Novel barnacle underwater adhesive protein is a charged amino acid-rich protein constituted by a Cys-rich repetitive sequence

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Barnacle cement is an underwater adhesive that is used for permanent settlement, and is an insoluble protein complex. A method for rendering soluble the cement of *Megabalanus rosa* has been developed, and three major proteins have been identified in a previous study. To survey the *M. rosa* cement proteins in a lower molecular mass range, the cement proteins were separated by reversed-phase HPLC and a previously unidentified protein named 20 kDa *M. rosa* cement protein (Mrp-20k) was found. Mrp-20k cDNA was cloned to reveal its primary structure. This cDNA was 902 bp long and encoded a 202 amino acid-long open reading frame, including 19 amino acids of the signal sequence. The molecular mass in the disulphide form was calculated to be 20357 Da and the isoelectric point of the mature polypeptide was 4.72. Mrp-20k was characterized by an abundance of Cys residues and charged amino acids. The most common amino acid

was Cys (17.5%), with Asp (11.5%), Glu (10.4%) and His (10.4%) following in order of magnitude. The alignment of the Cys residues indicated the primary structure of this protein to consist of six degenerated repeats, each about 30 residues long. Mrp-20k has no intermolecular disulphide bonds and no free thiol groups of Cys in the insoluble cement complex. Abundant Cys is thought to play a role in maintaining the topology of charged amino acids on the molecular surface by intramolecular disulphide-bond formation. The possible function of abundant charged amino acids, including the interaction with a variety of surface metals on the substratum, is discussed.

Key words: adsorption, cement, metal, sessile organism, settlement.

INTRODUCTION

Various sessile organisms abound in the marine environment, some of them producing a proteinaceous adhesive for their permanent attachment to rocks and other surfaces. Barnacles have long intrigued researchers for their fouling activity and their capability for tenacious underwater adhesion to the substratum by a proteinaceous cement substance [1,2]. The insolubility of the underwater adhesive after its secretion on to an attachment site and the unavailability of a specific method for measuring the underwater adhesive's activity have been obstacles to analytical studies on the substance. Some researchers have attempted to render soluble the insoluble cement, and have noticed the suitability of a reductive treatment [3–5]. An extended method has recently been developed to render soluble more than 90% of *Megabalanus rosa* cement, and three major proteins, named 100 kDa *M. rosa* cement protein (Mrp-100k), Mrp-52k and Mrp-68k, have been successfully identified by electrophoretic separation [6] (the cement proteins are referred to by the prefix Mrp followed by the apparent molecular mass, estimated by SDS/PAGE). Mrp-100k and Mrp-52k are characterized by their high hydrophobicity and insoluble nature after being secreted to the attachment site, and Mrp-68k is characterized by its unusual amino acid composition, 57% of the total residues being constituted by Ser (16%), Thr (14%), Gly (15%) and Ala (12%) [7]. The primary structure of Mrp-100k has been deduced from its cDNA sequence [6]. Underwater bio-adhesives successfully solve several problems; e.g. they prevent random aggregation at the gland [8,9], retain solubility during their transportation to the site for attachment, displace bound water

at the attachment site and undergo condensation during their interaction with the substratum and subsequent curing [10]. It is suggested that each cement protein fulfils a distinct and specific role in underwater adhesion, and that firm barnacle adhesion is achieved co-operatively by these cement proteins. Understanding the specific role of each cement protein will help to provide a better understanding of barnacle settlement and of synthetic polymer mimics, including underwater adhesives. Identification and characterization of all the cement components would be a major step forward.

In our previous study [6], *M. rosa* cement was separated into three fractions according to solubility: guanidinium chloride (GdmCl)-soluble fraction 1 (GSF1), GdmCl-soluble fraction 2 (GSF2) and a GdmCl-insoluble fraction (GIF). In that study, only SDS/PAGE separation was employed to identify the components in the *M. rosa* cement, and the investigation of lower-molecular-mass cement proteins was insufficient. In the present study, the separation of GSF1 by reversed-phase HPLC (RP-HPLC) indicated the presence of a previously unidentified cement protein. We report here that this novel 20 kDa cement protein, named Mrp-20k, is rich in charged amino acids, and consists of six repeats of a Cys-rich sequence.

EXPERIMENTAL

Chemicals

All chemicals were of the highest grade available, and most were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Takara Shuzo Co. (Shiga, Japan).

