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Ferreascidin: A Highly Aromatic Protein Containing 3,4-Dihydroxyphenylalanine from the Blood Cells of a Stolidobranch Ascidian†

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ABSTRACT: A method of isolation and purification was developed for the major protein from the blood cells of the stolidobranch ascidian *Pyura stolonifera*. The protein, called ferreascidin because of its strong iron binding capacity (two Fe^{3+} per molecule), is the most aromatic protein that has been characterized, with 67% aromatic amino acids including 42% tyrosine and 17% 3,4-dihydroxyphenylalanine. It is a glycoprotein with an apparent sedimentation coefficient of 1.3 S determined by sucrose gradient centrifugation, corresponding to a molecular weight of 10000 which is in agreement with that obtained from sedimentation equilibrium analysis (9800 ± 100). The presence of 3,4-dihydroxyphenylalanine, an amino acid involved in sclerotization of outer body tissues of invertebrates, supports the early proposition that the blood cells are involved in the formation of the test of the ascidian. Circular dichroism of whole cells indicated that the protein was isolated without major structural change.

The chemical constituents of the blood cells of the Ascidiacea, benthic marine invertebrates of the phylum Chordata, have been investigated since early this century (Henze, 1911, 1912). Only one protein has been characterized previously, a protein from the Phlebobranch, *Phallusia mamillata*, known as hemovanadin because of its association with intracellular vanadium (Califano & Caselli, 1948; Henze, 1912; Bielig & Bayer, 1954; Bielig et al., 1966; Boeri & Ehrenberg, 1954; Baltsceffsky & Mendia, 1958; Gilbert et al., 1977).

Endean (1955a) first showed that "morula" blood cells of an ascidian (*Pyura stolonifera*) contain significant concentrations of iron, and more recently, iron has been found generally throughout the Ascidiacea (Swinehart et al., 1974; Biggs & Swinehart, 1976). Research in this laboratory has sought to isolate ligands that bind the iron. This paper reports an unusual protein called ferreascidin that contains 3,4-dihydroxyphenylalanine (DOPA)[1] and has a strong iron binding capacity: it removes a total of two Fe^{3+} ions per molecule of protein from bis(nitrilotriacetato)iron(III) (C. J. Hawkins and Taylor, unpublished results). The catecholate group, a particularly strong chelating group for iron(III), is used by microorganisms to sequester iron (Raymond et al., 1984). DOPA-containing proteins have been shown to possess important functions in invertebrates as extracellular structural proteins (Rainsford, 1967; Degens et al., 1967; Knight & Hunt, 1974; Waite & Anderson, 1978, 1980) and are involved in the attachment of the invertebrates to solid surfaces (Waite & Tanzer, 1980, 1981; Waite, 1986; Mascolo & Waite, 1986; Benedict & Waite, 1986a,b).

Although this is the first report of a DOPA protein from an ascidian or any other chordate, tyrosinase activity has been reported in ascidian embryos (Jeffrey, 1985), and Endean (1955b) has reported the presence of tyrosine-containing proteins in the cells of *P. stolonifera* through staining with Millon's reagent.

Experimental Procedures

Collection of Animals. Specimens of *P. stolonifera* were collected from rocky outcrops below the high-tide level along the shoreline near Hastings Point in northern NSW, and

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[1] Abbreviations: CTAB, cetyltrimethylammonium bromide; DOPA, 3,4-dihydroxyphenylalanine; EDTA, disodium ethylenediaminetetraacetate; OPA, *o*-phthalaldehyde; PAGE, polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; Da, dalton(s).

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Noosa in southern Queensland. The animals were kept in salt water during transport to the laboratory, where they were placed in aerated aquaria containing rocky-shore fauna and denitrifying bacteria on a bed of shingle and oyster shell to assist in maintaining a stable ecosystem. Specimens of *P. stolonifera* survived for periods in excess of 20 weeks in this environment.

