ANTINEOPLASTIC AGENTS 78. ISOLATION OF PALYSTATINS 1–3 FROM THE INDIAN OCEAN *PALYTHOA LISCIA*[1]

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ABSTRACT.—The Western Indian Ocean *Palythoa liscia* Haddon and Duerden has been found to contain three cell growth (PS) inhibitory proteins with molecular weights ranging from 128,000 to $>2x10^7$. The lowest mass protein from this colonial Zoanthiniarian, palystatin 1 (PS, ED_{50} 1.3–5.5x10^{-4} μg/ml) may be a sub-unit of the two high mass cytotoxic (PS, ED_{50} 0.01 μg/ml) proteins palystatins 2 and 3. Isolation of palystatins 1–3 was guided by bioassay (PS *in vitro*) through a sequence of gel permeation chromatographic methods utilizing Sephadex LH-20 to Sepharose 4B.

The marine invertebrate order Zoanthiniaria (Coelenterata phylum, Anthozoa subphylum, Zoantharia class) contains a group of colonial encrusting animals resembling small anemones. In this order the family Zoanthidae is best known for its genus *Palythoa* which forms colonies on rocks and dead coral near the low tide level. Because of our long-term interest (2, 3) in exploring a broad variety of marine animal species as new sources of potentially useful cancer chemotherapeutic drugs, we undertook evaluation of *Palythoa liscia* Haddon and Duerden collected in Tombeau Bay off the Coast of Mauritius in the Western Indian Ocean. An ethanol extract of *P. liscia* produced a confirmed level of antineoplastic (41% life extension at a dose of 0.20 mg/kg) and cytotoxic (KB, ED_{50} 0.029 μg/ml) activity in the National Cancer Institute's (NCI) murine P388 lymphocytic leukemia (PS system) and KB (cell line from human nasopharynx carcinoma) systems.

When this first study of *P. liscia* constituents was initiated in 1972, Moore and Scheuer (4) had reported, a year earlier, the very important discovery of palytoxin in the Hawaiian (Maui) *P. toxica*.[2] Several years later palytoxin was found to be curative against the murine Ehrlich ascites carcinoma (at a dose of 0.34 μg/kg) and produce a 32% life extension (at a dose of 0.1 μg/kg) in the PS system (9). Unfortunately the LD_{100} in mice was 1.3 μg/kg and the LD_{50} in various species from mouse to monkey ranged from 0.033 to 0.45 μg/kg (10). The possibility that we might be pursuing palytoxin in extracts of the Mauritius *Palythoa liscia* was disconcerting until we were able to eliminate this obvious isolation pitfall. That our bioassay-guided separation (PS *in vitro*) was not leading to palytoxin was first evidenced by the high mass of active protein-like fractions whereas palytoxin was known to be nonpeptide and to have a molecular weight near 3,000 [now 2681 by ^{252}Cf plasma desorption mass spectrometry, (11)].

Successful isolation of the new protein cell growth inhibitory palystatins 1–3 was first achieved as follows. The ethanol extract of *Palythoa liscia* was partitioned between chloroform-water, and the chloroform phase was concentrated to a residue that was successively partitioned (12, 13) between ligroin-9:1 methanol-water, carbon tetrachloride-4:1 methanol-water, and chloroform-3:2 methanol-water. By this means the active components were concentrated in the 3:2 methanol-water solution and this fraction displayed a PS ED_{50} of <0.01 μg/ml. Sephadex LH-20

[1]Consult (1) for part 77.

[2]The toxic *limu-make-o-Hana* was first collected by the University of Hawaii Scheuer group near Hana, Maui on December 13, 1961. This *Palythoa* was protected by the ancient Hawaiian taboo (kapu) that would have disaster strike anyone who would remove the toxic limu. Interestingly, on the day of collection the U. of Hawaii, Institute of Marine Biology main laboratory building was destroyed by fire of unknown origin (4,5). In the spring of 1961 Ciereszko, Reeburgh and Attaway received a toxic reaction (severe chills and fever) from exposure to dust generated from dried *P. caribaeorum* collected near Jamaica and this observation led to an independent isolation of palytoxin from this species and the Caribbean *P. mammillosa* (6). Another independent isolation of palytoxin from the Indo-Pacific (Japan) *P. tuberculosa* Esper was made during the same decade by Hashimoto and co-workers (7,8).

