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Peptide Repeats in a Mussel Glue Protein: Theme and Variations†

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ABSTRACT: The adhesive protein from *Mytilus edulis* contains 75–80 closely related, repeated peptide sequences in its primary structure. These peptides can be resolved following digestion with trypsin by reversed-phase high-pressure liquid chromatography. The most frequently repeated sequence is the decapeptide Ala-Lys-Pro-Ser-Tyr-Hyp-Hyp-Thr-Dopa-Lys (peptide E). Variations of this occur in peptides B with Hyp-3 and Dopa-5, C with Dopa-5, and D with Hyp-3, respectively. Lesser amounts of hexapeptides (A and B′) that are lacking residues 4–7 also occur. Peptide A has the sequence Ala-Lys-Pro-Thr-Dopa-Lys, whereas B′ contains Tyr instead of Dopa. 4-Hydroxyproline occurs at positions 3 and 7 and occasionally at position 6 of the decapeptide; 3-hydroxyproline occurs only at position 6. Adhesiveness of the protein may be related to the repetition of Dopa residues, the catecholic moiety of which has strong hydrogen-bonding and metal-liganding capabilities.

The marine mussel *Mytilus edulis* is a sedentary mollusk whose survival depends largely on its capacity to attach expeditiously to solid objects under water. Attachment is mediated by the byssus, a bundle of silky threads that is proximally connected to the animal by a rootlike process and distally connected to a foreign surface by adhesive plaques (Brown, 1952). The plaques contain a glue called the polyphenolic protein (Tamarin et al., 1976; Waite & Tanzer, 1980). This protein is the product of an exocrine gland located in the foot of the mussel and seems to be applied as a foam by the foot to a foreign surface (Waite, 1983a).

Research has recently focused on the chemical nature of the polyphenolic protein as well as the mussel's adhesive delivery system because much needed synthetic underwater adhesives have not yet been successfully formulated by man. Thus far, the polyphenolic protein has been identified as a highly basic protein of intermediate molecular weight (125000). Eight residues account for 90% of the amino acids in the protein, i.e., lysine, hydroxyproline, alanine, serine, threonine, proline, tyrosine, and 3,4-dihydroxyphenylalanine (Dopa) (Waite & Tanzer, 1981). Evidence for repetitive sequences in the polyphenolic protein was obtained by trypsin digestion, which suggested that more than 75 repeats of a decapeptide having the sequence Ala-Lys-Pro-Ser-Tyr-Hyp-Hyp-Thr-Dopa-Lys (Waite, 1983b) occur in the protein. Although purification of the decapeptide was ostensibly accomplished by methods based on classical ion-exchange

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chromatography and gel filtration, the sequence analysis indicated a heterogeneity of the sort Pro/Hyp and Tyr/Dopa at positions 3, 5, and 9 of the peptide. The present research was aimed at resolving this heterogeneity so as to allow some insight into the specificity of the putative prolyl and tyrosyl hydroxylases involved.

Materials and Methods

The dissection of phenol glands and extraction of proteins therefrom have been described in detail previously (Waite, 1983b). Only modifications of the original procedure are described below. Phenol glands were dissected from mussel feet over slabs of dry ice so as to make the tissue more manageable. The glands were homogenized on ice with a 50-mL ground-glass tissue grinder (Kontes, Vineland, NJ) in 50 volumes of cold 1 M NaCl with 0.05 M tris(hydroxymethyl)aminomethane (Tris) (pH 7.5) and several enzyme inhibitors described previously. The homogenate was centrifuged at 5000*g* for 10 min at 4 °C, and the pellet was rehomogenized on ice in 5% acetic acid. This was centrifuged at 35000*g* for 1 h at 4 °C. The supernatant was dialyzed for 1 h against 100 volumes of distilled water and then overnight against 200 volumes of 0.1 M sodium borate, pH 8.5 at 4 °C. The dialysis against borate induces precipitation of collagens while keeping the polyphenolic protein soluble by complex formation between borate and Dopa residues (Waite, 1984). The borate-soluble fraction was then dialyzed against 100 volumes of 5% acetic acid for 5 h and adjusted to 5.5% guanidine hydrochloride and 0.001% Triton X-100. The volume was reduced to about 10 mL by ultrafiltration under N_2 using a PM-10 membrane (Amicon Corp.). The concentrate was applied to a column (1 × 25 cm) of SE-Sephadex C-50 equilibrated with 5.5% guanidine hydrochloride, 5% acetic acid, and 0.001% Triton X-100. After a 100-mL wash with the same buffer, the column was eluted with a linear gradient up to 15% guanidine hydrochloride. Fractions were assayed for conductivity, for absorbance at 280 nm, and for Dopa with nitrite–molybdate (Waite & Benedict, 1984). Those fractions containing peaks at 280 nm and Dopa were pooled and concentrated under N_2 to a final volume of 0.7 mL. The concentrate (5 mg of protein) was dialyzed against 2.5% acetic acid and purified by preparative reversed-phase chromatography using a C_8 column (RP-300 Brownlee) with acetonitrile in water and 0.1% trifluoroacetic acid. Four milligrams of purified polyphenolic protein was recovered by this procedure.

