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Marine Adhesive Proteins and Some Biotechnological Applications

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Introduction

For more than 5000 years the human race has used adhesive materials from nature as glues. Since the late 1930s, when synthetic polymeric materials were prepared by Carothers of du Pont, many kinds of synthetic petrochemical adhesive materials have taken the place of natural glues such as gelatin, starch, Arabian rubber, urushiol and so on. In some cases, rosin and polysaccharide glue makers to name but two, industries have been dealt a death blow.

Nature's powerful adhesives, secreted by marine mollusks such as mussels, oysters, and barnacles, which must routinely cope with the force of surf and tides, are simple proteins. These proteins insolubilize and adhere to the surfaces of a variety of substrates, such as rock, glass and plastics, in a watery environment. Some of them have been identified as L-β-3,4-dihydroxyphenyl-α-alanine (Dopa)-containing proteins (IUPAC, 1972), and they are also rich in Lys and Gly. The proteins are designated marine adhesive proteins and their adhesive properties have been investigated with regard to their use as adhesives for biotechnological (medical and dental) purposes (Waite, 1986; Benedict and Picciano, 1989), suggesting many challenging problems of bioadhesive formulations.

The research methods for marine adhesive proteins can be classified into the following nine approaches: (1) Phenomena and morphology in nature; (2) Purification and analysis of natural proteins; (3) Synthesis of adhesive proteins and their analogues (a) chemical synthesis, and (b) gene technology; (4) Insolubilization reactions and mechanisms; (5) Conformation and hierarchy; (6) Preparation of biohydrogels and biodegradation; (7) Surface characteristics; (8) Adhesive characteristics and bioadhesives; (9) Antifouling.

Apart from the adhesion and adhesives, which are of interest to chemists and

Abbreviations: Boc, N-α-*t*-butyloxycarbonyl; Z, benzyloxycarbonyl; Nps, *o*-nitrophenylsulfenyl; OMe, methyl ester; OEt, ethyl ester; ONp, *p*-nitrophenyl ester; Bzl, O-benzyl; Tos, *p*-toluenesulphonyl; DCCI, dicyclohexylcarbodiimide.

Biotechnology and Genetic Engineering Reviews – Vol. 13, December 1995
0264–8725/94/12/1–38 $20.00 + $0.00

material scientists, protein model compounds with simpler amino acid composition and repeated sequences have been investigated since the early 1950s for such medical purposes as antigenicity, antiviral, antibacterial, and antitumor activities, and growth-inhibitory effects, and also for practical uses such as synthetic fibers, water- and gas-permeable membranes, and artificial skins.

Several excellent reviews have been published by Waite (University of Delaware) who is a pioneer marine adhesive biologist and biochemist (Waite, 1990, 1992a). The objective of the present review is to describe the marine adhesive proteins and some biotechnological applications from the viewpoint of both polymer chemical and material science, summarising the above nine items in the order they appear above.

Phenomena and morphology in nature

Marine adhesive proteins secreted from invertebrates, such as mussels and barnacles, insolubilize and adhere to the surfaces of a variety of substrates in seawater. As early as the late 1940s a morphological study had been started by Brown (1949), and the structure and formation of the byssus attachment disc in *Mytilus* has been investigated in detail, using scanning electron microscopy, by Tamarin, Lewis and Askey (1976). The marine adhesive protein was also known as the polyphenolic protein, since the protein is produced and stored in the phenol gland of the mussel (Allen *et al.*, 1976).

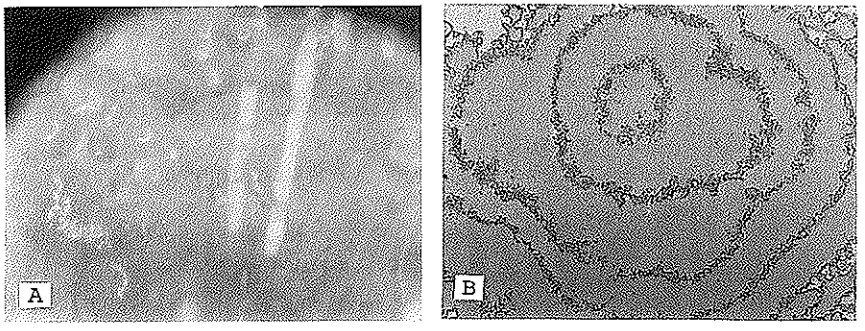


Figure 1. Barnacle attachment by the cement proteins. **A** droplets secretion by *Balanus eburnecus*; **B** cross section of the cement proteins of *Balanus albicostatus*. (Hematoxylin-eosin stained).

Historically, two different secretion and adhesion patterns have been observed and reported. We named the two patterns as (a) successive droplet secretion type and (b) thread (byssus) secretion type (Yamamoto, 1994). *Figure 1* and *Figure 2* show the two patterns. Barnacles secrete liquid droplets in a similar manner to a human perspiring, and as the barnacles mature, they secrete a greater amount of droplets in order to maintain their bigger shells (arthropod), exhibiting tree-like growth rings. Two typical barnacle photographs are shown in *Figure 1* (Taken by Dr Hiroichi Tsukamoto of Kobe Municipal Suma Aquarium). As for the thread secretion type, though the common blue mussel *Mytilus edulis* is well known, here the author shows the disc of the pearl oyster *Pinctada fucata* attached (a) to a glass wall and (b) to granite by the byssus in simulated sea water in this laboratory (*Figure 2*). Each byssus is secreted in 10 min in dark conditions.

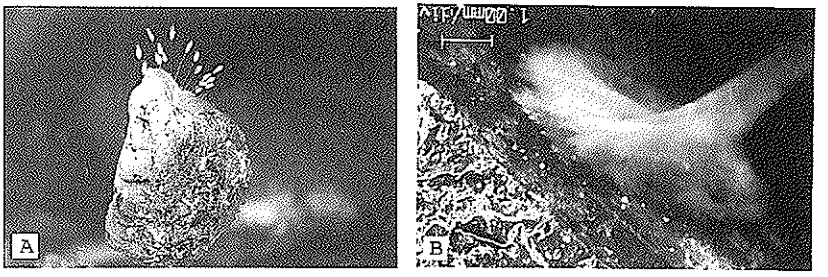


Figure 2. Pearl oyster attached **A** to glass wall and **B** on granite by the byssus.

Purification and analysis of natural proteins

The purification and chemical characterization of the amino acid Dopa from the periostracum was first reported by Waite and Anderson (1978). Two years later, Waite and Tanzer (1980) identified significant amounts of Dopa in hydrolysates of *Mytilus* byssal adhesive discs. More than three years later, Waite (1983) purified the *Mytilus* adhesive protein by a combination of ion exchange on sulfonylpropyl-Sephadex and gel filtration on low surface energy chromatographic media. From polyacrylamide gel electrophoresis, he found that the protein consists of two components, having a molecular weight of about 130 000. Tryptic digestion of the protein resulted in extensive degradation and the major tryptic peptide (80%) was found to contain a deca-repeated sequence of (Ala-Lys-Pro-Ser-Tyr-Hyp-Hyp-Thr-Dopa-Lys)$_{75}$. These outstanding results opened a new era of marine sessile material science. The initial finding has been expanded as peptide repeats were found in a mussel glue protein – theme and variations – (Waite, Housley and Tanzer, 1985). Following that, the primary sequences of some of the adhesive proteins have been determined (Waite, 1990). From the amino acid sequences of polyphenolic proteins, molecular diversity of marine glues and some structural motifs have been proposed (Rzepecki *et al.*, 1991).

Later, Chilean researchers (Burzio *et al.*, 1990; Pardo *et al.*, 1990) purified the adhesive polyphenolic proteins from the Chilean mussels (*Mytilus chilensia* and *Choromytilus chorus*) using a modification of the previous procedure described by Waite (1983). The major protein component in the shell matrix of the American oyster, *Crassostrea virginica*, was purified chromatographically and was shown to be highly acidic by analysis, which found 90% of the amino acids were comprised of Asp, O-phosphoserine and Gly in approximately equimolar amounts, showing an amino-terminal sequence consisting of NH_2-(P)Ser-Gly-(P)Ser-Gly-{(P)Ser},-Ile. . .-COOH (Rusenko, Donachy and Wheeler, 1991). More recently, one particular protein of 39 kDa, found in the cement of *Balanus perforatus*, was reported to have the *N*-terminal octadeca sequence, NH_2-Thr-Tyr-Phe-Pro-Val-Leu-Ser-Tyr-Gly-?-Ser-Ser-Ser-Leu-Ala-Pro-Val-Ile-, where the ? is mostly cysteine (Naldrett, 1993). In Asia, the adhesive proteins of the pearl oyster *Pinctada fucata* were purified (Yamamoto, Ikeda and Ohkawa, 1993). Most recently, a bioscience laboratory in Sweden purified a mussel adhesive protein from the common blue mussel *Mytilus edulis* following Waite's earlier work and intends to introduce two products on the Scandinavian market, and in Germany and Switzerland (private communication).

Most recently, the primary structures of the adhesive proteins of three mussels (*Mytilus edulis, Mytilus galloprovincialis, Mytilus trossulus*) and a barnacle (*Megabalanus rosa*) have been determined using a cDNA gene cloning method (orally presented at the 4th Marine Biotechnology Symposium, May 1995, Tokyo University of Fisheries) by researchers in the Marine Biotechnology Institute in Japan.

Synthesis of adhesive proteins and their analogues

Two approaches, polymer chemical and gene technology, provide competing strategies for preparing these marine adhesive proteins.