Abbreviations used: Mrp, *Megabalanus rosa* cement protein; GSF1 and GSF2, guanidinium chloride-soluble fractions 1 and 2 of *M. rosa* cement; RP-HPLC, reversed-phase HPLC; GdmCl, guanidinium chloride; MALDI-TOF, matrix-assisted laser-desorption ionization–time of flight; sp185, 185 kDa silk protein; Mgfp2, *Mytilus galloprovincialis* foot protein 2.

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The nucleotide sequence data reported have been submitted to the DDBJ, EMBL, GSDB and GenBank Nucleotide Sequence Databases under the accession number AB035415.

Purification and characterization of Mrcp-20k

GSF1 was prepared from the cement of *M. rosa* as described previously [6]. Cement of *M. rosa* was suspended in a 10 mM sodium phosphate buffer (pH 6.0) containing 6 M GdmCl, and the suspension was centrifuged at 200 000 *g* for 1 h at 20 °C. The protein fraction in the supernatant was GSF1, which was separated by RP-HPLC in a 3.9 mm-diameter $\times$ 150 mm μ -Bondasphere column (C_8 , 300 Å; Waters). SDS/PAGE [11] was used to confirm the purity and for mobility-shift analysis after adding some bivalent metals. The content of Mrcp-20k in *M. rosa* cement was estimated by using a Non-interfering Protein Assay[™] kit (Takara Shuzo Co.) for commercial protein quantification. Mrcp-20k was reduced and alkylated with dithiothreitol and 4-vinylpyridine [12] for the sequencing and the MS measurement. After the alkylation process, excess chemical was removed by dialysing against 2.5% (w/v) SDS/0.03 M Tris/HCl buffer (pH 6.8) in the dark at ambient temperature, the product being electrophoresed and electrophoretically transferred on to a PVDF membrane (Pro-Blott, PE-Biosystems). The excised band was applied to protein sequencing with a PSQ-2 protein sequencer (Shimadzu, Kyoto, Japan) for sequencing of non-alkylated Mrcp-20k or a Procise 494 cLC sequencer (PE-Biosystems) for sequencing of alkylated Mrcp-20k. MS measurement was attempted by RP-HPLC as already described, after removing excess chemical used for alkylation, although the recovery of the protein was extremely poor. The exact molecular mass was determined by matrix-assisted laser-desorption ionization–time-of-flight (MALDI-TOF) MS with a Voyager-DE STR instrument (PE-Biosystems) incorporating a 337 nm nitrogen laser operated in the linear mode at an acceleration voltage of 20 kV. Saturated sinapinic acid dissolved in 30% (v/v) acetonitrile containing 0.05% (v/v) trifluoroacetic acid was used as the MALDI matrix. The MS data were calibrated with myoglobin and adrenocorticotrophic hormone-(7–38) (Sigma).

The sample used for investigation of free thiol groups in native Mrcp-20k was prepared from freshly collected cement [7] suspended in a solution consisting of 0.1 M EDTA, 6 M GdmCl and 0.5 M Tris/HCl buffer (pH 6.8). This was allowed to stand overnight at 4 °C and then alkylated with 4-vinylpyridine and no reagent for reduction at ambient temperature for 4 h in the dark [12]. GSF1 was recovered by centrifugation and then dialysed against 0.1% (v/v) acetic acid in the dark at 4 °C. After the alkylation treatment, Mrcp-20k was purified by RP-HPLC as already mentioned and then analysed by MALDI-TOF MS to detect the increase in mass. A mobility-shift analysis was conducted by SDS/PAGE with the addition of different metal ions, as described by Kröger et al. [13], to investigate the metal-binding ability of Mrcp-20k. Mrcp-20k was incubated in the presence of a 10 mM solution of each salt at ambient temperature for 30 min before being subjected to SDS/PAGE by pretreatment at ambient temperature, but without addition of reducing reagent. Four bivalent cations, Mg^{2+} , Mn^{2+} , Ca^{2+} and Zn^{2+} , were used, and the effect compared with an EDTA treatment as the control.