Extraction of Protein. The animals were sliced through the test towards the base of the stolon exposing the major blood vessels. A depression was cut into the test of the upended animal, and the blood that welled into the hollow was collected with a Pasteur pipet into 50-mL centrifuge tubes on ice. In a typical preparation 10 animals were used, yielding approximately 190 mL of blood and 2.5 g of wet cells. The cells were centrifuged at 12000*g* for 10 min, and the plasma was discarded. The blood cells were resuspended in 100 mL of cold buffer A (0.1 M sodium acetate, pH 5, 10 mM EDTA, and 5 mM ascorbic acid). The cells were lysed in a Dounce homogenizer, nuclei and cell debris were removed by centrifugation, and the supernatant was stored at −70 °C to prevent oxidation of the material.

Sephadex G50 Chromatography. The extract, concentrated to 10 mL by ultrafiltration through a YM5 membrane, was loaded onto a Sephadex G50 column (13 × 1.2 cm) that had been equilibrated at 25 °C with buffer B (0.01 M sodium acetate, pH 5, 0.1 mM EDTA, 0.1 mM ascorbic acid, and 0.02 M sodium chloride). The material was eluted with buffer B at a flow rate of 2 mL/min. The column eluate was monitored by recording repetitive spectra using a Hewlett-Packard HP8450A diode array UV–visible spectrophotometer. Fractions containing absorption bands at about 280 and 350 nm were pooled and concentrated to 2 mL by ultrafiltration as described above.

Phenylboronate Affinity Chromatography. A Matrix gel PBA-10 column from Amicon (bed volume 5 mL) was equilibrated at 25 °C with buffer B at a flow rate of 1 mL/min. The concentrated ferreascidin fraction (1 mL) was loaded onto the PBA-10 column, and the column was washed with buffer B (5 mL), followed by buffer C (0.01 M Tris-HCl, pH 7.5, 0.02 M NaCl, 0.1 mM EDTA, 0.1 mM ascorbic acid, 1 mM sodium borate, and 0.2 M sorbitol) which elutes glycoproteins and nonspecifically bound material while inhibiting oxidation. Ferreascidin was eluted with buffer D (buffer B adjusted to pH 3.0). Any material remaining on the column was removed by washing with 0.25 M acetic acid, pH 2.5. The ferreascidin was dialyzed against buffer B at 25 °C for 12 h and concentrated by ultrafiltration to 2 mL.

Polyacrylamide Gel Electrophoresis. Because ferreascidin is precipitated by SDS, CTAB–PAGE was used as described by Mòcz and Bàlint (1984). The samples were prepared for PAGE analysis by diluting 1:1 with CTAB loading buffer (20% sucrose, 5% β-mercaptoethanol, 1.0% CTAB, and 0.1 M sodium acetate, pH 4.5). Bovine serum albumin, ovalbumin, and lysozyme were used as standards.

Sedimentation Equilibrium Studies. The protein solution (approximately 1.3 mg/mL) was dialyzed for 16 h at 4 °C against 0.1 M sodium acetate buffer, pH 4.5, and then subjected to equilibrium sedimentation at 11 000 rpm and 20 °C in a Beckman Model E ultracentrifuge: the resulting Rayleigh interference pattern was measured on a Nikon two-dimensional comparator.

For the determination of the partial specific volume, samples (75 μg) were subjected to isopycnic density gradient centrifugation in an SW41 rotor of the Beckman ultracentrifuge at 38 000 rpm for 24 h. Fractions were collected and assayed spectrophotometrically for protein. Each fraction was also analyzed refractometrically to determine the CsCl density. A partial specific volume of 0.736 mL/g was obtained by reciprocating the protein density.

Velocity Sedimentation. Linear sucrose gradients [5–20% (w/v)] were prepared in 0.1 M sodium acetate, pH 4.5, using a modification of the method of Britten and Roberts (1960), and protein samples (200 μL) were layered onto the freshly prepared sucrose gradients. Protein samples loaded included lysozyme (10 mg/mL) and bovine serum albumin (5 mg/mL) as well as ferreascidin (1.2 mg/mL). Gradients were centrifuged in a Beckman SW-41 swinging-bucket rotor at 38 000 rpm at 3 °C for 24 h. The rotor was accelerated very slowly and decelerated with the brake off to eliminate disturbances to the gradient. Fractions were collected and monitored at 280 nm using an LKB 2138 Uvicord. The distances migrated by the sample and standards were determined, and $s_{20,w}$ was calculated by reference to the standards according to the method of Martin and Ames (1965).