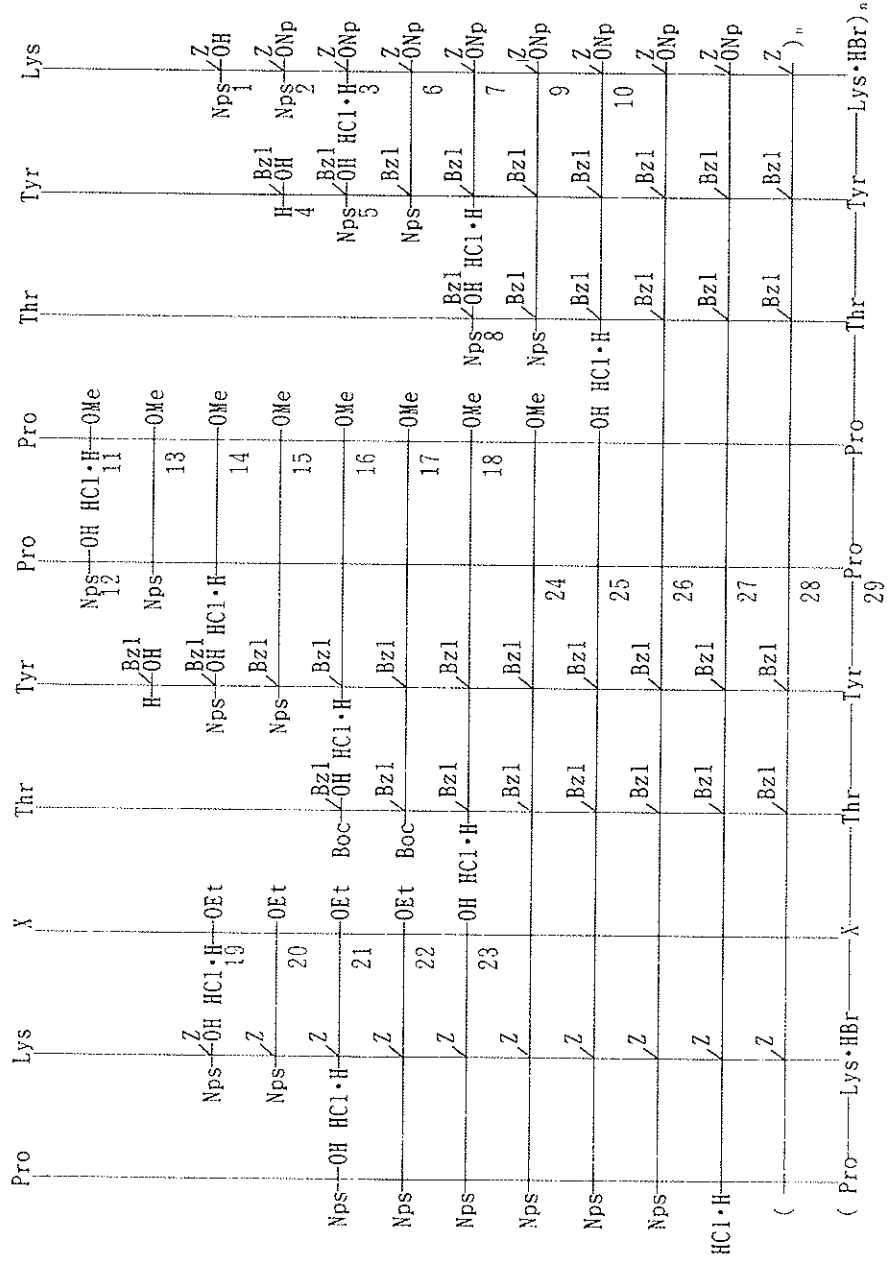
As early as 1976, synthesis of poly(Dopa) was accomplished (Yamamoto and Hayakawa, 1976, 1977, 1982). Based on the collected experience of Dopa chemistry, the present author first synthesised a polyphenolic decapeptide (Ala-Lys-Pro-Ser-Tyr-Hyp-Hyp-Thr-Dopa-Lys)$_n$ containing two Dopa residues from the blue mussel *Mytilus edulis* by polycondensation (Yamamoto, 1987a), and two groups prepared a precursor form, Ala-Lys-Pro-Ser-Tyr-Pro-Pro-Thr-Tyr-Lys, containing two Tyr residues using genetic engineering technology (Maugh, 1984; Strausberg *et al.*, 1989; Filpula *et al.*, 1990). The latter genetic product was converted to the final adhesive protein by a modification reaction using a tyrosinase oxidase.

More recently, among these marine adhesive proteins whose amino acid compositions and sequences have already been determined, the barnacle adhesive arthropodin proteins with random sequences (Yamamoto and Nagai, 1992), the adhesive proteins (Ala-Gly-Dopa-Gly-Gly-X-Lys) (X: hydrophobic amino acids) of a Chilean mussel *Aulacomya ater* Molina (Yamamoto, Yamauchi and Ohara, 1993), the cuticle collagens of the polychaete (*Nereis japonica*) (Yamamoto and Takimoto, 1991) and, although it is not of marine animal origin, the adhesive protein (Gly-Gly-Gly-Tyr-Gly-Gly-Tyr-Gly-X)$_n$ (X; Lys, His, or Arg) of the vitellaria of the liver fluke (*Fasciola hepatica*) have also been synthesized (Yamamoto and Ohkawa, 1993).

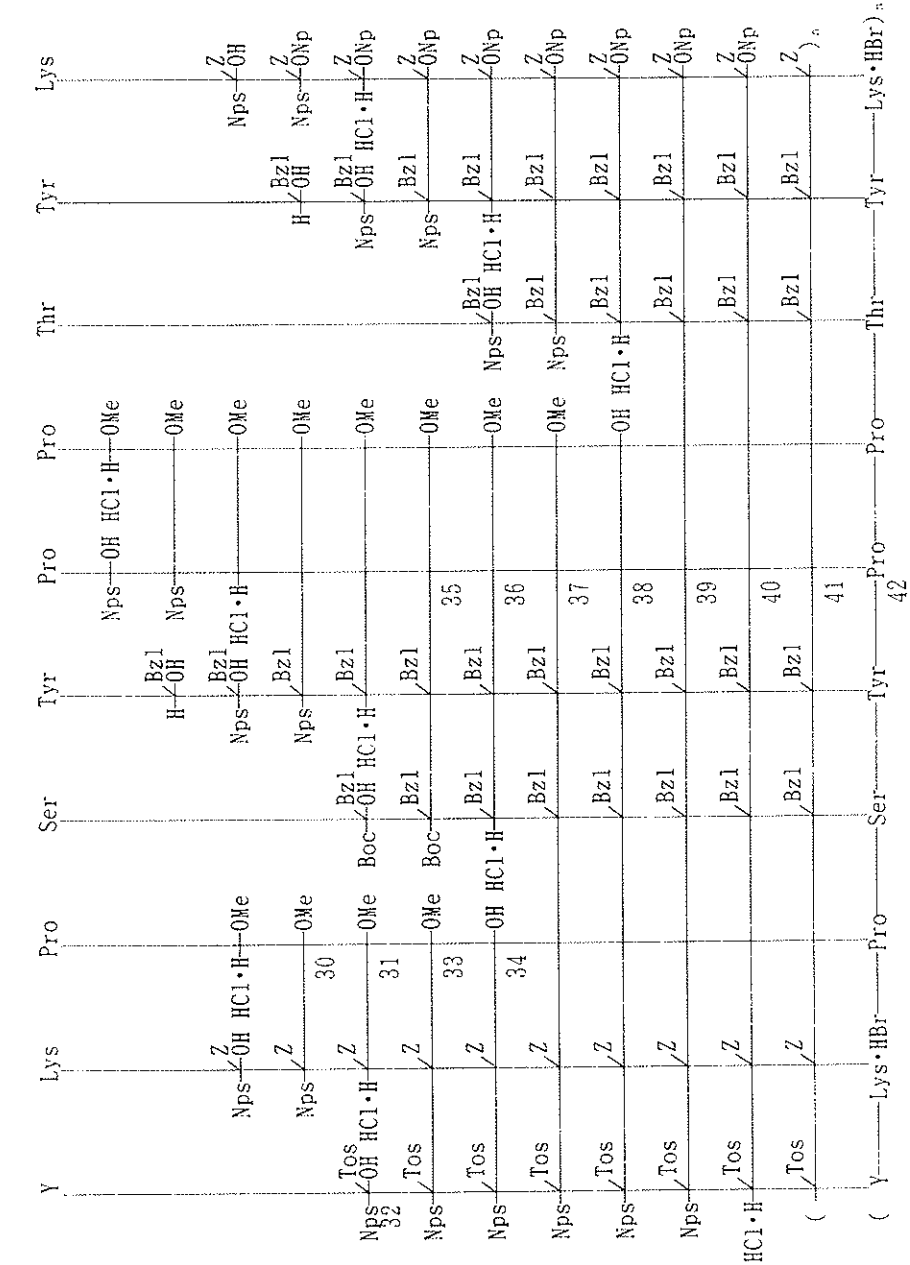
CHEMICAL SYNTHESIS

This section describes the improved synthetic route of the precursor polydecatapeptides of two mussel species, (Pro-Lys-X-Thr-Tyr-Pro-Pro-Thr-Tyr-Lys)$_n$ (X: Gly, Phe, and Ile) and (Y-Lys-Pro-Ser-Tyr-Pro-Pro-Thr-Tyr-Lys)$_n$ (Y: Ala and Arg), which are precursor sequences of the Californian and blue mussel adhesive proteins, and can be converted to final adhesive proteins by tyrosinase.

The synthesis of the Californian and blue mussel adhesive proteins is outlined in *Scheme I* and *Scheme II*. The synthesis has been done by two different strategies, in which all the reactions were the standard coupling method (Tatehata and Yamamoto, 1993; Yamamoto, 1995). The fragment couplings were accomplished through the use of DCCI in dioxane or chloroform. All the reactions followed the standard coupling method monitored by tlc on precoated silica gel plates. *Table 1* summarizes the chemically synthesised marine adhesive proteins and their precursors, together with their molecular weights by viscometry. In these synthetic mussel adhesive proteins, the estimated repeating units (26 at the highest) are considerably lower than that of the native blue mussel adhesive protein with a molecular weight of 130 000 (80 repeating units), of Californian mussel adhesive proteins with molecular weights of 85 000 (67



Scheme I. Synthesis of Californian mussel adhesive proteins (X, Gly, Phe, Ile).



Scheme II. Synthesis of blue (Y, Ala) and Californian mussel (Y, Arg) adhesive proteins.

Table 1. Chemically synthesized marine adhesive proteins and related adhesive proteins

Species (*scientific name*)	Amino acid sequence
Barnacle	
Balanus hameri	Random copolypeptides
Balanus balonoides	Random copolypeptides
Mussel	
Mytilus edulis	Ala-Lys-Pro-Ser-Dopa-Hyp-Hyp-Thr-Dopa-Lys
	Ala-Lys-Pro-Ser-Tyr-Pro-Pro-Thr-Tyr-Lys
Mytilus californianus	Phe-Lys-Ile-Thr-Tyr-Pro-Pro-Thr-Tyr-Lys
	Phe-Lys-Phe-Thr-Tyr-Pro-Pro-Thr-Tyr-Lys
	Arg-Lys-Pro-Ser-Tyr-Pro-Pro-Thr-Tyr-Lys
	Phe-Lys-Gly-Thr-Tyr-Pro-Pro-Thr-Tyr-Lys
Aulacomya ater	Ala-Gly-Tyr-Gly-Gly-Ala-Lys
	Ala-Gly-Tyr-Gly-Gly-Ile-Lys
	Ala-Gly-Tyr-Gly-Gly-Leu-Lys
	Ala-Gly-Tyr-Gly-Gly-Phe-Lys
	Ala-Gly-Tyr-Gly-Gly-Val-Lys
Polychaete	
Nereis Japonica	Ala-Gly-Glu-Hyp-Gly-Gly
	Hyp-Gly-Gly-Glu-Ala-Gly
Liver fluke	
Fasciola hepatica	Gly-Gly-Gly-Tyr-Gly-Gly-Tyr-Gly-Lys
	Gly-Gly-Gly-Tyr-Gly-Gly-Tyr-Gly-His
	Gly-Gly-Gly-Tyr-Gly-Gly-Tyr-Gly-Arg
Caddis worm	
Stenopsyche griseipennis	Random copolypeptides

repeating units), and of Chilean mussel adhesive proteins with molecular weights of 100 000 (155 repeating units). Native cuticle collagen from the marine worm *Nereis japonica* was reported to have a high molecular weight of 1 700 000, while synthetic cuticle collagens were estimated to have molecular weights of 9400–10 000.