Isolation of mRNA

Barnacles (*M. rosa*) measuring about 4 cm in diameter at the calcareous base were collected from Miyako Bay in Iwate Prefecture, Japan. Total RNA was extracted from the basal tissue of the barnacle using a Total RNA Separator Kit (Clontech). Poly(A)⁺ RNA was isolated by using oligo(dT)-latex Super (Takara Shuzo Co.). cDNA was prepared from *M. rosa* mRNA with a Zap-cDNA synthesis kit (Stratagene) according to the instructions of the supplier.

Preparation of the probe

The fully degenerate sense primer, 5'-CAY GAR GAR GAY GGN GTN TG-3', and antisense primer, 5'-TT YTT NGC YTC RCA RTC RAA-3', were designed from the N-terminal sequence of Mrcp-20k. Position X in the amino acid sequence was supposed to be a Cys residue. The probe for screening the cDNA library was generated by PCR using these primers. The 100 μ l reaction mixture, containing 3 μ g of each primer, 200 μ M dNTPs, 1 $\times$ *Tth* buffer, 4 units of *Tth* DNA polymerase (Toyobo, Osaka, Japan) and 0.3 μ g of *M. rosa* cDNA, was subjected to amplification by 29 thermal cycles of 95 °C for 1 min, 58 °C for 30 s and 70 °C for 2 min. The PCR products were resolved by electrophoresis with 3% (w/v) NuSieve 3:1 agarose gel (Takara Shuzo Co.), and a product of the expected size (110 bp) was purified from the gel. The 110 bp DNA fragment was subcloned in the pT7 Blue T-Vector (Novagen). The 110 bp DNA amplified by PCR was labelled with [α -³²P]dCTP by using a random-primer DNA-labelling kit (Takara Shuzo Co.), with oligonucleotide primers 5'-CACGAGGAGGACGGAGTGTG-3' and 5'-TTC-TTGGCTTCACAGTCAAA-3' complementary to the 110 bp DNA being used for priming instead of the random hexameric primers. The labelled probe thus obtained was used for screening of the *M. rosa* cDNA library.

Screening of the cDNA library

An oligo(dT)-primed cDNA library, which had been prepared from 5 μ g of mRNA from *M. rosa* basal tissue, was constructed by using the λ Zap II cloning kit (Stratagene). The library (7×10^5 plaques) was screened with the DNA probe already described. Ten positive clones were picked up, and the cDNA inserts were subcloned into the multicloning site of pBluescript II SK(–) according to the manufacturer's specification for the ExAssist system (Stratagene). The molecular size of each insert was determined by agarose-gel electrophoresis after digestion with an appropriate restriction enzyme.

DNA sequencing and computer analysis

The 110 bp DNA fragment amplified by PCR and the Mrcp-20k cDNA corresponding to the longest cDNA insert of the 10 positive clones were sequenced from both strands with a PRISM dideoxy sequencing kit and 373A DNA sequencer (PE-Biosystems). A homology search was performed on the SwissProt and Protein Information Resource databases by using the FASTA or BLAST programs. The isoelectric point [14] was predicted by using Genetyx-Mac version 7.0.1.

RESULTS

Purification and characterization of Mrcp-20k

Mrcp-20k was eluted from the reversed-phase column at an acetonitrile concentration of approx. 26% (v/v) (Figure 1). Mrcp-20k purified by RP-HPLC gave a single band by SDS/PAGE after being treated with a reductant (Figure 2). SDS/PAGE without reduction resulted in Mrcp-20k giving a band of slightly lower apparent molecular mass than that with reduction. After the reduction and pyridylethylation processes, removing the excess chemicals gave extremely poor recovery of the protein, except for co-existing SDS, probably due to the increase in hydrophobicity of the protein by the alkylation of abundant Cys. Carboxymethylation similarly gave poor recovery of the protein. The molecular mass of native Mrcp-20k was determined to be 20373 Da by MS. Pyridylethylated Mrcp-20k gave

Figure 1 RP-HPLC pattern for GSF1

Chromatographic conditions: column temperature, ambient; flow rate, 0.7 ml/min. The column was developed with a linear 30 min gradient from 0 to 40% solvent B, where solvent A was 5% (v/v) acetonitrile containing 0.06% (v/v) trifluoroacetic acid, and solvent B was 80% (v/v) acetonitrile containing 0.052% (v/v) trifluoroacetic acid. Absorbance was measured at 215 nm. The major peak at approx. 21 min corresponds to Mrcp-20k and was collected.