Carbohydrate Analysis. The carbohydrate content of ferreascidin was determined essentially by the colorimetric assay described by Dubois et al. (1956), D-glucose being used as the standard at a concentration of 35 μg/mL in Milli-Q water. The identification of the carbohydrate was made by the measurement of the ^1H and ^{13}C NMR spectra of a solution formed by the hydrolysis of ferreascidin in 6 M hydrochloric acid at 108 °C for 2 h with a Bruker CXP 300 Fourier-transform spectrometer.

Amino Acid Analysis. The lyophilized protein was hydrolyzed in vacuo at 108 °C for 48 h in 6 M constant-boiling HCl. Amino acid analysis was performed by using a Waters amino acid HPLC system with postcolumn fluorescence detection of the OPA adducts. Tryptophan was determined by the method of Simpson et al. (1976) and asparagine and glutamine by the method of Soby and Johnson (1981). Half-cystine was measured as cysteic acid after oxidation of the protein by performic acid (Moore, 1963). Disulfide analysis used the method of Anderson and Wetlaufer (1975). The Pierce-H amino acid standard to which DOPA had been added was used for quantification. The amino acid composition was calculated to give the best fit to the molecular weight. Samples of ferreascidin were also analyzed separately for DOPA and tyrosine according to the method of Waite and Benedict (1984) and Edelhoch (1962), respectively.

UV–Visible and Circular Dichroism Spectroscopy. The solution absorption spectra were measured with a Hewlett Packard 8450A diode array spectrophotometer and all circular dichroism spectra with a Jobin Yvon III Dichrograph. The UV–visible spectra of the blood cells were measured with a Shimadzu multipurpose spectrophotometer which has an end-on photomultiplier adjacent to the cell position so that the effects of scattering could be minimized. To decrease further the effects of scattering in spectral measurement of the cells, the cells were filtered to remove agglutinated cells and other large particles, and the cells were suspended in a poly(ethylene glycol) solution with a refractive index close to that of the cells (1.365): the latter was measured by the method of Barer et al. (1953).

Since the poly(ethylene glycol) solution with a refractive index of 1.365 precluded measurement of circular dichroism spectra below 300 nm, more dilute poly(ethylene glycol) solutions (with refractive indexes as low as 1.344) were used so that measurements could be made down to about 200 nm.

For the suspended cell experiments, approximately 50 mL of blood was centrifuged at 1000 rpm, and the supernatant

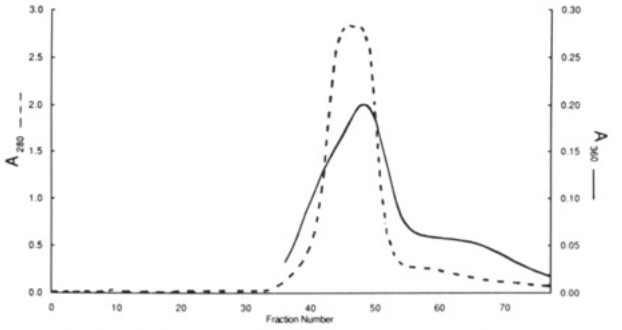

FIGURE 1: Elution profile of blood cell extract on Sephadex.

was discarded. The cells were gently shaken with about 5 mL of appropriately diluted poly(ethylene glycol) solution, and the resulting suspension, with washings, was filtered through coarse filter paper. Spectra were run immediately, and also after various time intervals up to 48 h to ensure that the spectra resulted from whole cells and not extracted compound. The whole cell spectra decreased in intensity with time as the cells settled.

RESULTS

Ferreascidin Extraction and Purification. Ferreascidin was extracted from *P. stolonifera* blood cells and purified by chromatography on Sephadex G50 and PBA-10.

The gel chromatographic behavior of the ascidian extract on Sephadex G50 monitored at 280 and 360 nm is given in Figure 1. The ferreascidin, which absorbs at 360 nm, is separated from higher molecular weight species that elute at the void volume. A comparison with standard proteins indicates that the ferreascidin elutes far behind the position expected for its molecular weight. This retarded elution behavior presumably is a consequence of adsorption to the polysaccharide matrix, a phenomenon in keeping with its high content of aromatic amino acids. Across the band containing the ferreascidin, the ratio of the absorbance at 360 and 280 nm varied, showing a lack of homogeneity. Since phenylboronate columns bind catechol-containing compounds tightly providing a simple method for separating catechol compounds (Hawkins et al., 1986), affinity chromatography of ferreascidin on Matrix Gel PBA-10 was used as an additional purification step.