GENE TECHNOLOGY

The microbial production of derivatives of the polyphenolic protein was investigated using recombinant DNA technology (Maugh, 1984). A microbial host such as *E. coli*, *S. cerevisiae* or *B. subtilis* was used. Also, the genetic engineering and development of yeast strains for production of mussel adhesive have been described. Experimentally, mRNA isolation and clone bank preparation, hybridisation screening of the clone bank, DNA sequence analysis, yeast genetics, purification of the polyphenolic protein from yeast, and final conversion of the microbially produced pre-adhesive to an adhesive protein were reported. Future research to develop genetically engineered molluscan adhesives has also been suggested (Strausberg *et al.*, 1989).

Insolubilization reaction and mechanism

Table 2 summarizes the insolubilization and adhesion mechanism of biopolymeric materials, proposed by biologists and polymer chemists. Among these, in the motif A, which has been called auto (or quinone)-crosslinking mechanism, the Tyr/Dopa and Lys residues are essential amino acids (Rzepecki *et al.*, 1991). In the motif B, which has been called the cystine-crosslinking mechanism, the CysH residues are essential. In *Table 2*, the amino acids, X, Y and Z denote amides, hydrophobic amino acids and imino acids, respectively.

The motif A auto-crosslinking mechanism of the settlement of watery adhesive proteins secreted mainly from mussels has been proposed by Lindner and Dooley (1973, 1976) and Lindner (1984), as has that of one family of byssal precursor proteins characterised in a number of mytilid species as already described. The auto-crosslinking occurs between the tyrosyl residues of protein side-chains and free amino or other reactive groups of another protein molecule with the aid of the phenolase enzyme, forming an inter-molecular crosslink. In this mechanism the enzyme oxidase secreted from the byssus and enzyme glands of mussels initially plays a primary role in first oxidizing and then insolubilizing the proteins, followed by adhesion (Crisp, 1973; Waite, 1987). Despite the occurrence of adhesive and hardening phenomena in nature, however, which consists of many complex factors, the auto-crosslinking mechanism described above has only been confirmed using catechols (Lindner and Dooley, 1976; Waite, 1985) and a simple synthetic poly(Lys Tyr) (Nagai and Yamamoto, 1989) by the use of tyrosinase. Recently, we have reported the oxidative reaction mechanism, by the use of tyrosinase, toward five synthetic mytilid bivalve marine adhesive protein precursors of two mussel species. We used (Pro-Lys-X-Thr-Tyr-Pro-Pro-Thr-Tyr-Lys)$_n$ (X: Gly, Phe and Ile) and (Y-Lys-Pro-Ser-Tyr-Pro-Pro-Thr-Tyr-Lys)$_n$ (Y: Ala and Arg) as the substrates, which are precursor sequences of the Californian and blue mussel adhesive proteins.

Table 2. Primary structures and insolubilization motifs of marine adhesive proteins

Animal (*scientific name*)	Amino acid sequence
(A) Dopa/Tyr motif consensus	
Blue mussel (*Mytilus edulis*)	Ala-Lys-Pro-Ser-Dopa-Hyp-Hyp-Thr-Dopa-Lys
California mussel (*Mytilus californianus*)	Hyp-Lys-Y-Thr-Dopa-Hyp-Hyp-Thr-Dopa-Lys
	Arg-Lys-Pro-Ser-Dopa-Hyp-Hyp-Thr-Dopa-Lys
Chilean mussel (*Aulacomya ater*)	Ala-Gly-Dopa-Gly-Gly-Y-Lys
Ribbed mussel (*Geukensia demissa*)	X-Thr-Gly-Dopa-Y-Z-Gly-Dopa-Lys
Reef-building polychaete (*Phragmatopoma californica*)	Val-Gly-Gly-Dopa-Gly-Dopa-Gly-Ala-Lys
Liver fluke (*Fasciola hepatica*)	Gly-Gly-Gly-Dopa-Gly-Gly-Dopa-Gly-Lys
	Gly-Gly-Gly-Dopa-Asp-Ser-Dopa-Gly-Lys
(B) Cystine motif	
Blue mussel (*Mytilus edulis*)	Cys-Tyr-Cys-Val-Gly-Gly-Tyr-Ser-Gly-Pro-Thr-Cys-Gly-Glu-Asn-Ala-Cys-Lys-Pro-Asn-Pro-Cys
Barnacle (*Balanus perforatus*)	NH_2-Thr-Tyr-Phe-Pro-Val-Leu-Ser-Tyr-Gly-Cys?-Ser-Ser-Ser-Leu-Ala-Pro-Val-Ile-

The possibility of the motif B crosslinking mechanism of the settlement of cement matrix proteins secreted from barnacles (*Balanus hameri* and *Balanus crenatus*) has been suggested from the cystine (6–7 mol%)-containing amino acid compositions (Walker, 1972). Recently, characterization of a cystine-rich polyphenolic protein family has been reported. In *Mytilus edulis*, a second structural family of Dopa proteins of about 42–47 kDa, which contain 2–3 mol% Dopa and are also enriched in the disulphide-containing amino acid cystine (6–7 mol%), have been found (Rzepecki, Hansen and Waite, 1992). The crosslinking occurs between the sulfhydryl moieties of CysH residues of protein side-chains with the aid of many kinds of oxidants such as oxygen, forming an inter-molecular crosslink. As for the second cystine motif B, the detailed investigation has just started.

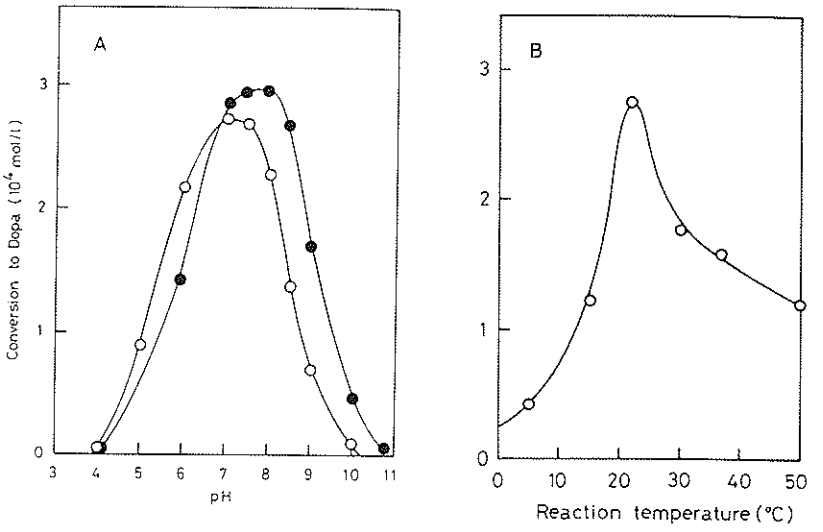


Figure 3. pH and temperature dependence profile of the oxidation reaction of synthetic Californian and blue mussel adhesive proteins with tyrosinase. **A** open circles, Californian mussel adhesive protein 3-Gly; filled circles, blue mussel adhesive protein at 22°C; **B** Californian mussel adhesive protein 3-Gly at pH 7.

TYROSINASE REACTION KINETICS

The oxidation reaction mechanism outside the secreting cells of mytilid bivalves was investigated using five authentic blue and Californian mussel adhesive precursor proteins with five different amino acids in the sequential decapeptide units as substrates, as occurs in nature. Oxidative activity was measured spectrophotometrically using tyrosinase from mushrooms (Nakamura, Sho and Ogura, 1966). Aromatic amino acids exhibit the characteristic absorption bands in seawater at pH 7 and 25°C; that is, Tyr with $\varepsilon_{275} = 1300$, Dopa with $\varepsilon_{283} = 2660$, and Dopa quinone derivative with $\varepsilon_{360} = 3870$, respectively.

When tyrosinase was added to Californian mussel protein precursor (CMP 3-Gly), poly(Pro-Lys-Gly-Thr-Tyr-Pro-Pro-Thr-Tyr-Lys), in simulated seawater at pH 7 and 22°C, the absorption peak of Tyr residues observed at 278 nm shifted to 283 nm after 5 h, to which the Dopa residues have been assigned, thus yielding the final chemical features of CMP adhesive protein (3-Gly), together with an additional shoulder in the 330–360 nm region. After 24 h, the new broad absorption band was observed to be centered at 350 nm. The band at 350 nm has been assigned to the Dopa quinone residues due to a successive oxidation action by tyrosinase (Yamamoto *et al.*, 1990). All synthetic adhesive protein precursors of mytilid mussels exhibited almost the same spectroscopic behavior upon oxidation by tyrosinase.

Figure 3 shows the temperature dependence and the pH dependence of CMP and blue mussel adhesive protein (BMP) oxidation by tyrosinase. The enzyme activity occurs in a rather wide pH range of 5–9, exhibiting optimal pH at 7 in the case of all CMP and at pH 8 in the case of BMP. The enzyme activity occurs in a rather narrow temperature range centered around 22°C.