(2:3 chloroform-methanol) was used to chromatograph the latter material, and the excluded fraction was subjected to further gel permeation chromatography in the following order: Sephadex G–50 (water), G–100 (99.5:0.5 water-*n*-butanol) and Sepharose 4B (0.1 M ammonium bicarbonate). The highest molecular weight (>2 x 10^7) protein obtained by the Sepharose 4B separation was designated palystatin 3 (PS ED_{50} 0.01 μg/ml) and a lower molecular weight (about 5 x 10^5) companion palystatin 2 (PS ED_{50} 0.01 μg/ml). Subsequent modification of this method, as described below, led to palystatin 1 (mol wt 128,000±12,000, PS ED_{50} 1.3–5.5 x 10^{-4} μg/ml) which may be a fundamental unit of the larger proteins palystatins 2 and 3.

As soon as the possible carcinogenicity of chloroform became known about four years ago, we replaced this solvent with methylene chloride in all solvent partitioning and chromatographic procedures. The safer methylene chloride has been found in this study, and a large number of related programs in our Institute, to routinely give results quite comparable to those obtained with chloroform. Indeed, in the present partition sequence, methylene chloride led to a greater concentration of active material. Here it should be noted that the use of chloroform-methanol-water systems, where the upper layer is nearly all methanol-water and the lower layer almost all chloroform, for the extraction of lipids of biological origin was based on work beginning about thirty years ago (*cf* 12) and nicely developed into a practical technique for extracting marine vertebrate lipids by Bligh and Dyer in 1959 (14). In addition to improving safety considerations, the substitution of methylene chloride eliminated side-reactions attributable to carbene, hydrogen chloride and phosgene formation in chloroform. Thus, a 1975 recollection of the *Palythoa liscia* was reinvestigated by replacing chloroform with methylene chloride in the separation procedures, the Sephadex steps were conducted with 0.02 M Tris hydrochloride buffer (pH 7.0) containing 0.5 M sodium chloride (desalted with Sephadex G–10) and the G–100 excluded fraction was further separated by use of Sephadex G–200 or Sephacryl S–300 prior to the Sepharose 4B procedure. The penultimate modification provided a convenient approach to palystatin 1.

The partition behavior of palystatins 1–3 in favor of chloroform or methylene chloride over water suggests that these substances may be lipoproteins. And in accordance with other marine animal antineoplastic proteins we have isolated (*cf* 1) palystatin 1 appears to have carbohydrate (12%) segments. The similar amino acid compositions of palystatins 1–3 indicates that palystatin 1 may be a sub-unit of the 2 and 3. But the elemental analyses (CH or N) appear to differ, so to provide a definitive conclusion this observation will require a future detailed structural investigation. Among a variety of such interesting questions raised by the discovery of palystatins 1–3 is whether palytoxin or a related substance may be a minor (below the level of ultraviolet detection) lipid-type attachment to these proteins.

Biological evalution of palystatins 1–3 is being performed by the National Cancer Institute. To date palystatin 1 has been found toxic to 0.3 mg/kg in the murine PS *in vivo* system. A study of lower doses containing palystatins 1–3 is in progress.

EXPERIMENTAL[3]

ANIMAL COLLECTION AND PRELIMINARY EXPERIMENTS.—The initial collection of *Palythoa liscia* Haddon and Duerden (kindly identified by Prof. C. E. Cutress, University of Puerto Rico)[4] made in September-October 1972 and preserved in denatured[5] ethanol was received in

[3]Introduction to the experimental section of (1) provides a summary of general methods used in this study. In addition, all solvents were redistilled and we wish to thank Drs. J R. Cronin and A. M. Yates and Mrs. S. L. Ode for the amino acid analyses and Dr. M. L. Parsons for the trace metal analyses.

[4]A specimen has been deposited with the Smithsonian Institution.