The repeating decapeptides were prepared as follows: After removal of acetonitrile and trifluoroacetic acid by vacuum evaporation at 7 °C, purified polyphenolic protein was dissolved in 5 mL of 0.5 M sodium borate (pH 8.0) with 2 M urea. Trypsin (Boehringer-Mannheim) was added at an enzyme to protein ratio of 1:100, and digestion was carried out at 25 °C for 12 h under 25 psi of N_2. The reaction was terminated by acidification to pH 4.0 with glacial acetic acid, and the sample was flash evaporated to a final volume of 1 mL and applied to a column of LH-Sephadex 60 (1.5 cm × 70 cm) eluted with 0.2 M acetic acid. Having removed the undigested material and trypsin in this way, we purified the remaining Dopa-rich peak to apparent homogeneity on SP-Sephadex C-25 and LH-Sephadex 60 as described before (Waite, 1983b).

Further resolution of the decapeptide (3.8 mg) was achieved by reversed-phase chromatography (C_8) using a linear gradient of acetonitrile (5–11%) in water and 0.1% trifluoroacetic acid. Eluted fractions were monitored at 280 and 230 nm, and those with major peaks at both wavelengths were pooled and evaporated under vacuum for 12 h at 4 °C.

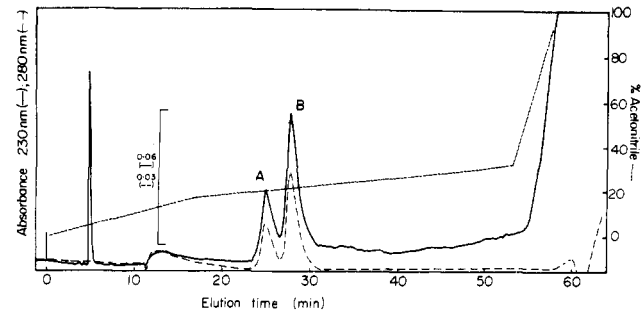

FIGURE 1: Preparative reversed-phase HPLC of polyphenolic protein prepurified by ion-exchange chromatography on SE-Sephadex. Two buffers used in the gradient system are buffer A (water with 0.1% trifluoroacetic acid) and buffer B (acetonitrile and 0.1% trifluoroacetic acid). The elution program consisted of linear gradients of 0–16% buffer B in 16 min and then increasing to 33% buffer B at 53 min. At 58 min, the column was washed with 100% buffer B.

Amino acid analysis was performed at conditions described previously (Waite & Benedict, 1984) following acid hydrolysis in 6 M HCl with 5% phenol in vacuo at 110 °C for 24 h. L-Dopa and 4-*trans*-hydroxy-L-proline standards were obtained from Sigma. 3-*trans*-Hydroxy-L-proline was supplied by Dr. Robert Trelstad *and* purified from telomycin (donated by S. Misiek of Bristol-Myers, Syracuse) following hydrolysis according to Sheehan et al. (1968).