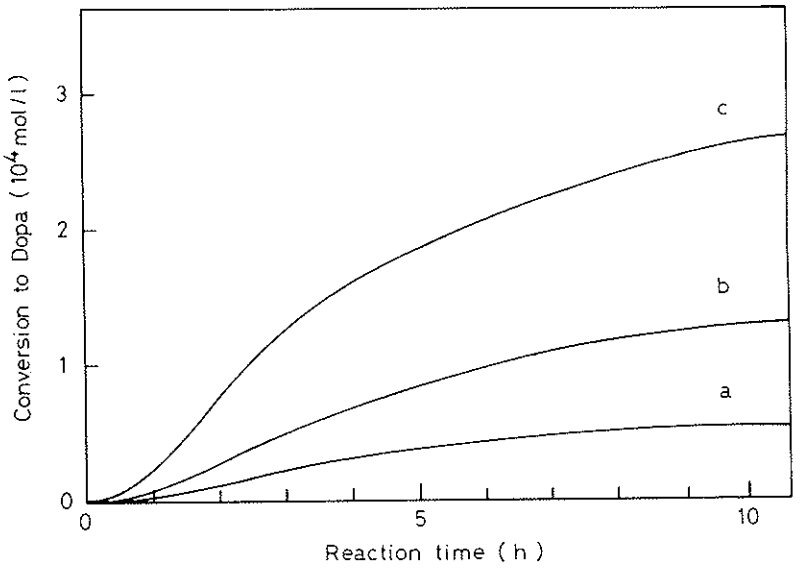


Figure 4. Salinity dependence of the oxidation reaction of synthetic Californian mussel adhesive protein 3-Gly at pH 7 and 22°C: curve a, in distilled water; curve b, in 20% seawater; curve c, in 100% seawater.

The relationship between the oxidation reaction of CMP 3-Gly by tyrosinase and salinity is shown in *Figure 4.* The conversion from Tyr to Dopa (and from Dopa to Dopa quinone) was slow in distilled water, at medium speed in 20% seawater, and the fastest in 100% seawater. This finding was the same as our earlier results on the

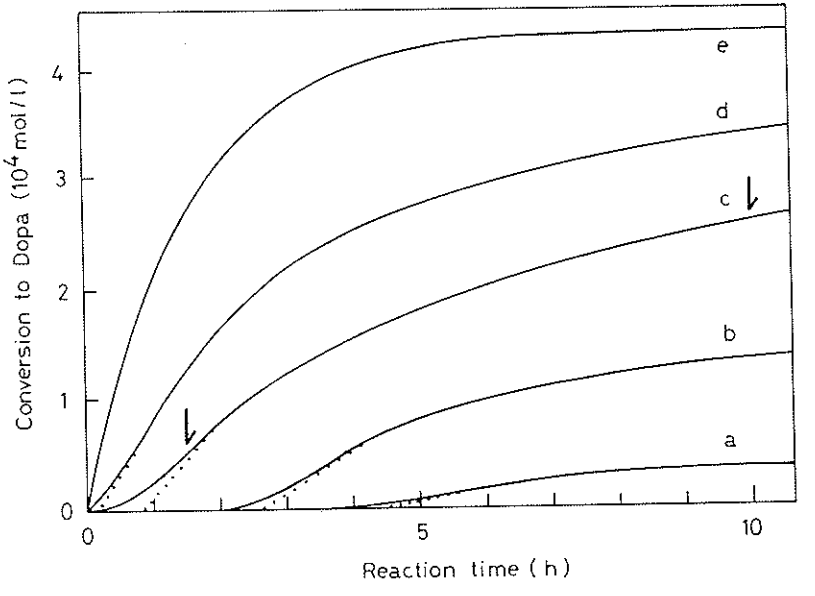


Figure 5. Effect of enzyme amount on the oxidation reaction of synthetic Californian mussel adhesive protein 3-Gly at pH 7 and 22°C. Added enzyme units: curve a, 4; curve b, 12; curve c, 40; curve d, 120; curve e, 400. Dotted lines extrapolated give the induction periods.

Chilean mussel adhesive proteins (Yamamoto, Yamauchi and Ohara, 1993). Thus the optimal condition of the oxidation reaction of CMP 3-Gly by tyrosinase was found to be pH 7, 22°C and seawater salinity.

Figure 5 shows the oxidation reaction of CMP 3-Gly by changing the tyrosinase concentration of the system, from 4 to 400 units, at the optimal condition. At low enzyme concentrations, the oxidation reaction by tyrosinase exhibited the induction period and, when the enzyme concentration was increased to 400 units, the induction period disappeared. The linear relationship between the induction periods and the $\log([S_{tyr}]/[E])$ was a typical kinetics profile of the autoreaction mechanism (Kagiya, 1973), which has been called auto-crosslinking in marine biology.

In order to obtain more convincing evidence of the successive reaction mechanism, the enzymic reaction mixture solutions after 1.5 h and 10 h (arrows in Figure 5), respectively, were added at the initial stage of its newly prepared reaction mixture of CMP 3-Gly and tyrosinase 40 units. Compared with the control experiment (no added reaction solution), neither reaction mixture exhibited the induction period (Yamamoto and Tatehata, 1995).

DOPA CONVERSION RATIOS AT VARIOUS TYR POSITIONS

Knowledge of amino acid composition and sequence in the oxidation reaction of the adhesive protein related peptides by tyrosinase was first reported by Marumo and Waite (1986), who elegantly employed the HPLC analysis method. However, the technique seems a very skilled operation. Again, the effect of the sequence of the adhesive proteins was examined. Since one Californian mussel adhesive protein poly(Arg-Lys-Gly-Thr-Tyr-Pro-Pro-Thr-Tyr-Lys) among five mussel adhesive proteins is barely soluble in seawater, the order of the oxidation rate of the five mussels is 1-Ala > 3-Phe > 3-Ile > 3-Gly > 1-Arg in 20% seawater using 5 units of tyrosinase. The blue mussel's adhesive protein (1-Ala) is oxidized faster than those of the Californian mussel. However, the 3-Arg adhesive protein of the Californian mussel insolubilized in the shortest time, exhibiting precipitation at about 2.5 h. *Table 3* summarizes the Dopa conversion ratios of various Tyr residues in synthetic deca repeating units. In seawater salinity the two Tyr residues in four mussels' adhesive

Table 3. Dopa conversion ratios of various Tyr residues in synthetic mussel adhesive protein precursors at pH 7 and 22°C.

X or Y	Salinity (seawater)	Enzyme units	Conversion to Dopa[a] (%)
Gly	1/5	5	3.5
	1	40	61.8
Ile	1/5	5	5.7
	1	40	62.7
Phe	1/5	5	7.7
	1	40	66.4
Ala	1/5	5	8.3
	1	40	67.3
Arg	1/5	5	1.5[b]

[a] After 20 h.
[b] After 2.5 h.

protein precursors oxidized to Dopa residues giving a 62–67 conversion %. When we compared our results in *Table 3* with the other HPLC analysis method employed by Marumo and Waite (1986), our present results coincided with those reported by Waite, judging from the fact that the former is based on proteins and the latter on oligopeptides with chain lengths from 1 to 10. The spectrophotometrical methodology is a much easier method for estimating the Dopa contents in the adhesive proteins (Waite and Tanzer, 1981).

ACTIVATOR AND INHIBITORS

The activation and inhibition of the oxidation reaction of mussel adhesive proteins catalyzed by tyrosinase was examined. Added Dopa enhanced the oxidation reaction at its initial stage. Likewise, simple catechol enhanced the reaction. On the other hand, added Dopa quinone depressed the enzyme reaction exhibiting partial precipitation in the reaction mixtures.

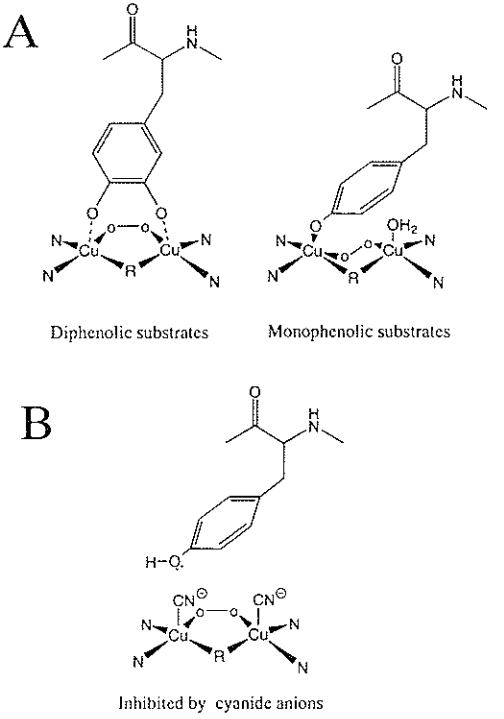


Figure 6. **A** The enzyme-substrate complex structures. **B** Inhibition of the active sites by cyanide.

Thio- and chloro-compounds, amines, and cyanide all inhibited the oxidation reaction of the Californian mussel adhesive proteins in the presence of tyrosinase. The inhibition ratios of the oxidation activities of CMP 3-Gly in the presence of tyrosinase are summarized in *Table 4* at molar ratios of inhibitor/Tyr residues from 1/15 to 1. The highest inhibition was caused by potassium cyanide (0%–11%). The second highest inhibition was obtained by mercaptoethanol (32%). The next highest inhibition was

obtained with phenylhydrazine (47%) and, when the amount of phenylhydrazine was increased from 1/15 to 1, the inhibitory effect increased to 14%. *Figure 6A* shows the ES complex structures of tyrosinase. The reason for the inhibition is thought to be because the inhibitors coordinate with the copper located at the active center of this metalloenzyme and the coordination prevents the catalytic oxidation-reduction ability of the copper atoms as shown in *Figure 6B*. The inhibition by cyano-, thio-, chloro- and amine compounds gives clues on prevention of biofouling by marine invertebrates such as mussels (see paragraph 9).

Table 4. The effect of various inhibitors on the oxidation of the Californian mussel adhesive protein precursor (3-Gly) by tyrosinase

Inhibitor	Equivalent (mol/Tyr mol)	Tyrosinase activity[a] (%)
Control		100
Cysteine	1/15	70
Methionine	1/15	86
Mercaptoethanol	1/15	32
Thiourea	1/15	73
Sodium Thiosulfonate	1/15	81
Potassium Thiocyanate	1/15	86
Ethylenediamine	1/15	93
	1/1	58
Methylhydrazine	1/15	77
	1/1	35
Phenylhydrazine	1/15	47
	1/1	14
Nor-epinephrine	1/15	87
	1/1	59
L-Chloroalanine HCl	1/15	76
DL-Chloroalanine HCl	1/15	82
Potassium cyanide	1/15	11
	1/1	0

[a] Ratios of the activities with and without inhibitor after 10 h.

INSOLUBILIZATION

The crosslinking reaction of copoly (Lys Tyr) and CMP (3-Gly and 1-Arg) with tyrosinase in simulated seawater systems was examined. The polydecapeptide systems with tyrosinase exhibited a precipitate after 1–3 h (in seawater) – 2.5 h (in 20% seawater) and the turbidity of the systems increased gradually when observed from the decrease of the transmittance at 650 nm (not shown here). Thus the synthetic mussels' adhesive proteins become insoluble in aqueous systems and, therefore, are promising as bioadhesive materials (Yamamoto *et al.*, 1992; Yamamoto, 1994).