[5]With ligroin (2% by volume) and pyridine (1% by volume) as denaturant.

March 1973. Biological evaluation of the shipping solution gave confirmed levels of activity against the NCI PS (T/C 141 at 0.20 mg/kg, 15) and KB (ED_{50} 0.029 μg/ml) systems. Extraction of the animals with refluxing ethanol provided an extract with comparable activity (PS T/C 152 at 3 mg/kg and KB ED_{50} 0.33–2.9 μg/kg). When the shipping solution ethanol extract was triturated successively with ligroin, chloroform, methanol and water, the methanol soluble fraction led to a PS *in vitro* ED_{50} of 0.01 μg/ml. The water fraction was less active (PS ED_{50} 2 μg/ml) and the organic solvent fractions were inactive. Analogous separation of the hot ethanol extract gave a less cytotoxic series with, e.g., the methanol fraction displaying PS ED_{50} 2.4 μg/ml. The PS *in vivo* results for both sets of fractions showed only marginal activity (T/C 120) and/or toxicity.

Although a variety of methods were eventually utilized to separate both (ambient and hot) ethanol extracts, the original significant level of PS *in vivo* activity was never again encountered in these or later (see below) experiments. Consequently, the very reproducible PS *in vitro* system (16) was selected as the bioassay for all efforts beginning in 1976. The Procedure A route to palystatins 2–3 and the Procedure B path to palystatins 1–3 was guided by this bioassay. The former method was developed with a 1975 (received in February 1976) recollection (about 23 kg wet weight) and the latter with a 1978 recollection (about 125 kg wet weight) received in March 1979. All recollections were made in the same general location (Tombeau Bay) on the West Coast of Mauritius.

ANIMAL EXTRACTION.—The 23 kg (approximate wet weight) of *Palythoa liscia* recollected in 1975 (starting point for Procedure A) and the 125 kg recollected in 1978 (used for Procedure B) were initially processed in the same manner. The shipping solution (denatured[5] ethanol) was decanted, the animals were ground and placed in muslin bags, and extracted with hot ethanol employing a modified (stainless steel) Soxhlet apparatus. The combined shipping solution and hot ethanol extract was evaporated (at 40°, *in vacuo*). After extraction (as above) of the residual animal material with hot water and partial removal of solvent at room temperature, the concentrated aqueous solution was lyophilized. Typically, the 125 kg of wet *Palythoa liscia* afforded 4.53 kg of alcohol extract and 465 g of water extract.

Evaluation of these solvent extracts by the PS *in vitro* system showed the alcohol extracts to be quite active with ED_{50} <0.01 μg/ml. The water extracts were found to be inactive.

SOLVENT PARTITION SEQUENCE.—The 835 g ethanol extract from the 1975 recollection was partitioned between chloroform (1.5 liters)-water (1.0 liter) to provide 190 g in the chloroform phase and 645 g (PS inactive) in the water phase. The chloroform fraction was successively partitioned between 9:1 methanol-water-ligroin, 4:1 methanol-water-carbon tetrachloride and 3:2 methanol-water-chloroform as previously described (12). Removal of solvent from the ligroin, carbon tetrachloride, chloroform and methanol-water solutions gave, respectively, 127 g (PS and KB inactive), 3.4 g (PS ED_{50} 4.2, KB 27 μg/ml), 24 g (PS ED_{50} 0.74, KB 2.3 μg/ml), and 33 g (PS ED_{50} 0.09, KB 2.1 μg/ml) fractions.

Analogous (methylene chloride replacing chloroform) separation (see fig. 1) of the ethanol extract (4.53 kg) from the 1978 recollection again gave a very active (PS ED_{50} <0.01 μg/ml) and more concentrated methanol-water fraction. The 1975 and 1978 active methanol-water fractions were used in the following experiments for, respectively, Procedures A and B. Possibly the chloroform and methylene chloride active fractions would yield additional amounts of the palystatins, but this point was not evaluated in detail.

