Hemisphaericin-D, a dialysable and polymerizable protease found in Bromelia hemisphaerica[1]

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Summary. Proteolytic activity was detected outside dialysis bag filled with Bromelia hemisphaerica fruit juice. The dialysable protease was concentrated and purified from small molecular weight contaminants on Sephadex G-10 columns. Acrylamide gel electrophoresis of the dialysable protease, in the presence of SDS and 2-mercaptoethanol, demonstrated a single protein band of about 8000 daltons mol. wt. The same single band with identical mobility was shown with Hemisphaericin, the enzyme retained inside the dialysis bag. The small protease, named Hemisphaericin-D was antigenic in rabbits and the antibodies cross-reacted fully with Hemisphaericin. Hemisphaericin-D appears not to be a degradation product of Hemisphaericin.

Del Castillo and Castañeda have studied Hemisphaericin, the proteolytic enzyme present in the fruit juice of Bromelia hemisphaerica. It was described as a thiol enzyme with apparent mol. wt of around 24,000 daltons[3]. Isoelectric focussing revealed multiple molecular forms with valine as the only N-terminal and serine the only C-terminal aminoacids[4]. These results were interpreted as due to natural internal aminoacid substitution leading to multiple molecular forms[3]. According to our own data, presented in this paper, it appears that Hemisphaericin is not comprised of slightly different polypeptide chains but the results of polymerization of a much smaller homogeneous dialysable polypeptide named Hemisphaericin-D (this work).

Methods. Juice was obtained by squeezing through a cheesecloth Bromelia hemisphaerica fruit collected in the State of Morelos, México. The juice was dialyzed using cellophan tubing (Visking) against several changes of 0.02 M citrate buffer, pH 5.5, containing 10 mM phenylmercuric acetate. After each change, the buffer was kept for the determination of protease activity and replaced by an identical volume of fresh buffer. Dialysis of protease activity from intact fruits was obtained by immersion of the fruits into known volumes of the same buffer. Enzymatic activity in aliquots of the diffusates were measured using as substrates either Remazol brilliant blue hide-powder azure (Calbiochem, USA), casein or hemoglobin. The procedure used for the respective substrates were those of Rinderknecht et al.[5], Kunitz[6] and Anson[7]. Diffusates from 200 ml of Bromelia hemisphaerica juice containing 40 mg total nitrogen, as estimated by a Nessler procedure[8] were concentrated, dissolved in 5 ml of water, and filtered through Sephadex G-10 (2.5 × 60 cm) columns to remove small mol. wt components, like sugars and ninhydrin positive contaminants. Deionized and glass destilled water was used as eluant. The samples displaying proteolytic activity were excluded in the column void volume and were pooled, liophylized, dissolved in 0.01 M Tris-glycine buffer, pH 8.1, and applied to acrylamide gel electrophoresis columns described by Clarke[9].

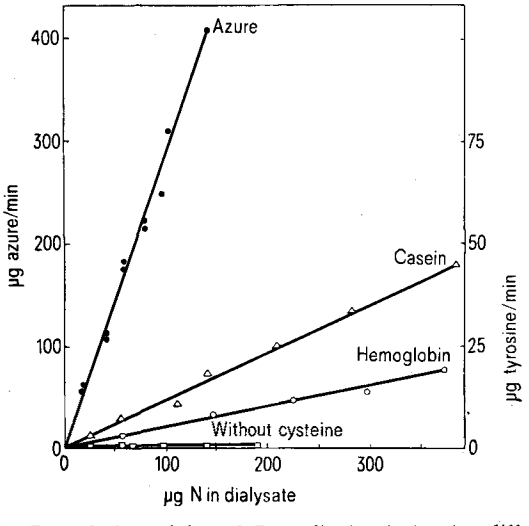

Fig. 1. Proteolytic activity of Bromelia hemisphaerica diffusates assayed with Remazol brilliant blue hide-powder azure, casein and hemoglobin, in the presence of 0.01 M cysteine. Abcissa indicates amount of dialysable nitrogen estimated by Nessler method.

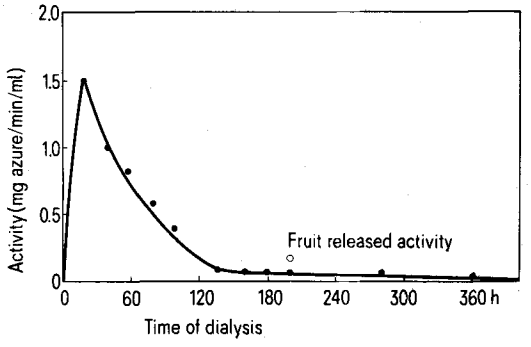

Fig. 2. Kinetics of Hemisphaericin-D dialysis B. hemisphaerica juice was dialyzed using cellophan (Visking) bags against 0.02 M sodium citrate, pH 5.5 buffer with 10 mM phenylmercuric acetate, at room temperature. At times indicated (-●-●-●-) the buffer was replaced and proteolytic activity assayed in each sample. Circle (○) represents activity released from one whole intact B. hemisphaerica fruit at time indicated in a separate experiment.