From the total experimental results described in this paragraph, the insolubilization reaction mechanism of marine adhesive proteins is summarized in *Figure 7*.

Conformation and hierarchy

Both native adhesive protein from blue mussels and a genetically synthesized nonhydroxylated analog (20 deca-repeats) have been analyzed by far-UV circular

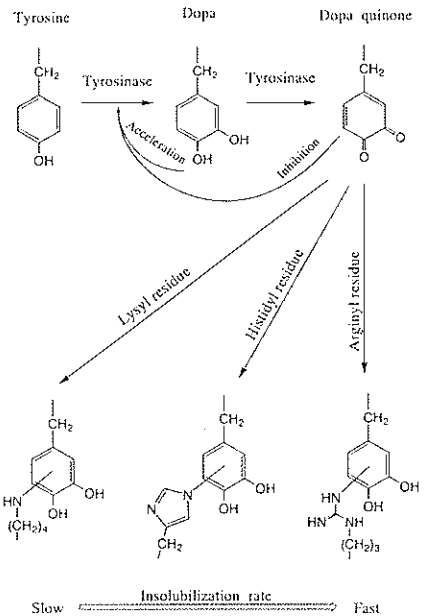


Figure 7. Insolubilization reaction mechanism of the quinone crosslinking of marine adhesive proteins.

dichroism (CD) under a variety of conditions (Williams *et al.*, 1989). Analysis of the CD spectra strongly suggested a primarily random coil structure for both forms of the protein. However, β-turns (~ 20%) were predicted to be present in the 5-Tyr region of high probability in 0.6M NaCl, 0.6M KF, and 6M guanidine hydrochloride. These data suggested that the marine adhesive proteins in solutions have only small amounts of secondary structure.

Conformations of model peptides of the adhesive protein of the mussel *Mytilus edulis* were investigated using molecular mechanics. The conformation was analysed to be more stable than 3_1 and α-helical conformations. Adjacent reverse β-turn structures have little conformational preference. The overall protein can possess a significant random coil nature, yet have a highly ordered embedded conformational component (Nagy *et al.*, 1991). From a different point of view, the conformation of PLL from DP 1 to 640 in aqueous solutions toward wettability has been studied. The conformation of the DP 32 PLL takes the most closed packed random coil structure. When the DP was larger than 32, the PLL molecules take more rigid extended conformation as DP increased (Ogawa and Yamamoto, 1995a, b). In this connection, among the three tertiary structures of PLL, the β-structural conformation enhanced the adhesability of the surfaces. Additionally, β-structural conformation and crosslinking caused by tyrosinase enhanced the adhesiveness of the surfaces (Ohara, Ohkawa and Yamamoto, 1993).

As for hierarchy, the morphology and mode of formation of some structural proteins of *Mytilus edulis* have been examined, by using standard histological and histochemical techniques using polarized light and X-ray diffraction data (Brown,

1952). More recently, the formation of mussel byssus has been reviewed by Waite from the point of view of the anatomy of a natural manufacturing process (Waite, 1992b). The latter review was stimulating and raised many points for both biologists and polymer chemists.

Preparation and biodegradation of biohydrogels

Mytilid mussels secrete a soft semi-transparent gel thread emerging side-ways from a flattened ovoid disc. This is an example of a biohydrogel. The transparent thready gels turn successively white, turbid, then pale yellow after a few hours, brownish red overnight, and finally into a green strong thread after several days, with a high tensile strength of 200–1000 kgf/cm^2. This hardening process is due to auto-cross linking. In this way, mussels attach to the surfaces of substrates by way of byssus.

For many reasons, crosslinked polymers in watery systems have long been an important class of materials, and are used in a diverse assortment of applications as hydrogels, including medical wound dressing. Progress has been made in developing approaches to the description of the molecular structure of crosslinked polymers (Dickie, Labana and Bauer, 1988).

BIOHYDROGELS

Biohydrogel formation

Organic crosslinking agents such as dialdehydes and diketones, insolubilized water-soluble cationic Lys- and Orn-containing polypeptides, show in most cases a rapid precipitation or gel formation which was observed using spectroscopy (Yamamoto *et al.*, 1992). In the pH region above 10.5, when the equivalent molar ratios of glutaraldehyde (GA) to amino groups are low (i.e. 1/10–1/5), the crosslinking reaction took over 2 h; on increasing the ratio to 1/2 the reaction is almost completed after about 0.5 h, in both cases forming a solid gel. Likewise, the crosslinking reaction using 2,5-hexanedione (HD) is almost completed after about 4 h. However, when comparing the dialdehydes and the diketones, the crosslinking reactions using dialdehydes were much faster than those using diketones (Yamamoto and Tanisho, 1993).

When allowed to stand without mixing or stirring, the cationic polypeptides and dialdehyde systems formed soft and solid gels after 20–40 h. When the ratios of the added glutaraldehyde to amino acid residues are less than 1/10, soft gels formed, and when the ratios are more than 1/2, solid gels formed. In cases where excess aliphatic dialdehydes were used as a cross-linking agent for copoly(Orn^1 Tyr^1) in distilled water, solid gels were also obtained. The copolypeptide gels formed with glutaraldehyde are brownish red, while the copolypeptide gels formed with glyoxal are almost colourless. The cationic polypeptides also formed soft gels with diketones (HD and 2,4-pentanedione), while the copoly(Orn^1 Tyr^1) did not.

System pH levels play roles in the gel formation. The gel formation in the alkaline pH region was much faster, but even in the strongly acidic pH region the gel formed very slowly. Accordingly, the pH values are not very effective in the gel formation.

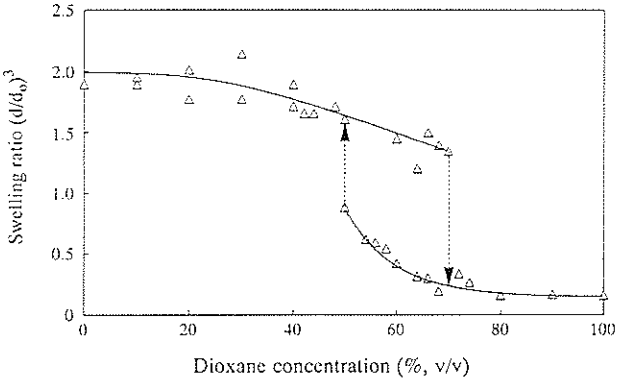


Figure 8. Swelling ratio of the PLO-GA (1/10) gel plotted as a function of dioxane content in dioxane-water mixtures.

Swelling properties

The dry crosslinked PLO-GA (1/20) gel with 5 mm diameter swells to a 16-fold apparent surface area (20 mm diameter) in distilled water. The swelling degree of the PLO-GA gel changes by changing the sunken medium, water to organic solvents (acetone, ethanol, or dioxane) and inversely, changing organic solvents to water, exhibiting time-dependent behaviours. The cationic polypeptide-GA gels exhibited mostly reversible expansion-contraction when immersed alternately in water and in organic solvents media. *Figure 8* shows the change of the swelling degree of the PLO-GA gel after swelling equilibrium by changing the solvent composition in water-dioxane mixed media. The shrunken gel with a swelling degree of about 0.2 in dioxane started to swell at about 70% dioxane and expanded at dioxane concentration 50 vol%, and, conversely, the swollen gel with a swelling degree of about 2 in water gradually contracted and shrank at 70 vol % dioxane, returning mostly to the initial swelling degree. This expansion-contraction was reversible but exhibited a hysteresis in the water-dioxane or acetone mixed solvent systems.

The low DP PLO (DP 290)-HD (1/1) gel was fragile, and the high DP PLO (DP

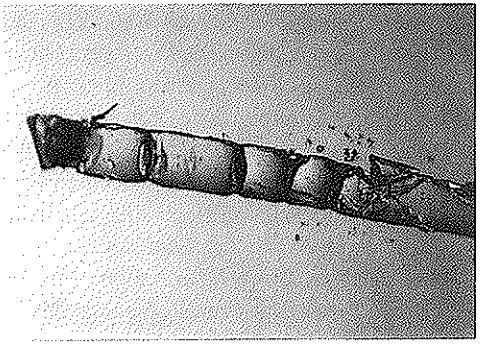


Figure 9. Bamboo-like pattern of the PLO-GA (1/10) gel.

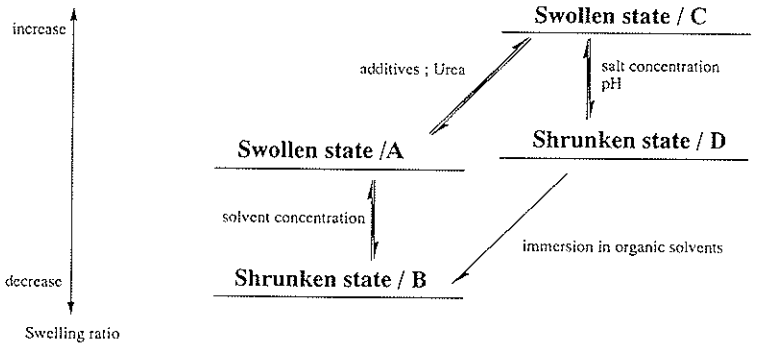


Figure 10. Phase diagram of the crosslinked cationic polypeptide gels.

720)-HD gel was much stronger, but both gels exhibited a swelling gel property. As one of the patterns in shrinking gels (Matsuo and Tanaka, 1992), a bamboo-like pattern in a cylindrical PLO-GA (1/10) gel, 0.8 mm in diameter, is shown in *Figure 9*.

Finally, the phase diagram of crosslinked cationic polypeptide gels is summarized in *Figure 10*.

ADSORPTION PROPERTIES

The selective amino acid adsorption of anionic Asp and Glu, neutral Ala and Trp, and cationic Lys in the matrix of cationic crosslinked polypeptide-GA gels was studied. When Asp and Glu were added to the ω-amino groups of the cationic gel matrices, the adsorption of the anionic amino acids reached a final maximum value after 10 min. This was predominantly due to free ω-COO^- with higher pKa_2 values. When half these amounts each of Asp and Trp were added to the above gel, the adsorption of the Asp and Trp reached final values after 10 min and 2 min, respectively, and 63% of Asp and 5% of Trp were adsorbed (Yamamoto and Tanisho, 1993; Yamamoto and Hirata, 1995a). From the independent adsorption results using benzoic acid and the PLO-GA gel, the Donnan equilibrium between the ionic solute and the cationic gel is estimated to participate in the adsorption to a small extent. These findings suggest that the matrix of the cationic gels could be anticipated to adsorp acidic polysaccharides originating from marine plants and microorganisms in seawater.

