of activity was found to vary tremendously among the preparations which is a reflection of handling conditions.

While the distribution of proteolytic activity among several protein components is quite different for the latices of several varieties of *F. carica* (11), the specific activity was remarkedly constant. It would appear therefore that the presence of multiple components with proteolytic activity is not an attempt by the plant to vary the amount of proteolytic activity. These various enzyme components do not appear to have widely different substrate specificities although there appears to be some significant differences among the components of *F. carica* variety Kadota (5) and *F. glabrata* (unpublished work) in this respect.

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Literature Cited

1. Condit, I. J. 1955. Fig varieties: a monograph. Hilgardia 23 (11): 323–538.
2. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of the serum proteins by means of the biuret reaction. J. Biol. Chem. 177: 751–66.
3. Hooker, J. D. and B. D. Jackson. 1895. Index Kewensis and supplements. Oxford at the Clarendon Press, London.
4. Hutchinson, J. 1959. The Families of Flowering Plants. 2nd Ed., Vol. I. p 201.
5. Kramer, D. E. and J. R. Whitaker. 1964. *Ficus* enzymes II. Properties of the proteolytic enzymes from the latex of *Ficus carica* variety Kadota. J. Biol. Chem. 239: 2178–83.
6. Krause, O. 1930. Cytological studies of the Urticales. Deutsch. Bot. Ges. Ber. 48: 9–13.
7. Kunitz, M. 1947. Crystalline soybean trypsin inhibitor II. General properties. J. Gen. Physiol. 30: 291–310.
8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 265–75.
9. Peterson, E. A. and H. A. Sober. 1956. Chromatography of proteins I. Cellulose ion-exchange adsorbents. J. Am. Chem. Soc. 78: 751–55.
10. Robbins, B. H. and P. D. Lamson. 1934. Further studies on the proteolytic enzyme content of latex from the fig and related trees. J. Biol. Chem. 106: 725–28.
11. Sgarbieri, V. C., S. M. Gupte, D. E. Kramer, and J. R. Whitaker. 1964. *Ficus* enzymes I. Separation of the proteolytic enzymes of *Ficus carica* and *Ficus glabrata* latices. J. Biol. Chem. 239: 2170–77.
12. Tauber, H. 1949. The Chemistry and Technology of Enzymes. John Wiley and Sons, New York.