ISOLATION OF PALYSTATINS 1–3.—*Procedure A.* A 19 g portion of the 33 g active 3:2 methanol-water fraction (see above) was chromatographed in 2:3 chloroform-methanol on a column of Sephadex LH–20 (6.5 x 240 cm). After the first 1.1 liters of eluate was discarded, 24 ml fractions were collected. Fractions 1–80 were combined to give 3.9 g of brown material that was dissolved in water and chromatographed on a column of Sephadex G–50 (5 x 117 cm). The first 720 ml of eluate was discarded. When fractions (7 ml each) 1–33 were combined and lyophilized 0.10 g of tan powder was obtained. A solution of this high molecular weight fraction (0.10 g) in 99.5:0.5 water-*n*-butanol was chromatographed on Sephadex G–100 (2.7 x 89 cm), and the first 180 ml of eluate was eliminated. The first fourteen fractions (7 ml each) were combined and lyophilized to yield 87 mg of yellow powder that was further purified by gel filtration on Sepharose 4B (2.5 x 78 cm) in 0.1 M ammonium bicarbonate. Fractions of 6 ml were collected, the first 75 ml was discarded. Fractions 3–7, when combined and lyophilized, provided palystatin 3 (7 mg) as a pale tan fluffy powder (PS ED_{50} 0.1–0.01 μg/ml). Palystatin 3 was found to be a protein (see table 1 for amino acid analysis) with a mass estimated (Sepharose 4B behavior) to be at least $2x10^7$. Metal analyses revealed only trace amounts of aluminum, copper, iron, lead, magnesium, silicon and sodium.

Anal. Found: C, 50.15; H, 7.01; N, 2.89.

Continuation of the gel permeation chromatography on Sepharose 4B, collection and combination of fractions 22–40, and lyophilization gave 65 mg of yellow powder which was finally purified by chromatography in 0.1M ammonium bicarbonate on Sephadex G–200 (2.5 x 85 cm). After removal of the first 70 ml of eluate, fractions (5.8 ml each) 7–25 were combined and lyophilized; 55 mg of palystatin 2 was obtained as a pale yellow powder containing trace amounts of aluminum, copper, iron, magnesium and sodium. Sepharose 4B was used to estimate the mass of this cytotoxic protein (table 1 for amino acid analysis) to be about 2 to $5x10^5$.

Anal. Found: C, 57.33; H, 8.44; N, 5.79.

Procedure B. With the 1978 recollection of *Palythoa liscia* as a starting point the isolation of palystatins 3 and 2 was further developed and expanded to include the isolation of palystatin 1. Here a 44 g amount of the 54 g active 3:2 methanol-water fraction was used to prepare (refer to Procedure A) the Sephadex LH–20 (8.5 x 116 cm) active fraction (30.5 g, by combination of the 20.5 ml fractions 4–53 after 1.6 liter of clear eluant was discarded). A 3.1 g portion