The gel formation might not be directly related with the natural observation of the biological adhesion process described in the Introduction. However, the results might offer some clues to understand biological adhesion, which clearly includes the cross-linking and gel formation process.

BIODEGRADATION

The attachment byssus of mussel *Mytilus edulis* was biodegraded by a bacterium of the genus *Alteromonus*, which is an aerobic, polarly flagellated, gram-negative and rod-shaped organism which plays a role in keeping marine ecology clean. Extracellar proteinases produced by this *Alteromonus* strain were partially purified (Dohmoto *et*

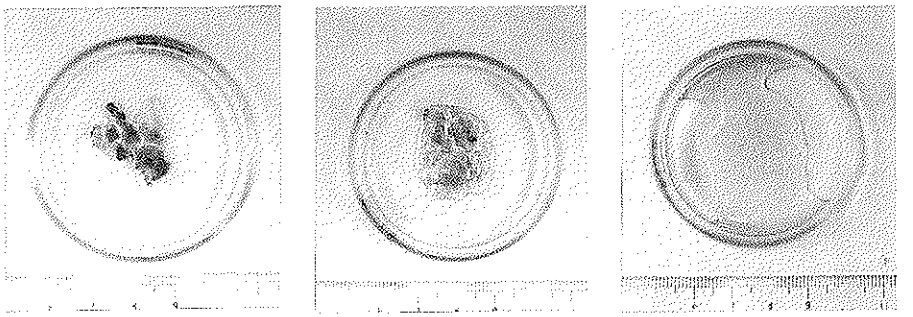


Figure 11. Photographs of the enzymatic degradation process of the copoly(Lys Tyr)-GA (1/5) gel by chymotrypsin. **a**, 0 h; **b**, 6 h; **c**, 10 h.

al., 1993). In this connection the biodegradation characteristics of the cationic cross-linked polypeptide hydrogels has been studied using proteolytic enzymes (Yamamoto and Hirata, 1995b).

Figure 11 shows the photographs of the biodegradation process of the copoly(Lys^{l} Tyr^{l})-GA gel by 3000 units of chymotrypsin. The gel was degraded to liquid after 10 h by chymotrypsin and after 6 h by trypsin. The rapid degradation to final liquid was completed within 10 h, and the slower or partial degradation within 60 h. Each enzyme specifically recognized the gels as substrates with different amino acid compositions, and digested the peptide bonds. Papain, which has low substrate specificity, exhibited a slower biodegradation ability toward the copoly(Lys^{l} Tyr^{l})-GA gel than did trypsin and chymotrypsin, and exhibited no degradation towards PLL-GA gels. Among the three enzymes used, the degradation ability of trypsin is the highest toward cationic polypeptide-gels containing Lys. PLO-GA gel and copoly(Orn^{l} Tyr^{l})-GA gel exhibited no degradation by the three enzymes. This is due to the Orn residues; Orn is non-coded amino acid residue and cannot be digested by proteases.

Figure 12 shows the enzyme concentration dependence of the degradation of the PLL-GA microgels by trypsin at 25°C and pH 7. When 100 units of trypsin were added, it took over 48 h to degrade the microgels. Increasing the trypsin amount from 500 to 1500 units shortened the degradation time from 48 to 2 h. Thus 1000–1500 units of trypsin were found to be a suitable amount for a fast degradation of the PLL microgels: since lesser amounts of the enzyme, such as 100 units, completely digested and degradated the microgels after several days, the biodegradation ability of the trypsin toward the PLL-GA gels seems very high and specific.

Between pH 7 and pH 11, the tryptic degradation proceeded quickly (4 h), but degradation was slow below pH 5, which coincides well with the optimal pH 8 of this enzyme. The degradation time of the PLL-GA microgels by 500 units trypsin was 2 h in 0.5 M NaCl. When the NaCl concentrations were increased to 4 M, the degradation times increased to 24 h. However, the degradation time of the microgels by trypsin in 4 M NaCl was shorter than the tryptic degradation time of the same microgels in salt-free water. It was clear that the presence of NaCl in the systems enhanced the degradation of the PLL-GA gels, and the optimal salt concentration of the degradation of the PLL-GA gels was 2 M NaCl. In the swollen state, trypsin easily penetrates into

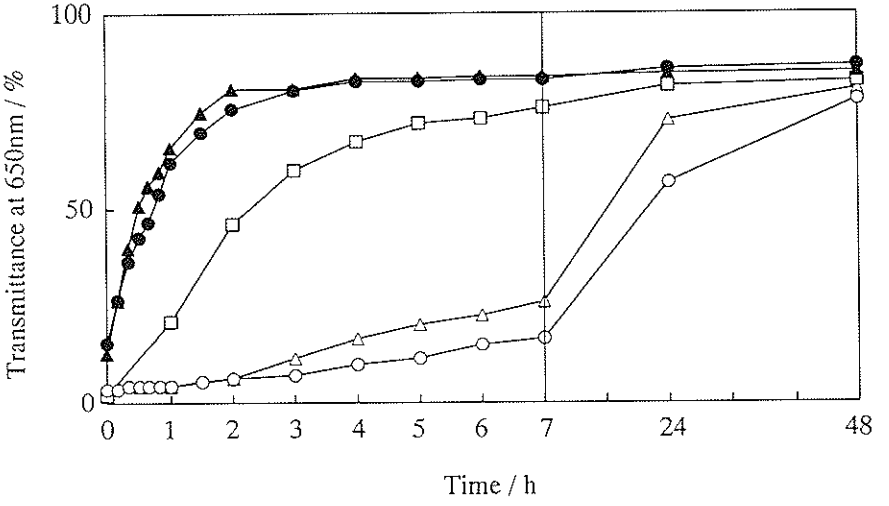


Figure 12. Trypsin amount dependence of the degradation of the PLL-GA (1/10) gels. Filled triangles, 1500 units; filled circles, 1000 unites; open squares, 800 units; open triangles, 500 units; open circles, 100 units.

the gels and degrades them (pH 7–11), but when the NaCl concentration in the system at pH 7 was increased from 0.5 to 4 M, the gels were in their contracted state. The enzymes could not then penetrate them, and degradation time of the PLL-GA gels became much longer, from 2 to 24 h. Thus, tryptic digestion toward the PLL-GA gels was activated by increased NaCl concentrations and was also activated by NaBr, KCl, and NH_4Cl, exhibiting no significant salt species dependence.

The degradation time decreased when temperatures were raised. The PLL microgels were degraded after several days at 5°C, 48 h at 25°C, 24 h at 40°C, and 1 h at 60°C, respectively. As opposed to other common enzymes, it is known that trypsin can display some activity even at temperatures over 80°C. Due to this temperature characteristic, trypsin degrades the PLL microgels according to rate kinetics rather than enzyme kinetics.

By changing the internal amino acid compositions, controlled degradation by enzymes was examined using some copolypeptide-GA gels, into which non-coded amino acids were incorporated. In order not to change greatly, or lose, the favorable PLL-GA gel characteristics, cationic Orn, which is a lower homologue of Lys, was chosen. *Figure 13* shows the degradation profile of the PLO-GA and copoly(Lys^1 Orn^1)-GA microgels by 1000 units of trypsin at pH 7. PLO-GA microgels were not subject to biodegradation. Trypsin degraded copoly(Lys^1 Orn^1)-GA slightly more slowly than did the PLL-GA. When the molar ratios of Orn residues in copoly(Lys Orn) and copoly(Tyr Orn) are enriched, the degradation rate can be slowed, thus exhibiting controlled biodegradation, depending on Orn content.

These findings may have the prospect of developing new biodegradable biohydrogel materials, including medical wound dressings with very high water content. In this connection, since the PLL-GA and PLO-GA gel matrices selectively adsorbed anionic molecules such as benzoic acid and acidic amino acids (Yamamoto and Tanisho, 1993), it may be possible to adsorb anionic medicines into the cationic cross-linked

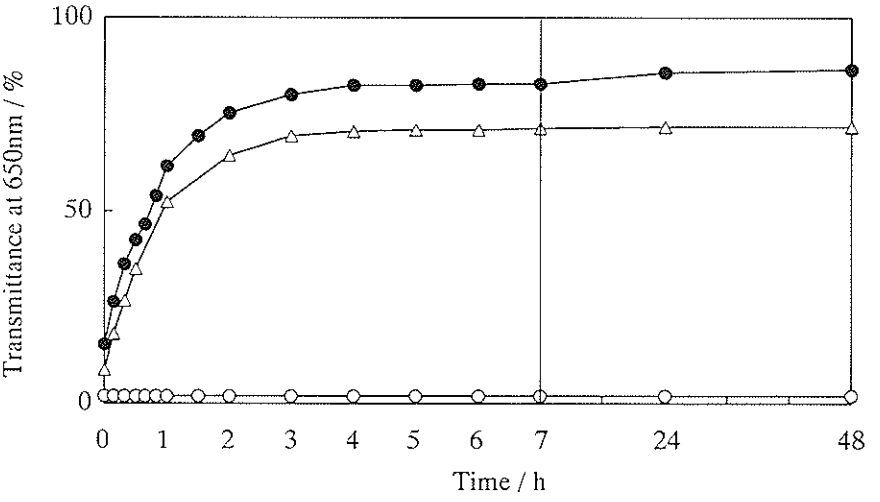


Figure 13. Degradation profile of the cationic polypeptide-GA (1/10) gels by trypsin. Open circles, PLO-GA; open triangles, copoly(Lys[1]Orn[1])-GA; filled circles, PLL-GA.

polypeptide gels. When the gels are biodegraded, the digested oligolysine fragments are expected to exhibit anti-infection action as reported earlier (Hadwiger, Loschke and Teasdale, 1977), and simultaneously, the medical materials released from the inside of the gels are also expected to be effective to cure injured tissues of the animal organism. Thus, PLL related hydrogels look very promising and we anticipate diverse medical applications.