Figure 2 SDS/PAGE pattern for Mrcp-20k with and without pretreatment with a reductant

SDS/PAGE (16.5% T/3% C) was used for the analysis of Mrcp-20k, where T denotes the total percentage concentration of both monomers (acrylamide and bisacrylamide) and C denotes the percentage concentration of the crosslinker relative to the total concentration T. Lanes 2 and 3 show Mrcp-20k with and without the reductive pretreatment respectively. Lane 1 shows molecular-mass standards (Bio-Rad). Numbers on the left indicate molecular mass (kDa).

a molecular mass of 23707 Da, and the alkylation of all Cys residues gave a molecular mass of 23689 Da. The N-terminal amino acid sequence of HEEDGVCNSNAPCYHCDANGEN-XSXNXLFDXEAKKP was detected by directly sequencing Mrcp-20k, X most likely having been Cys. The content of Mrcp-20k was estimated to have been at least 2% in lyophilized *M. rosa* cement. This protein was not detected in GSF2, which was the fraction rendered soluble from reductive treatment of the precipitate after removing GSF1 from the *M. rosa* cement [6]. Mrcp-20k that had undergone pyridylethylation of the thiol group without reduction treatment was co-eluted with untreated Mrcp-20k by RP-HPLC. The MS data indicated that the molecular mass of pyridylethylated Mrcp-20k without reduction also agreed with that of non-pyridylethylated Mrcp-20k. No mobility shift could be detected by SDS/PAGE after pretreating with the cations Mg^{2+} , Mn^{2+} , Ca^{2+} and Zn^{2+} when compared with the mobility by EDTA treatment.

Cloning of Mrcp-20k cDNA

The 110 bp DNA was amplified from *M. rosa* cDNA by PCR with the pair of PCR primers designed according to the N-terminal amino acid sequence of Mrcp-20k. The deduced amino acid sequence of the 110 bp cDNA was identical to the N-terminal amino acid sequence of Mrcp-20k. About 30 positive clones were obtained by screening the *M. rosa* cDNA library with ^{32}P -labelled copies of the 110 bp cDNA as a probe. Ten randomly selected clones were subcloned into pBluescript SK(–), each having DNA inserts of about 0.9 kb in size. Restriction-endonuclease analysis showed that they were almost identical (results not shown). A plasmid containing the largest cDNA insert was selected for sequencing.

Structural outline of the Mrcp-20k cDNA and encoded polypeptide

The sequence of Mrcp-20k cDNA determined in this study was 902 bp long (Figure 3). The coding region consisted of 202 amino acids. The first 19 amino acids had high hydrophobicity and are thought to comprise the signal peptide. The mature N-terminal sequence was confirmed from residue 20 onwards. The molecular mass and isoelectric point of mature Mrcp-20k were calculated to be 20357 Da and 4.72, respectively. All Cys residues were calculated to be of disulphide-bond form in the prediction of the mass. The alignment of the Cys residues indicates that the primary structure of Mrcp-20k consists of six repeated sequences (Figure 4), with the first four repetitive units having six Cys residues, and the last two units having four Cys residues. We have divided each repetitive unit into two regions to represent the sequence motif. Region I basically included three Cys residues. The interval between the first and second cysteines was five amino acids in all but the second unit, which had 12 amino acids in this interval. The interval between the second and third Cys residues was two amino acids, these usually being the basic amino acids, such as Arg or His, although Tyr and Trp were sometimes present. In the last two units, the third Cys was not present. Pro preceding the second Cys was invariant in all units. Region II of each unit represents the Cys-Xaa-Cys-Xaa-Cys motif. Although the amino acid at position Xaa was variable, Ser and charged amino acids were common. The motif Asn-B-B-His (in which B is Glu or Asp) was also found before the second Cys in three units. The lengths and sequences before region I and between regions I and II were variable, and clusters of such charged amino acids as HEED, EAKKPD, HEEDDGD, EHSHDHHDDD, DLKHD and EED were sporadically present. The insertion of such charged amino acid clusters seems to have caused degeneration of the alignment of each unit. In the last two units, Cys residue numbers 3 and 4, present in the first four units, were absent. The most abundant amino acid was Cys (17.49%), with the charged amino acids Asp (11.48%), Glu (10.38%) and His (10.38%) following. The charged amino acids Asp, Glu, His, Lys and Arg are estimated to comprise 40% of the total residues. A homology search analysis on the SwissProt and Protein Information Resource databases indicated limited similarity to the 185 kDa silk protein (sp185) of *Chironomus tentans*, which has Cys-Xaa-Cys-Xaa-Cys motifs approximately every 22–26 residues [15]. Partial similarity with metal-binding proteins such as metallothionein and with His-rich calcium-binding proteins was also suggested. Some partial amino acid sequences have been reported for the cement of *Balanus perforatus* [16] and *B. eburneus* [5], although no sequences similar to those of Mrcp-20k were found.