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3 M. T. Cruz, L. M. del Castillo and M. Castañeda-Agulló, Rev. latinoamer. Quim. *5*, 18 (1974).

4 R. Garduño, M. Soriano, E. Chavez, M. T. Cruz, L. M. del Castillo and M. Castañeda-Agulló, Rev. latinoamer. Quim. *5*, 243 (1974).

5 H. Rinderknecht, M. C. Geokas, P. Silverman and B. J. Heverback, Clin. chim. Acta *21*, 197 (1968).

6 M. Kunitz, J. gen. Physiol. *30*, 291 (1947).

7 M. L. Anson, J. gen. Physiol. *22*, 79 (1939).

8 F. Lanni, M. L. Dillon and J. W. Beard, Proc. Soc. exp. Biol. Med. *74*, 4 (1950).

9 J. T. Clarke, Ann. N. Y. Acad. Sci. *121*, 428 (1964).

Parallel samples were electrophoresed in SDS-polyacrylamide gels containing 0.1% 2-mercaptoethanol[10]. The samples were heated for 1 h at 100 °C in the presence of 1% SDS and 1% 2-mercaptoethanol, prior to electrophoresis. Egg albumin, bovine serum albumin, cytochrome-C and bovine hemoglobin (Sigma, Chem. Co., USA) and insulin (Eli Lilly Co.) were treated similarly and used as standards of known molecular weights.

The dialysable and purified protease, named Hemisphaericin-D, was mixed with Freund's adjuvants (Difco Co., USA) and injected 6 times i.m. into rabbits at weekly intervals. The animals were bled 6 weeks later and the antisera tested against dialysable and non dialysable forms of Hemisphaericin using immunodifussion gel plates according to Ouchterlony[11].

Results. Bromelia hemisphaerica diffusates contained proteolytic activity towards casein, hemoglobin and hide-powder azure. Activity was demonstrated only when 0.02 M cysteine or 2-mercaptoethanol were present (figure 1). The enzyme dialyzed rapidly and 140 h later activity was scanty outside the dialysis bag. The shape of the release curve suggested a limited process leading to depletion of the low mol. wt enzyme stores inside the bag. The dialysis kinetics at pH 5.5 are displayed in figure 2.

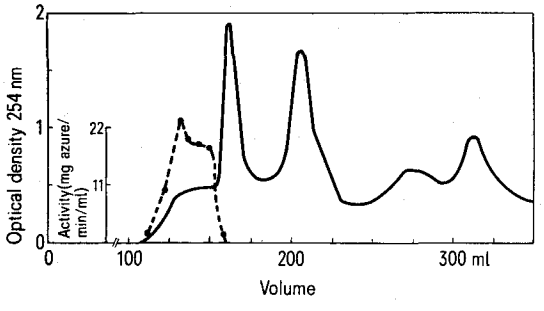

Fig. 3. Effluent diagram of 40 mg concentrated Bromelia hemisphaerica diffusates over a 2.5×60 cm Sephadex G-10 column eluted with deionized water. Black dots indicate proteolytic activity assayed with 'azure' (small scale).

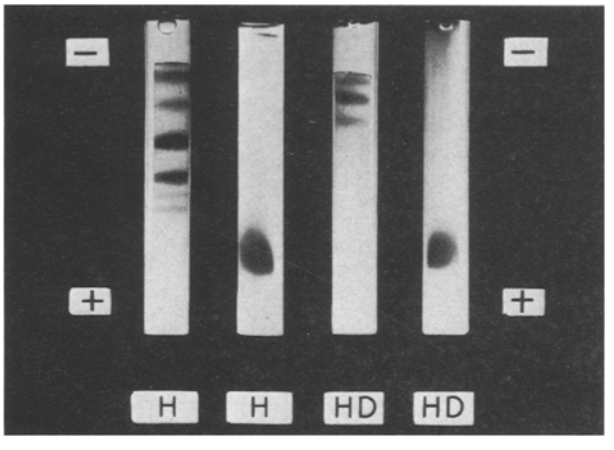

Fig. 4. Photograph of Hemisphaericin (H) and Hemisphaerin-D (HD) run in standard polyacrylamide gels (left side in each pair) and same samples run in polyacrylamide gel with SDS and 2-mercaptoethanol (right side in each pair). In the latter case, a single band with identical mobility is present in both instances.