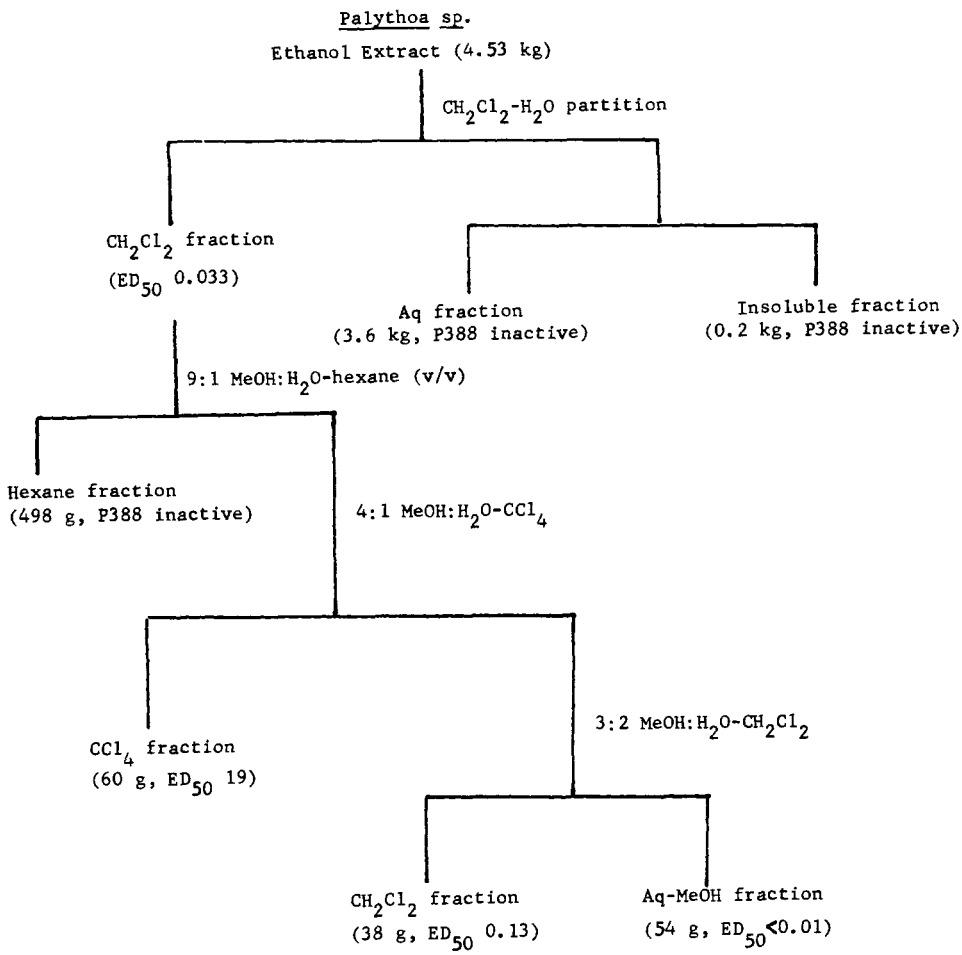

FIG. 1. Solvent partition sequence.

TABLE 1. Amino acid compositions (Mole %) of palystatins 1–3.

Amino Acid	1	2	3
Ala	6.2	6.9	7.7
Arg	4.9	4.0	4.3
Asx	7.9	7.7	7.7
Cys	0.8	1.1	0.7
Glx	7.5	8.8	7.3
Gly	20.6	19.4	21.3
His	0.8	1.0	0.7
Ile	4.6	3.9	3.7
Leu	7.0	6.3	6.4
Lys	7.1	8.3	7.8
Met	1.3	1.1	1.5
Phe	3.3	3.3	3.3
Pro	9.3	8.9	7.5
Ser	5.6	7.6	7.3
Thr	5.6	5.4	5.8
Tyr	2.4	2.1	2.4
Val	5.1	4.2	4.6