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      10      20      30      40      50      60      70      80
GAATTCGGCAGGAGCCGCCATCCACAACGAGCACCATGAAGTGGTTTCTGTTTGTAAACACCGCTGTCTGGCGGCA
      M K W F L F L L T T A V L A A
GTTGTCTCGGCGCACGAGGAGGACGGAGTGTGTAACTCTAACGCCCCGTGCTACCACTGCGATGCGAACGGCGAGAACTG
V V S A H E E D G V C N S N A P C Y H C D A N G E N C
CAGCTGCAACTGTGAACCTTTGACTGTGAAGCCAAGACCGGACGGATCGTACGCCACCCCTTGCCGCGCTGTGAGC
S C N C E L F D C E A K K P D G S Y A H P C R R C D A
CGAACACATCTGCAATGCAGCTGCACCGGATTTCGTGCAACGAGGATCACCCGTGCCATCACTGTACAGGAGGAGAC
N N I C K C S C T A I P C N E D H P C H H C H E E D
GATGGTGATACTCACTGTCACTGTTCTGTGCGAGCATTCCCATGACCACACGATGACGACACGACGGGAATGTACCAA
D G D T H C H C S C E H S H D H H D D D T H G E C T K
GAAAGCGCGTGTGGAGATGCGAGTATAATGCCGACCTCAAGCATGATGTTTGGCGCTGTGAGTGCAGCAAGTTGCCAT
K A P C W R C E Y N A D L K H D V C G C E C S K L P C
GTAAACGACGACCCCTGTTACCGAAAGGAAGGAGCGTGTGAAGTTGTGACTGCAAAACAATCATGTAAACGAAGAT
N D E H P C F Y R K E G V V S C D C K T I T C N E D
CATCCATGCTACCATTCATATGAAGAAGACGGTGAACGAAGTCAAGTGTGACTGCGAGCACTCACCTGGCCCTTCAGA
H P C Y H S Y E E D G V T K S D C D C E H S P G P S E
ATAAGCCTTGCAGTAAGAACAGAGGCAACGGAATGAAGCACGCCAGCAAAACAGGCAGTGACACTTGGGAACGTATATTT
*
CTGACGTCAATGAGTGTGGCTTCTCTTTTCGTAATAATTCTATACATTTGTTGAGCATGGGTATTAGAACTACGATGCT
TTTGTGTGACCACGACTTAGGCAATTGTGTATGCCGACGATTTTATCTAGCTTGCACTGCTTCGTTTCATGAAATAATAAA
AATTGCGGGTTTCAGGTCAAAAAAAAAAAAAAAAAAAAA

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Figure 3 Nucleotide and predicted amino acid sequences of Mrsp-20k

Nucleotides are numbered above the sequence. The termination codon, TAA, is indicated by an asterisk. The putative poly(A)⁺ site is shown by a dotted underline. The N-terminal amino acid sequence of Mrsp-20k, as determined by the results of the protein sequence analysis, is underlined.

Figure 4 Representation of the repetitive unit of Mrsp-20k by the alignment of Cys residues

The alignment of Cys residues indicates six repetitions in the Mrsp-20k sequence. All the Cys residues are boxed in black. Charged amino acids clusters and the motif Asn-B-B-His are shown with solid and dotted underlines, respectively.

DISCUSSION

SDS/PAGE, with the addition of 6 M urea, was used in the previous study [6] to identify the major cement proteins due to the high capacity to keep the proteins soluble during the process of separation. Although the three proteins Mrsp-100k, Mrsp-68k and Mrsp-52k were identified as the major constituents of *M. rosa* cement, no cement proteins of lower molecular mass were apparent in that study. In the present study, however, a further component, Mrsp-20k, was identified by RP-HPLC investigation of GSF1 from *M. rosa* cement.