Peptide sequencing and amino-terminal analysis were done on a Beckman 890c sequenator with the 0.1 M Quadrol program in the presence of polybrene and glycylglycylglycine, which was subjected to three precycles of Edman degradation (Ozols & Heinemann, 1982). Cleavage of the anilinothiazolinone derivatives was done with anhydrous heptafluorobutyric acid. Conversion of these to phenylthiohydantoin (PTH) derivatives was carried out in methanolic HCl, and the PTH derivatives were identified by high-pressure liquid chromatography (HPLC) on a C_{18} silane column using a 5–45% linear gradient of methanol in aqueous 0.1% acetic acid. Identification of amino acids was corroborated by amino acid analysis following hydrolysis of the PTH derivatives in HI vapor (Ozols et al., 1976). Two amino acids, serine and threonine, are usually destroyed by PTH derivatization; these are identifiable as alanine and α-aminobutyric acid, respectively, following HI hydrolysis. PTH standardization with regard to Dopa, 3-*trans*-hydroxyproline, and 4-*trans*-hydroxyproline was done with lysine–Dopa copolymer (Yamamoto & Hayakawa, 1982), free 3-hydroxyproline, and poly(4-hydroxyproline) (Sigma), respectively.

Polyacrylamide gel electrophoresis of the polyphenolic protein was performed in 5% acid–urea gels (Panyim & Chalkley, 1969).

Results

It is evident that much of the previously observed nonspecific binding of the polyphenolic protein to common hydrophilic resins, e.g., Sephadex, Bio-Gel P, Sepharose, and Sephacryl (Waite, 1983b), can be circumvented by reversed-phase HPLC without a significant loss in yield (Figure 1). Chromatography of polyphenolic protein (partially purified by ion-exchange chromatography) on a C_8 silica column yields two peaks, A and B. Peak B represents about 80% of the total protein applied. Evaluated by polyacrylamide gel electrophoresis, peak A contains contaminants of lower molecular weight and polymerized B, and B contains the two closely running bands typical of the polyphenolic protein on acid gels (polyphenolic protein is insoluble in sodium dodecyl sulfate containing so-

FIGURE 2: Polyacrylamide gel electrophoresis of HPLC peak B in acid–urea gels. Gels contained 5% acrylamide, 2 M urea, and 5% acetic acid. The tracker dye methylgreen was run off the gel for 1 h prior to cessation of electrophoresis at 0.18 mA/mm^2.

Table I: Amino Acid Composition of Polyphenolic Protein HPLC Peak B

amino acid	residues/1000	amino acid	residues/1000
3-hydroxyproline	30	methionine	0
4-hydroxyproline	118	isoleucine	7
aspartate	22	leucine	11
threonine	113	Dopa	138
serine	102	tyrosine	40
glutamate	9	phenylalanine	1
proline	81	histidine	7
glycine	18	lysine	210
alanine	81	arginine	2
half-cystine	0	total	1000
valine	10		

lutions) (Figure 2). Amino acid composition (Table I) also suggests high purity on the basis of the low levels of Gly, Asx, Glx, Cys, and Met reflecting the virtual elimination of contaminating byssal collagens. Whether the remaining detected Gly does indeed belong to the protein is not clear and demands further scrutiny. The limited degradation of polyphenolic protein by clostridial collagenase (Waite, 1983b) tends to support the presence of Gly in -Pro-Gly-X-Y sequences. The electrophoretic doublet of the polyphenolic protein is a persistent feature of its purification from bulk mussel extractions, and no attempt to separate the two components has yet succeeded. PTH-alanine is the only identifiable N-terminal amino acid of the doublet. Notably, both the Dopa and 4-hydroxyproline contents in Table I are 20% higher than those in earlier analyses (Waite, 1983b). There is some evidence (J. H. Waite, unpublished results) that the levels of Dopa and hydroxyproline present in the polyphenolic protein of individual mussels can vary by as much as 50%. The reason for this variation is not known but may apply to populations of mussels as well as individuals.