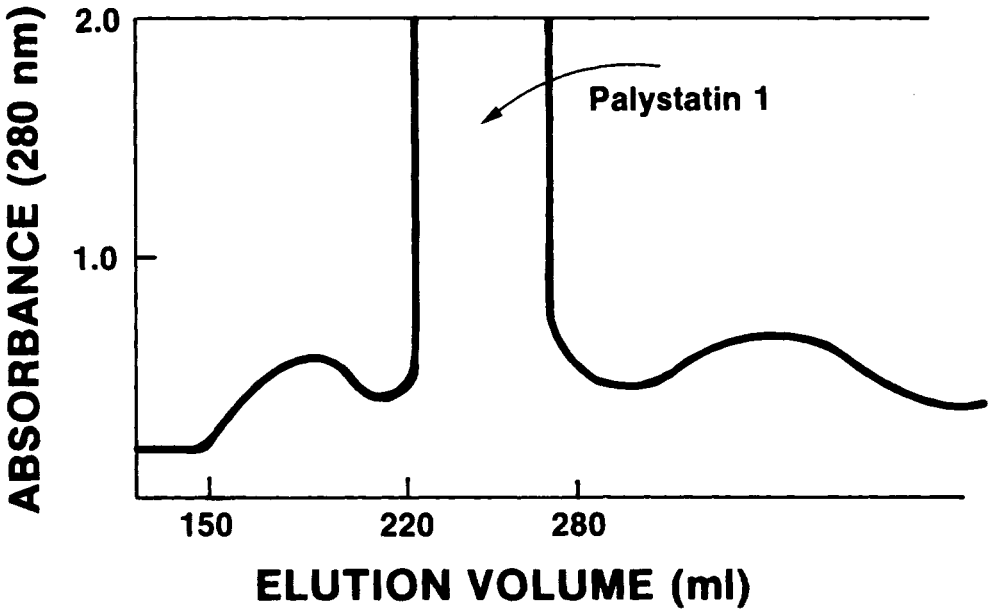

FIG. 2. Separation of Palystatin 1 on Sephadex G-200 (2.5 x 88 cm) using 0.02 M Tris-hydrochloride buffer (pH 7.0) containing 0.5 M sodium chloride.

of this material was chromatographed in 0.02M Tris hydrochloride buffer (pH 7.0, containing 0.5M sodium chloride) on a column of Sephadex G-50 (5 x 75 cm). After the first 350 ml was discarded, the next 210 ml was collected, dialyzed against distilled water, and lyophilized to yield 1.12 g of active (PS ED_{50} <0.01 μg/ml) fraction. Further separation of this material (1.12 g) on a column of Sephadex G-200 or Sephacryl S-300 (2.5 x 88 cm) in the same buffer gave (first 150 ml discarded) in the included fractions 10-26 (7 ml each, desalted by dialysis) 0.54 g of palystatin 1 (PS ED_{50} 1.3-5.5 x 10^{-4} μg/ml) as a tan fluffy powder. The use of Sephadex G-200 (1.2x46 cm) with the same buffer as above and with catalase, bovine serum albumin and cytochrome *c* as standards (17) palystatin 1 was found to have a molecular weight of 128,000±12,000. Metal analyses indicated trace (<0.01%) amounts of aluminum, calcium, copper, iron, magnesium, manganese and sodium.

Anal. Found: C, 56.6; H, 8.5; N, 3.2; P, 2.1.

The nitrogen content (3.2%) of palystatin 1 suggests a 20% protein (refer to table 1 for amino acid analysis) content (based on a 16% nitrogen composition for the average protein),

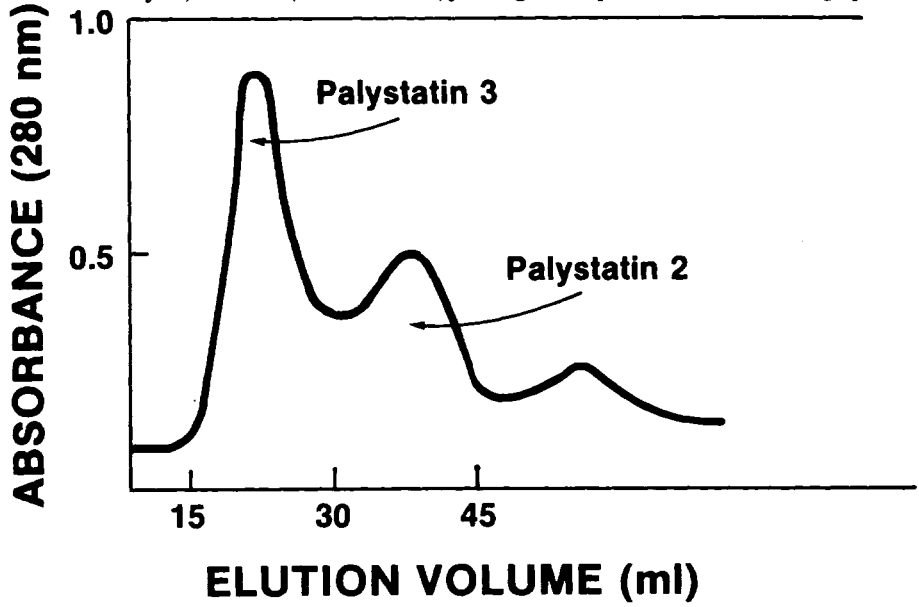

FIG. 3. Separation of Palystatins 2 and 3 on Sepharose 4B (1.6 x 29 cm) using 0.02 M Tris-hydrochloride buffer (pH 7.0) containing 0.5 M sodium chloride.

and a Lowry protein assay (1) indicated 23% protein. A phenol-sulfuric acid (glucose as standard) estimation (1) for carbohydrate resulted in a value of 12%.