Although the function of Mrsp-20k in underwater adhesion is unclear, abundant Cys residues and charged amino acid contents are an unusual and notable characteristic of this protein. Mrsp-20k was recovered from the insoluble cement complex without reduction treatment, gave a band corresponding to the monomer

without reduction treatment and MS measurement gave the mass of the monomer. Mrsp-20k thus has no intermolecular disulphide bonds after being secreted at the attachment site. This absence of thiol groups in the Cys residues was also suggested by the lack of any reaction with an alkylating agent without reduction treatment, although the presence of hidden thiol groups cannot be discounted. The abundant Cys residues are likely to form intramolecular disulphide bonds. The SDS/PAGE data show that Mrsp-20k with native disulphide bonds migrated faster than Mrsp-20k with reduced disulphide bonds, and this might be indicative of native Mrsp-20k being a compact molecule. It can be predicted from the cDNA that Mrsp-20k has abundant hydrophilic amino acids and a small number of hydrophobic amino acids. These hydrophilic amino acids would be enhanced by being placed on the surface of such a compact molecule. The elution of Mrsp-20k at a low concentration of organic solvent from the reversed-phase column might support this proposition. The abundant disulphide bonds may thus play a role in maintaining the topology of the charged amino acids on the molecular surface in an extracellular environment. An even number of Cys residues was maintained in each repetitive unit of Mrsp-20k, even in the last two, which may indicate that disulphide bonds were formed as intra-repetitive unit cross-links.

We have no data to indicate the oxidation state of this protein in the secretory organs. The Cys-Xaa-Cys-Xaa-Cys motif in region II of Mrsp-20k is known to be repeated in sp185 of the aquatic midge larvae of *Chironomus* species, which is used in the construction of the larval tube. It has also been proposed that sp185 may prevent random aggregation of other components in the gland [15]. The contribution of disulphide bonds to stabilizing Mrsp-100k and Mrsp-52k in the insoluble cement complex has been indicated [6], although it is not known whether disulphide bonds are formed intermolecularly or intramolecularly. Such a complex may be stabilized after being secreted at the attachment site. It is also suggested that Mrsp-20k could play a role in preventing other components, such as Mrsp-100k and Mrsp-52k, from randomly aggregating before their secretion from the gland.

The abundance of charged amino acids is another extreme characteristic of Mrcp-20k that might be significant for the role of this protein in underwater adhesion. Although the homology search analysis suggests that abundant charged amino acids have the potential to interact with metal ions, the mobility-shift analysis by SDS/PAGE suggests no strong ability towards the bivalent cations Mg^{2+} , Mn^{2+} , Ca^{2+} and Zn^{2+} . However, this result does not preclude an ability for weak interaction with a variety of metal ions, which would be required for good underwater adhesion to a range of substrata. Holding of some metals by cement has been reported [2,17], so the interaction with surface metals on a substratum could be a role of the abundant charged amino acids in Mrcp-20k. The similarity between Mrcp-20k and *Mytilus galloprovincialis* foot protein 2 (Mgfp2) could be noteworthy [18]. Mgfp2 is the major component of the adhesive plaque that anchors mussels to the substratum in water, and its primary structure consists mainly of 11 repetitions of an epidermal growth factor-like motif in which six Cys residues are conserved. None of the amino acid sequences implicated in the role of cell–cell interaction of the epidermal growth factor family were conserved in Mgfp2. Both Mrcp-20k and Mgfp2 contain six Cys residues in each repetitive unit. Clusters of acidic amino acids are also present in Mgfp2, and some of the Tyr residues are converted to dopa by a peptidyl tyrosyl hydroxylase, which has an affinity for multivalent metals [19]. It has been suggested that Mgfp2 undergoes protein aggregation due to reduction of the negative charge of the acidic cluster by the positive charge of basic amino acids in its central region. Although no sequence similarity was found in this study, the functional relationship in underwater adhesion between these two proteins should be noted.

Mrcp-20k had characteristics distinct from the other three major cement proteins, Mrcp-100k, Mrcp-52k and Mrcp-68k. This means that barnacle underwater adhesion should be explained by the product of the functions of these proteins. This study has identified a novel cement protein, thus providing further functional analysis to elucidate the phenomenon of barnacle underwater adhesion.

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