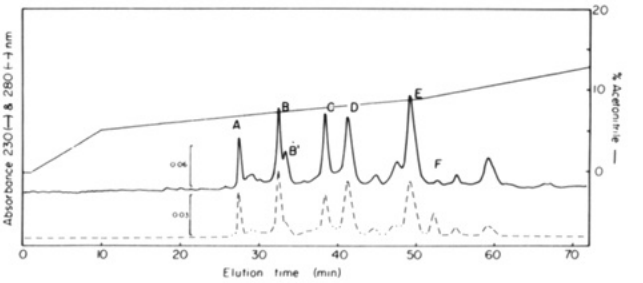

FIGURE 3: Preparative reversed-phase HPLC of trypsin-digested polyphenolic protein. Gradient was made from buffers described in Figure 1. The elution program consisted of 0% buffer B for 1 min increasing to 5% at 10 min, a second linear gradient to 9% buffer B ending at 50 min, and a third linear gradient to 13% buffer B ending at 74 min, followed by a 100% buffer B wash at 80 min.

Peptide A. Ala-Lys-Pro-Thr-Dopa-Lys

Peptide B. Ala-Lys-4-Hyp-Ser-Dopa-X-4-Hyp-Thr-Dopa-Lys

Peptide B'. Ala-Lys-Pro-Thr-Tyr-Lys

Peptide C. Ala-Lys-4-Hyp-Ser-Tyr-X-4·Hyp-Thr-Dopa-Lys

Peptide D. Ala-Lys-Pro-Ser-Dopa-X-4·Hyp-Thr-Dopa-Lys

Peptide E. Ala-Lys-Pro-Ser-Tyr-X-4·Hyp-Thr-Dopa-Lys

X is mostly 3-Hyp with occasional 4-Hyp variants

FIGURE 4: Sequence of HPLC tryptic peptides resolved by HPLC.

As reported earlier, the tryptic digestion of the polyphenolic protein results in its collapse to a single major peptide species as determined by ion-exchange and gel filtration chromatography (Waite, 1983b). Previous sequence analysis of the Dopa-containing peptide reflected anything but homogeneity, particularly in positions 3, 5, and 9 of the decapeptide. At these positions, both the prehydroxylated amino acid and its hydroxylated derivative were detected, i.e., proline and 4-hydroxyproline at position 3 and tyrosine and Dopa at positions 5 and 9. Additional purification of this heterogeneous decapeptide by reversed phase on a C_8 column produced as many as seven to eight major components (Figure 3). The amino acid compositions of six of these (Table II) illustrate distinct similarities as well as differences in the relative abundance of some of the residues. Peptides A and B′ are unique in lacking both hydroxyprolines and serine. These proved to be hexapeptides containing the first and last three amino acids of the decapeptide (Figure 4). These probably were not previously detected in the decapeptide mixture since they constitute only 18% by weight. The destruction of threonine during PTH derivatization and poor recovery of lysine would also tend to limit detection of this in the mixture. B′ clearly represents the prehydroxylated form of A. Hydroxyproline-containing versions of A or B′ have yet to be found. Peptides B–E are

Table II: Amino Acid Composition of Dopa-Containing Peptides Isolated by HPLC from Trypsinized Polyphenolic Protein

	residues/1000 (residues/peptide)						
amino acid	A	B	B′	C	D	E	pre-HPLC peptide
3-hydroxyproline	0	68 (1)	0	64 (1)	70 (1)	46	33
4-hydroxyproline	0	208 (2)	0	206 (2)	110 (1)	115	150
threonine	159 (1)	103 (1)	169 (1)	111 (1)	107 (1)	113	107
serine	0	104 (1)	0	109 (1)	115 (1)	106	101
proline	181 (1)	0	163 (1)	0	117 (1)	113	100
alanine	173 (1)	109 (1)	171 (1)	106 (1)	103 (1)	100	115
Dopa	164 (1)	176 (2)	0	106 (1)	170 (2)	110	142
tyrosine	0	0	167 (1)	86 (1)	0	92	52
lysine	322 (2)	233 (2)	330 (2)	212 (2)	208 (2)	205	210
rel abundance (% wt)	9	12	8	15	15	30	