The purified ferreascidin gave a single band with CTAB–PAGE (Figure 2), indicating a single species. However, the broad nature of the band indicated some microheterogeneity. This characteristic was always observed irrespective of the source of the specimen and the age or the degree of oxidation of the protein. An apparent molecular weight of approximately 20 000 is inferred from the migration rate during CTAB–PAGE at pH 3.5.

The electrophoresis of the cell lysate shows that ferreascidin is the major cellular protein, estimated to be about 27% of the total cellular protein by densimetric tracing, and greater than 90% of the protein extracted in buffer A.

Low-speed sedimentation equilibrium analysis (Van Holde & Baldwin, 1958) was carried out with ferreascidin. A molecular weight of 9800 ± 100 was estimated from the linear position of the plot of ln $j(r)$ against r^2. Furthermore, isokinetic sucrose gradient ultracentrifugation yielded a sedimentation coefficient ($s_{20,w}$) of 1.3 S, which is consistent with a value of 10 000 for the molecular weight. Some heterogeneity of the preparation is indicated by the slight upward curvature of the above plot and also by a comparison of the above molecular weight with the overall weight-average value [M_w(cell)] of 11 400. Aggregation of the 10 000-Da entity is considered to

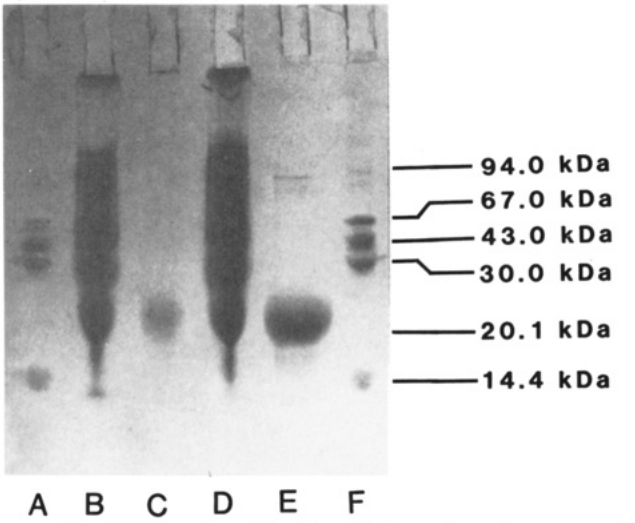

FIGURE 2: CTAB–polyacrylamide gel electrophoresis pattern of ferreascidin. Gels were stained with Coomassie blue. Lanes A and F, standard protein markers; lanes B and D, samples of crude lysate; lane C, 3 μg of purified ferreascidin; lane E, 30 μg of purified ferreascidin.

Table I: Amino Acid Composition of Ferreascidin (Residues per Hundred)[a]

amino acid	composition	amino acid	composition
Asp	1.7 (0.4)	Ile	3.6 (0.7)
Thr	2.0 (0.7)	Leu	6.3 (0.8)
Ser	2.4 (0.6)	DOPA	16.9 (2.1)
Glu	1.0 (0.4)	Tyr	41.9 (3.5)
Gly	4.4 (1.1)	Phe	8.5 (1.2)
Ala	0.5 (0.5)	His	7.4 (1.1)
Val	0.4 (0.6)	Lys	2.8 (1.0)
Met	0.7 (0.7)		

[a] Mean analysis for 12 preparations with standard deviation in parentheses.

be the most likely source of this heterogeneity.

Carbohydrate Analysis. A value of two hexose units per 10 000 Da was obtained for the carbohydrate content of the protein. The ^{1}H and ^{13}C NMR spectra of the 6 M hydrochloric acid hydrolysate were identical in all respects with the spectra of authentic D-glucose prepared under identical conditions. The amino acids were sufficiently insoluble under these conditions not to contribute to the spectra.