The proteolytic activity remaining inside the dialysis sac was due to Hemisphaericin[3,4]. Upon filtration of the diffusate, the enzyme displayed charge heterogeneity since 3 bands of activity were observed upon electrophoresis (figure 4). In spite of this ionic heterogeneity, these samples gave only a single band on electrophoretic gels containing SDS and mercaptoethanol. The unique band migrated to a position corresponding to a mol. wt of around 8000 daltons, significantly less than the value of 24,000 reported for Hemisphaericin on the basis of thiol group content[3]. However, Hemisphaericin isolated by the procedure described[4] and by our own very similar method[13] when similarly analyzed in the presence of SDS and mercaptoethanol, is also a single 8000 daltons component but yields a multiple pattern in electrophoresis free of denaturing and reducing agents (figure 4). Thus, the dissociated monomer appears identical in the dialysable and in the non-dialysable enzymes.

Discussion. The presence of dialysable activity is not explained by autolysis of the non-dialysable enzyme for the following observations: a) Proteolytic activity of juice is absent at pH 3.5, natural pH of more than 20 different Bromelia hemisphaerica samples, but when the pH is raised and reducing agents added, the juice shows strong proteolytic activity. Thus autolysis is very unlikely to occur during collection and storage of the fruits. b) Enzymatic activity is released by simple immersion of ripe and green intact Bromelia hemisphaerica fruits in citrate buffer made isotonic with 0.25 M sucrose. This latter condition seems to preclude enzyme release due to tissue disruption. Nevertheless, the enzyme always appears in the buffers surrounding the fruits. This active fraction is again Hemisphaericin-D. c) The dialysis kinetics at pH 5.5 depict a fast initial release of Hemisphaericin activity followed by diminishing amount until depletion of dialysable protease. This type of kinetics is not predicted by autolysis (or leaky dialysis bag).

The simplest and most compelling conclusion is shown from the results of polyacrylamide gel electrophoresis using SDS and reducing agents. By this method of analysis, both dialysable and non-dialysable forms of the enzyme were identical. In addition, the finding of immunological identity between Hemisphaericin and Hemisphaericin-D proves that the similarity in mol. wt is not simply fortuitous; the 2 sources of enzyme activity are one and the same protein (or family proteins). Thus the non-dialysable Hemisphaericin previously assigned a mol. wt of about 24,000 daltons[3] is in fact a trimer of a basic subunit of 8000 daltons mol. wt. We must assume the

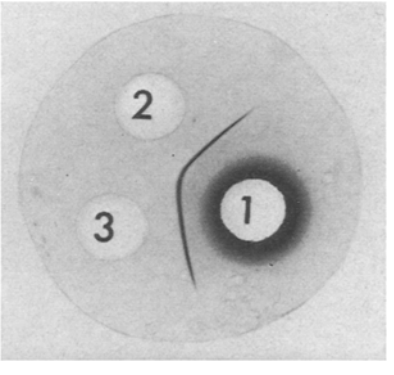

Fig. 5. Immunodiffusion plate with: 1. Rabbit antiserum against Hemisphaericin-D; 2. Hemisphaericin-D; 3. Hemisphaericin[3,4].

presence of the basic subunit within the intact fruit, which forms the dialysable form of the enzyme and is in fact one of the smallest natural proteases so far reported[12]. The exact mode of disulphide and non-covalent interactions responsible for polymer formation in this enzyme, remains to be investigated.

10 K. Weber and M. Osborn, J. biol. Chem. *244*, 4406 (1969).
11 O. Ouchterlony, in: Progress in Allergy, vol. 5, p. 1. Karger, Basel (1958).
12 M. Dixon and E. C. Webb, in: Enzymes, p. 672. Longmans Green, London (1964).
13 M. Reyes, C. Agundis and F. Córdoba, work in preparation.

Isoperoxidase spectra of single tulip cultivars and their parrot mutants

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Summary. The isoperoxidase spectrums of 2 single tulip cultivars and their parrot mutants were analyzed by disc electrophoresis in leaves and tepals i.e. petals plus sepals excised from within the bulbs. It was found that the anodal isoperoxidase pattern of parrot mutant tepals differs distinctly from tepals of their mother cultivars,but no differences were observed in cathodal isoperoxidases. Both the anodal and cathodal isoperoxidase spectra of parrot mutant leaves and leaves of their mother cultivars were similar.

All cultivars of tulips are classified into many groups and published as 'The Classified List and International Register of Tulip Names'. 1 of the groups is Parrot tulips which are characterized by laciniate tepals (sepals and petals of Tulipa are indistinguishable and are collectively referred to as tepals)[1]. It is known that most parrot tulip cultivars formed as vegetative mutants (sports) from other cultivars belonging to non-parrot groups[2].