Figure 14 shows the biodegradation of cationic PLL-GA related hydrogels by micro-organisms. After 22 days the gels were degraded completely. Two bacteria strains separated are aerobic, non-flagellated, and gram-negative. One is rod-shaped, and the other is spherical. The degradation of the cross-linked cationic polyamino acid-GA gels by proteolytic enzymes and micro-organisms offers some clues to

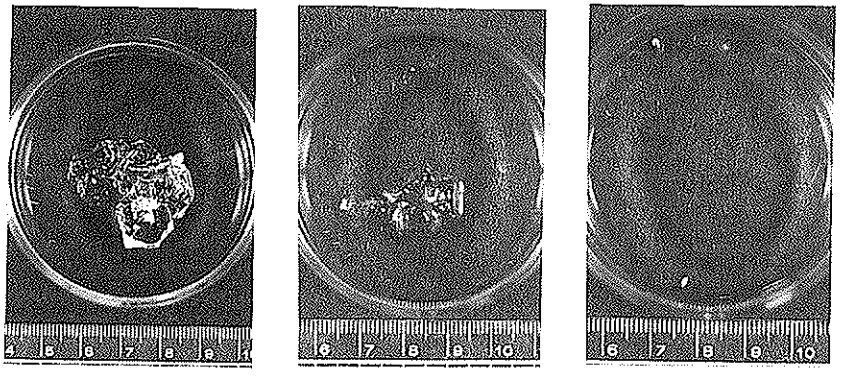


Figure 14. Photographs of the microbial degradation process of the PLL-GA (1/10) gel. **a**, 0 days; **b**, 12 days **c**, 22 days.

understanding degradation in biological adhesion, which includes the protein biodegradation process by micro-organisms and enzymes (Yamamoto and Amaike, 1995).

Surface characteristics

Composition of mussel and barnacle deposits at the attachment interface on Teflon was described by Cook (1970). As an advanced surface chemical approach, the force of adhesion of the mussel byssus disc together with a characteristic finding of the contact angles and the bonding strengths on the substances in sea water was first described by Crisp *et al.* (1985) and a hypothetical explanation has been given (Waite, 1987). However, the characteristics of the biological adhesion have not been examined from the standpoint of molecular structures such as amino acid species and their sequences.

The wettability and adhesion characteristics of marine adhesive proteins in water has been investigated (Yamamoto, Ogawa and Nishida, 1995; Yamamoto, Ogawa and Ohkawa, 1995), using (a) lysine peptides with different defined molecular weights (degrees of polymerization, DP, 1–32), (b) homo- and copolypeptides, (c) Lys containing sequential polypeptides, (d) mussel adhesive proteins such as blue mussel, Californian mussel and Chilean mussel, and (e) liver fluke adhesive proteins. Purified pearl oyster adhesive proteins and chitosan were also used.

THEORY FOR SURFACE CHEMISTRY

From the advancing contact angles ($\theta°$) on the substrates and the surface free energy (surface tension $\gamma_{L,V}$) of the aqueous solutions, the work of the adhesion (W_A) was calculated by the following Young-Dupré equation (Zisman, 1964; Hata, 1983):

$$W_a \doteqdot \gamma_{L,V}(1 + \cos\theta) \qquad \text{Eq. (1)}$$

The surface excess concentrations Γ of PLL aqueous solution were calculated from the surface free energy-concentration curves using the Gibbs isothermal adsorption equation

$$\Gamma = -(1/\upsilon RT)(d\gamma_{L,V}/d\ln C) \qquad \text{Eq. (2)}$$

where υ is 2 (Adamson, 1976). By the combination of the extended Fowkes's equation and the Young–Dupré equation, the dispersion and polar components of the surface free energies of proteins were calculated according to the following equation

$$\gamma_L(1 + \cos\theta) / 2\sqrt{\gamma_L^d} = \sqrt{\gamma_S^d} + \sqrt{\gamma_S^p}\,(\sqrt{\gamma_L^p} / \sqrt{\gamma_L^d}) \qquad \text{Eq. (3)}$$

Experimentally the contact angles of the droplets of pure organic solvents such as *n*-octane and glycerine, whose dispersion and polar components have been determined (Dann, 1970; Kitazaki and Hata, 1972), were measured on a PLL film coated glass slide. From the contact angles and surface free energies of the solvents above and Eq. (1), the $\sqrt{\gamma_S^d}$ and $\sqrt{\gamma_S^p}$ values of adhesive proteins were calculated (Fowkes, 1964).

As for the protein molecules adsorbed on the solid surfaces, the work of adsorption (W_{ads}), was calculated from the equation below, taking into consideration the interactions between water-protein and water-solid.

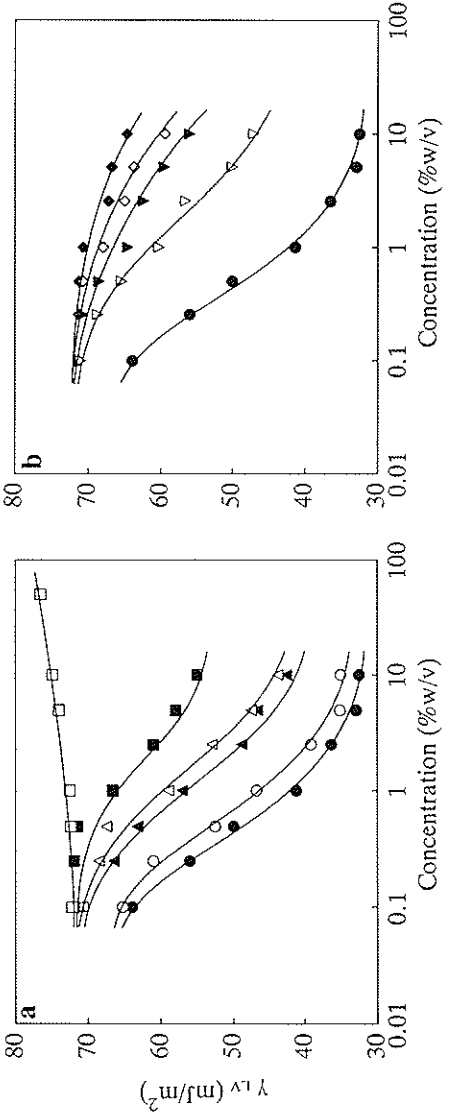


Figure 15. Concentration dependence profile of the surface free energies of PLL solutions. **a**, open squares, DP 1; filled squares, DP 2; open triangles, DP 4; filled triangles, DP 8; open circles, DP 16; filled circles, DP 32. **b**, filled circles, DP 32; inverted open triangles, DP 84; inverted filled triangles, DP 640; open diamonds, DP 1310; filled diamonds, DP 3260.

$$\Delta G_{ads} = -W_{ads} = \gamma_{pros} - \gamma_{prow} - \gamma_{sw} \quad \text{Eq. (4)}$$

where γ_{pros}, γ_{prow}, and γ_{sw} are the surface free energies of protein-solid, proteins-water, and solid-water, respectively.

$$W_{ads} = 2[(\sqrt{\gamma_{pro}}^{d} - \sqrt{\gamma_{w}}^{d})(\sqrt{\gamma_{s}}^{d} - \sqrt{\gamma_{w}}^{d}) + (\sqrt{\gamma_{pro}}^{p} - \sqrt{\gamma_{w}}^{p})(\sqrt{\gamma_{s}}^{p} - \sqrt{\gamma_{w}}^{p})] \quad \text{Eq. (5)}$$

where γ_w^d and γ_w^p are the dispersion and polar components of surface free energy of water. The W_{ads} were calculated by substituting the components from Eq. (3) in Eq. (5).

The surface free energies of substrates used were polytetrafluoroethylene (Teflon, 19 mJ/m^2), polyethylene (PE, 33 mJ/m^2), nylon (45 mJ/m^2), iron (56 mJ/m^2), soda glass (67 mJ/m^2) and alumina (40 mJ/m^2).

WETTABILITY OF ADHESIVE PROTEINS AND RELATED MODEL COMPOUNDS

The surface free energies of PLL solutions in the 0.1%–10% concentration decreased gradually with time. In the lower concentration range below 0.25%, the decrease of the surface free energies was slow, while in the high concentration range above 2.5% the decrease was much steeper, reaching constant values after 60 min. *Figure 15* shows the concentration dependence of the surface free energies of the PLL samples at the equilibrium state by changing their DPs. The decrease of the surface free energies of lysine polypeptide solutions enhanced the adsorption of PLL molecules by moving to the surface at the air-water interface. The surface free energy of the PLL solutions decreased when the DP was increased from 2 to 32. This reached the minimum values at DP 32, and then actually increased up to the DP 3260 sample. The reason for the decrease of high molecular weight PLL is the charge repulsive extended conformation of polypeptide, causing the adsorption barrier sterically at the air-water interface. The surface free energies, the contact angles, the work of adhesion, and the surface excess concentration of PLL solutions at the air-water interface has been investigated in detail (Yamamoto, Ogawa and Nishida, 1995).

The work of adhesion as a function of Tyr content of two different copoly(Tyr^l Lys^x) and copoly(Tyr^l Glu^y) also was examined. The work of adhesion depended on the substrates, and the order in both copolypeptides was glass > nylon > PE > Teflon. When the molar ratios of the Tyr residues in copolypeptides were increased from zero to 50 mol%, the work of adhesion increased proportionately with increasing amounts of the Tyr residues. Furthermore, the work of adhesion of Lys copolypeptides were always larger than Glu copolypeptides. Additionally, β-structural conformation and cross-linking caused by tyrosinase enhanced the adhesiveness of the surfaces (Yamamoto *et al.*, 1993; Ohara, Ohkawa and Yamamoto, 1993).

These wettability results give additional importance to Lys residues in the marine adhesive proteins, since until now the Lys residues in marine adhesive proteins have been thought to act as the crosslinking bridgehead by reacting with dopa quinone in order to insolubilize the proteins (Lindner and Dooley, 1973).

Table 5 summarizes the surface chemical properties of sequential polypeptides containing Lys residues and synthetic marine adhesive proteins. Among these sequential polypeptides, polypeptides containing Gly exhibited higher work of adhesion in four different substrates with four different surface energies. This effect could be

Table 5. The surface free energy, the contact angle and the work of adhesion of 1% synthetic adhesive protein solutions

Sample	γ_{lv}	Contact angle (θ°)				Work of adhesion (mJ/m²)			
	(mJ/m²)	Teflon	PE	Nylon	Glass	Teflon	PE	Nylon	Glass
Water	72.8	108	90	57	21	50	73	112	141
$(Lys\text{-}Gly)_n$	72.1	116	71	19	19	41	96	140	140
$(Lys\text{-}Lys\text{-}Gly)_n$	67.1	114	69	29	21	40	91	126	130
$(Lys\text{-}Lys\text{-}Ala)_n$	54.7	96	62	33	20	49	80	103	106
$(Lys\text{-}Lys\text{-}Ser)_n$	50.9	108	67	21	23	35	71	98	98
Blue mussel	51.4	100	65		16	42	73		101
Californian mussel	48.9	103	64		15	38	71		96
Chilean mussel	61.7	133	55	51	25	20	97	100	117

attributed to the free rotation ability of the Gly residues, having the smallest side chains (proton only). Furthermore, when we compared poly (Lys-Gly) with poly (Lys-Lys-Gly), the works of adhesion of polypeptide were always larger than those of polytripeptides on the four substrates. The works of adhesion of the hydrophobic Ala polypeptide were smaller than the Gly polypeptides, and the hydrophilic Ser polypeptides exhibited the smallest work of adhesion among all four sequential polypeptides. These findingsj from sequential polypeptides suggest an additional important role of Gly residues in the adhesive proteins. Synthetic marine adhesive proteins supported the findings from the much simpler model polypeptides mentioned above (Yamamoto *et al.*, 1994; Yamamoto, Ogawa and Nishida, 1995).