Amino Acid Analysis. The average amino acid composition from the analysis of 12 separate samples by HPLC is given in Table I. Over 42% of the amino acids are tyrosine and a further 17% DOPA usually considered a non-protein amino acid. Together with phenylalanine (8%), the aromatic amino acids make up two-thirds of all the amino acids. The separate spectroscopic analyses of DOPA and tyrosine gave a ratio of 1:2.5 for these residues, in agreement with values from hydrolyzed material determined by amino acid analysis.

UV–Visible and CD Spectra. Figure 3A gives the spectra of a purified solution of ferreascidin in 0.1 M acetate, pH 5.0. The absorption spectrum shows peaks at 360 and 278 nm with a ratio of 1:2.4. The former peak originates from a chromophoric group attached to the protein, and the latter from tyrosine and DOPA. The CD spectrum shows a negative band at 365 nm characteristic of the ferreascidin in its natural state. Oxidation or exposure to low pH removes this band. Positive bands occur under the aromatic region and at higher energy. Figure 3B describes the spectra of the whole cells. The CD band of the chromophoric group of ferreascidin is present slightly shifted to 350 nm. The absorption corresponding to this band is not as clearly defined as in the solution spectrum

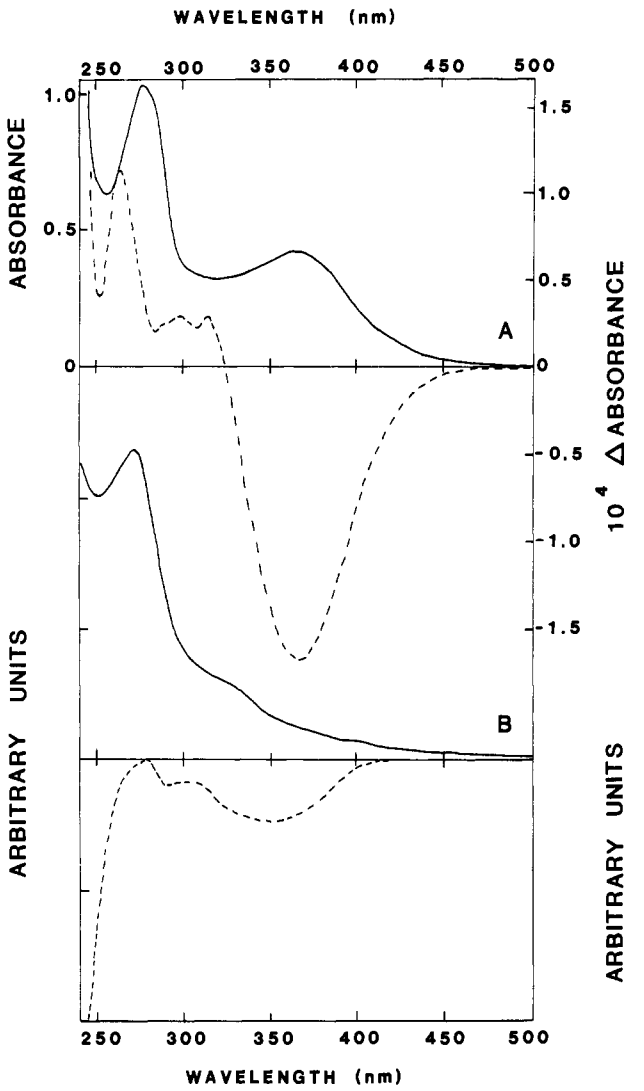

FIGURE 3: Absorption (—) and circular dichroism (---) spectra of (A) ferreascidin (A_{280} = 1.0) (1-cm cell) and (B) whole blood cells from *Pyura stolonifera*.

of the purified protein. The aromatic band is found at 271 nm.