The aim of the present work was to study isoperoxidase patterns of 2 single cultivars and their parrot mutants (cultivars) since it is well known that peroxidases play an important role in cell and organ differentiation[3–5].

Material and methods. For the experiments the parrot cv. Fantasy which was formed in 1910 as a mutant of cv. Clara Butt (both cultivars have red tepals) and parrot cv. Pitt's Parrot formed in 1945 from cv. William Pitt (both cultivars have pink tepals) were taken[6]. Cv. Clara Butt, Fantasy, William Pitt are diploids (2 n = 24 chromosomes) and the number of chromosomes in cv. Pitt's Parrot has not been determined to our knowledge[7]. For isoperoxidase spectrum analysis leaves and tepals were excised on November 11, 1975, from within the bulbs which had been kept in a storage room at 17–20 °C. Parrot cultivars had laciniate tepals at that time. Leaves and tepals from 15 bulbs of each cultivar were frozen and lyophylized, ground and then kept in a refrigerator at 4 °C. Soluble proteins were extracted from powder with cold 0.1 M Tris-hydrochloride buffer at pH 7.8, containing 6 mM ascorbic acid, 6 mM L-cysteine hydrochloride and 0.5 M sucrose[8], using 1.5 ml of buffer for 0.1 g of dry powder of leaves and tepals. Homogenates were centrifuged in a refrigerated centrifuge for 10 min at 20,000 × g. Disc electrophoresis on polyacrylamide gel was performed according to techniques described by Ornstein[9] and Davis[10]. Electrophoresis were conducted in

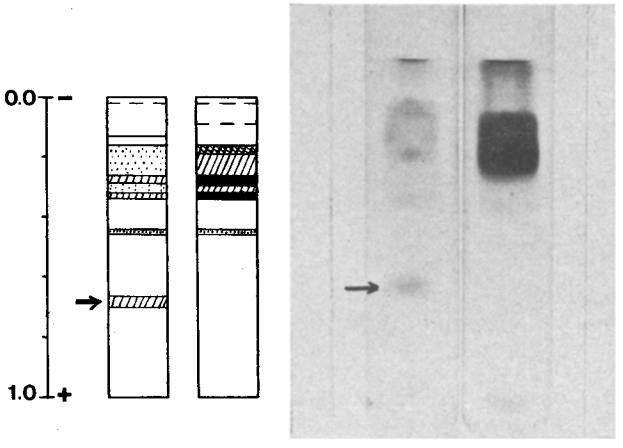

Fig. 1. Pictures and zymograms of anodal isoperoxidases of tepals of cv. Clara Butt (left) and its parrot mutant, cv. Fantasy (right).

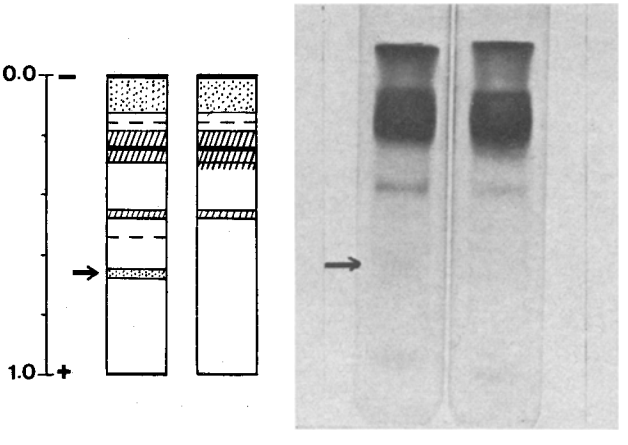

Fig. 2. Pictures and zymograms of anodal isoperoxidases of tepals of cv. William Pitt (left) and its parrot mutant, cv. Pitt's Parrot (right).

1 A. R. Rees, in: The Growth of Bulbs. Academic Press, London and New York 1972.
2 J. Dabrowski, Biuletyn Instytutu Hodowli i Aklimatyzacji Roślin 102/103, 29 (1971).
3 R. Borchert, Devl. Biol. *36*, 391 (1974).
4 A. M. Hirsch, C. r. Acad. Sci., Ser. D *280*, 829 (1975).
5 M. Mäder, P. Münch and M. Bopp, Planta *123*, 257 (1975).
6 J. Dabrowski and S. Dabrowska, Biuletyn Instytutu Hodowli i Aklimatyzacji Roślin 102/103, 5 (1971).
7 O. Plavcova, Acta prühon. *21*, 1 (1969).
8 M. Saniewski and J. Puchalski, Bull. Acad. pol. Sci. Sér. Sci. Biol. *23*, 725 (1975).
9 L. Ornstein, Ann. N. Y. Acad. Sci. *121*, 321 (1964).
10 B. J. Davis, Ann. N. Y. Acad. Sci. *121*, 404 (1964).