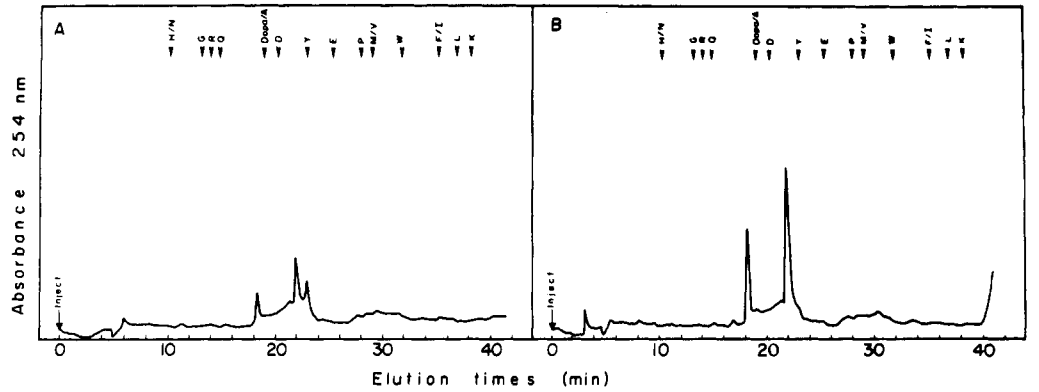

FIGURE 5: C_{18} reversed-phase HPLC of PTH cycles 6 (A) and 7 (B) of tryptic decapeptide E. Elution positions of standard PTH-amino acids are indicated overhead. Peak at 23 min is a tyrosine carry-over from the previous cycle. Absorbance range is 0.02. The 18- and 22-min peaks in cycle 6 of peptides B–D are less than one-fourth of the height shown for peptide E.

all variations on the decapeptide theme with or without additional hydroxylations at postitions 3, 5, and 9 (Figure 4). Peptide B is fully hydroxylated, having no proline nor tyrosine. Peptide C is fully hydroxylated with regard to proline; however, tyrosine-5 is not hydroxylated. Peptide D has only Dopa but is not hydroxylated at proline-3; the most abundant peptide, E, has both proline-3 and tyrosine-5. Curiously, Dopa-9 is present in all the decapeptides sequenced thus far despite the trace of tyrosine-9 detected in the mixture (Waite, 1983b). Perhaps one of the less prominent peaks in Figure 3 will prove to have a pattern of tyrosine-5/tyrosine-9, or even Dopa-5/tyrosine-9.

The patterns of hydroxylated proline in the decapeptide are more difficult to interpret because (1) hydroxyprolines are degraded by derivatization with phenyl isothiocyanate and (2) there are two isomers of hydroxyproline in the decapeptide (Table II). Amino acid analysis on sulfonated polystyrene resins is known to be able to separate all the cis and trans isomers of 3- and 4-hydroxyproline (Irreverre et al., 1963). The hydroxyprolines of the decapeptide coelute with standards for 3-*trans*-hydroxyproline and 4-*trans*-hydroxyproline by amino acid analysis. In our hands, 4-*trans*-hydroxyproline gives rise during Edman degradation to two PTH derivatives running at 18 and 22 min, which appear consistantly at position 7 of the decapeptides B–E and less frequently at position 3 (Figure 5). HI hydrolysis of the PTH derivatives leads to a single 570-nm peak coeluting with methionine by amino acid analysis. 3-Hydroxyproline, like serine and threonine, is hydroxylated on the β-carbon, and like these is predisposed to β-elimination. Standard free 3-hydroxyproline produced no detectable PTH derivatives, nor did HI hydrolysis of 3-hydroxyproline following Edman degradation yield detectable amino acids. Gryder et al. (1975) obtained similar negative results with 3-hydroxyproline by using anhydrous trifluoroacetic acid as the cleavage buffer. Thus, in sequencing a hydroxyproline-containing peptide, one would expect to see nothing at the position of 3-hydroxyproline and nothing following back-hydrolysis. As shown in Figure 5A, this is not entirely the case. Small peaks are visible for cycle 6 at 18, 22, and 23 min. The first two are indicative of 4-hydroxyproline, and the third is a carry-over from tyrosine-5. There are no detectable products following HI hydrolysis with the exception of trace tyrosine. The likely interpretation of this is that 3- and 4-hydroxyproline occur at position 6, and this heterogeneity is not resolved in peak E by HPLC of the decapeptide mixture. The 18- and 22-min peaks, however, were only barely detectable in peptides B–D. In order to corroborate this, the initial decapeptide mixture was subjected to 5 and 6 cycles, respectively, of automated Edman degradation, and the remaining peptides were recovered from the cup wash and hydrolyzed. The amino acids present in the hydrolysates are indicated in Table III. 3-Hydroxyproline occurs predominantly after cycle 5 (position 6) with some expected carry-over due to incomplete derivatization and cleavage. 4-Hydroxyproline is also associated with position 6 to some extent. These data indicate that the earlier assignment of 3-hydroxyproline to position 7 of the decapeptide (Waite, 1983b) is untenable.