DISCUSSION

This report describes the isolation and purification of ferreascidin, a protein from *P. stolonifera* which is remarkable not only because it contains the "non-protein" amino acid DOPA but also because it has by far the highest content of aromatic amino acids described to date for any natural protein. Aromatic amino acids make up 67% of the total content in ferreascidin. Of these, 42% are tyrosine and 17% DOPA residues. The insect arylphorin proteins, which were previously thought to be the richest in aromatic content, and which are essential to extensive exoskeleton formation accompanying metamorphosis, have in comparison up to 25% total aromatic amino acids (Wyatt & Pan, 1978), including about 10% tyrosine. High-tyrosine (11–20%) proteins have also been reported in wool (Marshall et al., 1980). No DOPA has been recorded in these proteins. A number of DOPA proteins have been identified and characterized by Waite and his co-workers. The periostracum of *Geukensia demissa* has 21% total aromatic amino acids with 11% DOPA and 15% tyrosine (Waite, 1977). Polyphenolic proteins, isolated from the "phenol gland" of mytilid bivalves, have for *Mytilus edulis* 19% aromatic amino acids with 11% DOPA and 7% tyrosine (Waite, 1983) and for *Mytilus californianis* 21% aromatic amino acids with 13% DOPA and 7% tyrosine (Waite, 1986). The soluble fraction from the periostracum of *M. edulis* has 18% aromatic amino acids, with 4% DOPA and 11% tyrosine (Waite & Anderson, 1980). DOPA proteins are also found in the byssal threads of bivalve molluscs such as *M. edulis* (Waite & Tanzer, 1980, 1981; Benedict & Waite, 1986a,b), *G. demissa*, *Atrina vexillum*, and *Atrina rigidex* (Mascolo & Waite, 1986). The DOPA proteins are involved in the formation of sclerotized tissue and in the adhesion of the animal to its substrate. Ferreascidin, which is localized in the blood cells of the ascidian *P. stolonifera*, could have analogous functions in the ascidian because it puts out stolonic threads to bind it to its substrate in the intertidal region, and there is evidence to link the blood cells with the formation of the ascidian's test (Endean, 1955b,c, 1960, 1961; Wardrop, 1970).

The pH 4.5 extraction of the blood cells produces the apoprotein and the iron(III)-coordinated protein. At this pH, the iron can be removed by dialysis with EDTA. A separate study in this laboratory of this coordination shows that the protein binds to iron progressively over the pH range 4.5–7.0. One mechanism that has been recognized for the curing of polyphenolic proteins involves the chelation of metals such as Fe^{3+} by DOPA residues (Avdeef et al., 1978).

The presence of DOPA in ferreascidin was proved by ^1H and ^{13}C NMR spectra of acid hydrolysates adjusted to pH 1.5. Lines identical with an authentic sample were present in the spectra.

The purification of ferreascidin was complicated by its facility to oxidize in slightly basic solutions and by the strength of its binding to most solid chromatographic media. Sephadex G50 successfully separated ferreascidin from some high molecular weight proteins, but the protein was retarded as if it had a molecular weight of about 1000, possibly due to the specific binding of protein to the medium. The apparent difference in the molecular weight between the value of 10000 obtained with sedimentation analysis and the value of 20000 from PAGE can also be explained by the interaction of the protein with the gel, even in the presence of CTAB. The phenylboronate affinity column provides a very efficient means of purifying ferreascidin (Hawkins et al., 1986). However, a low density of phenylboronate groups was necessary for successful elution because of the strength of the binding between ferreascidin and the affinity column, no doubt compounded by its hydrophobic nature.

The CTAB–PAGE of the purified ferreascidin showed a broad single band. The broadness of this band may be due to either (a) the binding of the protein to the polyacrylamide support due to the extreme hydrophobicity of the protein, (b) self-interaction during electrophoresis, or (c) microheterogeneity in the protein arising from multiple gene copies with some variation in sequence or in size due to processing from a polyprotein. In the latter case, it would bear some resemblance to ubiquitin which is synthesized as a polyprotein (Wiborg et al., 1985).

Because of the inherent instability of the protein to oxidation, it was important to determine whether the isolated protein was in the same form as the protein in its native state within the blood cells. The probe chosen was circular dichroism because of the observed sensitivity of the CD spectrum of ferreascidin to oxidation or denaturation. The negative CD band at about 360 nm is particularly sensitive to changes in structure, and this was monitored in the intact cells and during the various stages of isolation of the protein. Processes that removed this CD band were abandoned. The presence of other chiral compounds in the cells caused the whole cell spectrum

below 300 nm not to resemble the purified protein's CD spectrum.

Sequencing of this protein is under investigation, but insolubility of ferreascidin above neutral pH and the apparent lack of accessibility by proteolytic enzymes have hindered progress to date. However, partial acid hydrolyses have yielded a variety of fragments, which have been isolated by reverse-phase HPLC.

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Registry No. DOPA, 59-92-7.

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