Different preparations of palystatin 1 were found to aggregate in varying amounts depending on subtle changes in the procedure. The degree of aggregation caused the G–200 void volume fraction to vary in weight (fig. 2). In a typical experiment, 250 mg of the G–50 active fraction led to 15 mg of the G–200 excluded fraction. This fraction was separated by chromatography, in the Tris hydrochloride buffer (see above), on Sepharose 4B (1.6 x 29 cm). The first 14 ml was eliminated, and fractions (2 ml) 1–7 and 9–23 were each combined, desalted (dialysis) against distilled water and lyophilized; 3 mg of palystatin 2 and 4 mg of palystatin 3 (fig. 3) were obtained. The ultraviolet spectrum of palystatin 1 shown in figure 4 bears no resemblance to that recorded (7) for palytoxin.

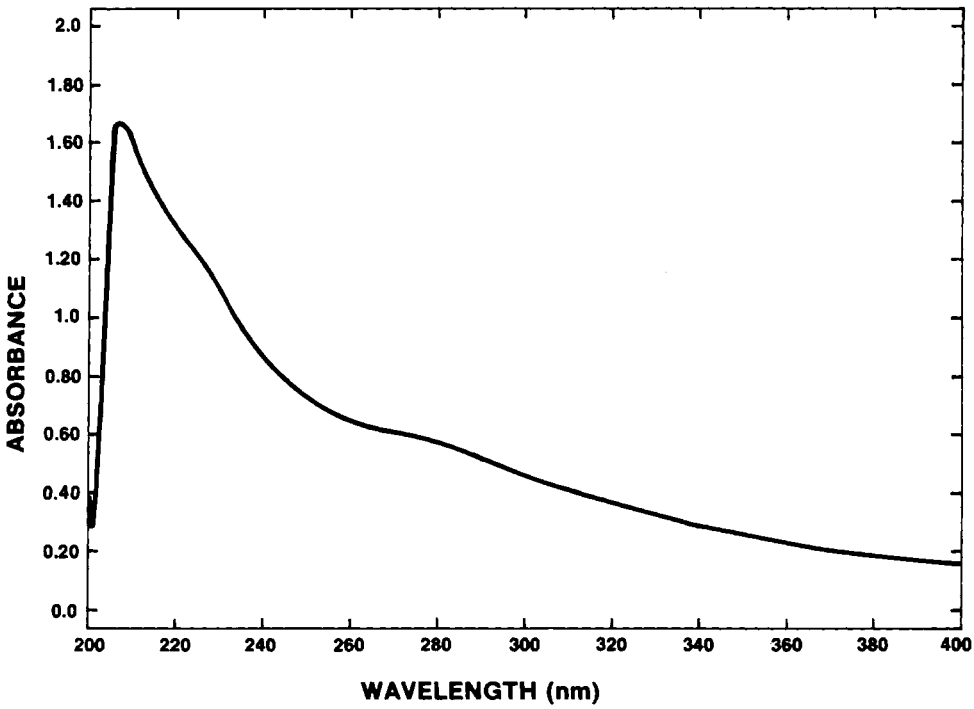

FIG. 4. Ultraviolet spectrum of Palystatin 1 (0.5 mg/3.0 ml of 0.02 M Tris-hydrochloride buffer (pH 7.0) containing 0.5 M sodium chloride).

AMINO ACID ANALYSES.—A solution of palystatin 1 (2.0 mg) in constantly boiling hydrochloric acid (0.5 ml, 6.1N) was sealed (*in vacuo*) in a glass tube. After 24 hr at 105°, the hydrolysate was evaporated, dissolved in 2.5 ml of citrate buffer (pH 2.2) and analyzed (18). Palystatins 2 (4.43 mg) and 3 (1.79 mg) were treated in the same manner. The results have been summarized in table 1 and suggest that palystatins 1–3 are related. Palystatin 1 may be the fundamental sub-unit of the higher molecular weight proteins palystatins 2 and 3.

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