Table III: Amino Acid Analysis of Mixed Dopa Decapeptides (30 nmol) Recovered from Sequenator Cupwash after Edman Cycles 5 and 6

amino acid	recovery (nmol)	
	cycle 5	cycle 6
3-hydroxyproline	8.71	1.21
4-hydroxyproline	17.13	12.80
threonine	18.32	17.90
Dopa	18.40	17.51
lysine	22.21	20.80

DISCUSSION

The adhesive polyphenolic protein consists largely of related hexa- and decapeptide repeats that contain among others the unusual amino acids Dopa, 3-hydroxyproline, and 4-hydroxyproline. Although it has not been possible to demonstrate that the repeats occur in a tandem fashion, this seems inevitable since at least 80% by weight of the intact protein is recoverable as Dopa-containing hexa- and decapeptides. The existence of other unrelated sequences in the chain termini of the intact polyphenolic protein is suggested by the amino acid composition (Table I) as well as the limited degradation by bacterial collagenase (Waite, 1983b), but this has eluded direct demonstration to date.

The origin of hydroxyproline and Dopa in this protein is difficult to explain except by posttranslational enzymatic hydroxylation. Dopa probably owes its existence to a protein-specific 3-tyrosylhydroxylase, although there is no known precedent for such an enzyme. An examination of sequences reflects an enzyme preference for tyrosine in the sequence Hyp-Hyp-Thr-Tyr or Pro-Pro-Thr-Tyr, depending on the priority of hydroxylations. Tyrosine is present almost entirely as Dopa in such sequences. Conversion to Dopa is lowered to 50% in the sequences Lys-Pro-Thr-Tyr and Lys-Pro/Hyp-Ser-Tyr. Hydroxyproline is detected as two trans isomers in sequences atypical of collagen. This distinguishes the polyphenolic protein along with elastin (Gray et al., 1973) and geographotoxins (Sato et al., 1983; Olivera et al., 1984) as the only known animal proteins with hydroxyproline in noncollagenous sequences. The putative 4-prolylhydroxylase

demonstrates specificity for the sequences Pro-Ser/Thr-Tyr and Pro-Ser/Thr-Dopa depending on the order (if any) of hydroxylation. Additional specificity is indicated by the fact that unhydroxylated proline is never detected in the second position of the sequence Hyp-Hyp-Thr-Dopa. Similarly, proline has never been detected in the first position of the same sequence. Present evidence suggests that this is the sole hydroxylation site of the putative 3-prolylhydroxylase.

The meaning of hydroxylation patterns in the decapeptide repeats is difficult to evaluate at this time because so little is known about sequence–structure relationships of such a curious assortment of amino acids. Clearly, the presence of Dopa in the repeats is an asset for adhesion since the catecholic moiety is a potent metal chelator (Kummert & Stumm, 1980; Pierpont & Buchanan, 1981) and can form irreverisible organometallic complexes on surfaces (Soriaga & Hubbard, 1982) and strong hydrogen bonds with proteins and other polar polymers (Hagerman & Butler, 1981). The impact of repeating Dopa-containing sequences in a polymer is best assessed by Silberberg (1984) as follows: "If a group which is frequently repeated along the chain possesses an interaction energy with a solid/liquid interface which is favorable, even if only slightly so, the large number of contacts that can be taken up simultaneously by the large molecule will effectively attach it to the surface." Moreover, Dopa, oxidized to *o*-quinone, may enhance the cohesive properties of the glue by way of covalent cross-links formed with lysine and cysteine (Waite, 1983c). Although the proposed cross-links have yet to be demonstrated, the byssus does contain significant amounts of an enzyme capable of converting Dopa to *o*-quinone (Waite, 1985).

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