ADSORPTION OF PLL AND ADHESIVE PROTEIN MOLECULES

Table 6 summarized the adsorption characteristics of the PLL samples, and synthetic mussel and natural pearl oyster adhesive proteins, together with chitosan, on the solid surfaces. When the total surface free energies were divided into the dispersion and polar components, the dispersion components exhibited a constant value of about 26–28 mJ/m² but the polar components changed from 3 to 14 mJ/m². Thus, the total surface free energies were affected by the polar component of polypeptides and bivalve proteins. The work of adsorption of adhesive proteins exhibited higher values (41–68 mJ/m²) on Teflon and PE, and the lowest values (6–9 mJ/m²) on glass.

Table 6. Surface energy components and work of adsorption of marine adhesive protein related compounds

Sample		γ_{Pro}^{d}	γ_{Pro}^{p}	γ_{Pro}	Work of adsorption (mJ/m²)				
				(mJ/m²)	Teflon	PE	Iron	Glass	Chitosan
PLL DP	4	26.3	7.9	34.2	54	53	18	7	52
	8	27.0	6.7	33.7	58	56	19	7	55
	32	27.7	2.9	30.6	68	66	23	9	66
	84	28.4	9.2	37.6	51	50	18	7	50
	640	27.8	14.3	42.1	43	41	14	6	41
Blue mussel		27.6	11.4	39.0	48	46	16	6	46
Californian mussel		28.2	11.8	40.0	47	45	16	6	45
Pearl oyster		27.5	10.4	37.9	50	48	17	6	47
Chitosan		27.9	1.1	29.0	77	74	26	10	73

Olivieri *et al.* reported the γ^d, γ^p and γ_c values (notation $\gamma_{pro}{}^d$, $\gamma_{pro}{}^p$, and γ_{pro} in this article) of commercial mussel adhesive protein Cell-Tak™ (1990) and its di to deca fragment peptides (Olivieri, Baier and Loomis, 1992). The $\gamma_{pro}{}^d$, $\gamma_{pro}{}^p$, and γ_{pro} values of Cell-Tak films were reported to be 27.2–32.2, 11.0–17.2 and 40.7–46.3 dyne/cm (mJ/m^2 in this article), respectively, which did not depend on the final surface concentrations of polypeptide. The three γ values of the deca fragment were reported to be 23–30, 22–31, and 52–54 dyne/cm, which depended on the film conditions. When we compare our blue mussel γ values in *Table 6* with the results by Olivieri groups, three $\gamma_{pro}{}^d$ (27.6 mJ/m^2), $\gamma_{pro}{}^p$ (11.4) and γ_{pro} (39.0) values are close to the results of Cell-Tak but do not agree with the results of deca fragment and shorter fragments. These results suggest that high molecular weight samples are essential to collect detailed knowledge of marine and indeed of any adhesive proteins.

The surface chemical features of chitosan have been investigated. Firstly, as an adhesive material, chitosan exhibited the similar $\gamma_{pro}{}^d$ value of 27.9 mJ/m^2 to proteins, but the $\gamma_{pro}{}^p$ value was smaller than PLLs and marine adhesive proteins, exhibiting the smallest γ_{pro} value (29.0 mJ/m^2). However, the calculated work of adsorption of chitosan was much higher than those of PLLs and marine adhesive proteins on four solid surfaces. This means that a polysaccharide chitosan adhered faster than marine adhesive proteins in water. Secondly, as a surface material, the PLLs and marine adhesive proteins adsorbed predominantly on the chitosan coated surfaces rather than teflon, PE, iron, and glass. Thus, when one immerses a solid substrate in sea water, polysaccharide adsorbs to the substrate surface first and the protein adsorption follows. In fact, biological adhesion in nature does occur in this order (Yamamoto, Ogawa and Ohkawa, 1995).

Adhesive characteristics and bioadhesives

BONDING STRENGTHS

The bonding strengths of biological adhesion in nature have been widely investigated and reported (for example, Smeathers and Vincent, 1979; Young and Crisp, 1982) and these high bonding strengths, up to 10 000 kgf/cm^2 in wet environments, interested scientists in basic and practical adhesion material science. Later, the bonding strengths (tensile and compressive shear) of a variety of synthetic polypeptides as a model for marine adhesive proteins in water or in organic solvent systems have been reported (Yamamoto, 1987b).

BIOLOGICAL ADHESION

The biological adhesiveness of marine and related adhesive proteins on surfaces precoated with synthetic adhesive proteins has also been examined. Sperm cells of the goby fish *Tridentiger kuroiwae brevispinis* from Sagami Bay in Japan were used. 50 µl of adhesive protein solutions in 0.02 M Tris · HCl buffer were coated on soda glass slides. 0.5 µl of suspended sperm cell solutions of the goby fish in Tris · HCl-NaCl buffer was dropped onto the precoated glass slides, and the motility and adhesion of the sperm cells were immediately video recorded microscopically. The number of the adhered cells was counted as schematically drawn in *Figure 16* (Si and Okuno, 1993; Morisawa, 1994).

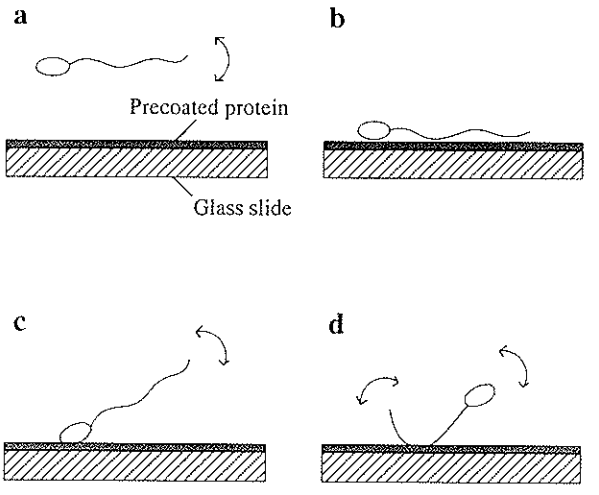


Figure 16. The sperm cell adhesion of the goby fish on the surface of a protein precoated glass slide. **a**, motile; **b**, immotile; **c, d**, adhered.

Table 7. Adhesion activity on glass surface of synthetic adhesive proteins to a goby fish sperm cells

Sample	Cells				
	Motile	Immotile	Adhered	Total	Adhesion (%)[a]
Control[b]	20	2	0	22	0
Poly(L-Lys)	0	9	1	10	10
Poly(L-Glu)	18	2	0	20	0
Copoly(Lys[1] Tyr[1])	0	17	0	17	0
Chilean mussel	2	6	4	12	33
Blue mussel	5	9	5	19	26
Liver fluke[c]					
9-Lys	7	3	2	12	17
9-Arg	10	4	1	15	7
9-His	7	11	3	21	14

[a] The ratio of (adhered cells)/(total cells).
[b] Adhesion of sperm cells on glass in 0.2M Tris HCl buffer at pH 7.
[c] $(Gly\text{-}Gly\text{-}Gly\text{-}Tyr\text{-}Gly\text{-}Gly\text{-}Tyr\text{-}Gly\text{-}X)_n$.

Table 7 summarizes the results of the goby fish sperm cell adhesion on soda glass plates. When the adhesion results between poly(Glu) and PLL were compared, cationic Lys residues are responsible for the sperm cell adhesion. Synthetic adhesive proteins exhibited the 7%–33% adhesion activity of the goby fish sperm cell on the glass plate. Among these, Chilean mussel adhesive protein exhibited the strongest adhesive ability and the second strongest was the liver fluke egg shell hardening protein. When we compared the sperm cell adhesion among three analogues of the liver fluke adhesive proteins containing 9-Lys, 9-Arg and 9-His, Lys adhesive protein ranked the highest with 17% adhesion activity. The fish sperm cell adhesion activity on the synthetic adhesive proteins was higher than that on the simpler model

polypeptides, showing the importance of the Lys residues and the primary structures in the marine and related adhesive proteins.

As mentioned by Baier (1982), it is very difficult to evaluate the interaction of living cells in flat sheets on glass slides. The data in *Table 7* may not be sufficient to draw statistically significant conclusions, since the experiment has been restricted for seasonal reasons. We can only collect the sperm of the goby fish in Summer (Middle June to Middle July in Japan).

Specific small peptides can mimic barnacle pheromone, which enhanced the rate of settlement and metamorphosis of larvae of *Balanus amphitrite* Darwin. The most effective peptides were Leu-Arg and His-Lys (Tegtmeyer and Rittschof, 1989). Most recently, catecholamines such as L-Dopa and dopamine hydrochloride were reported to act as settlement inducers of barnacle (*Balanus amphitrite*) larvae (Kon-ya and Endo, 1995).

MEDICAL ADHESION

Methods were described for the preparation and isolation of novel marine adhesive decapeptides by Waite (1986). Based on the decapeptide sequences, gene technology and adhesive materials for medical applications have been developed. Two groups described medical adhesives. Benedict and Picciano (1989) reported the research on two-part adhesive formulations for the bonding of tissues and on the immobilization of baby hamster kidney cells (BHK-21), human histocytic lymphoma (U-937) and bovine corneal endothelial cells (BCE). The attachment of adherent BHK cells and nonadherent U-937 cells to marine adhesive (trade name, Cell-Tak) was much better than to collagen, poly-D-lysine, fibronectin and laminin. Benedict and Picciano (1989) also evaluated the attachment of two bacteria, *Staphylococcus aureus* and *Escherichia coli*, and one yeast, *Saccharomyces cerevisiae*, to Cell-Tak coated plastics and uncoated plastics. For wound repair, the development of the overlapping corneal bond system *in vitro* has been examined in two test systems: cornea to cornea bond strengths with marine adhesive protein were measured, and a transparent hydrogel disc for use as a contact lens with marine adhesive protein was used to seal the thick corneal holes created in bovine eyes.

The protein-based medical adhesives from the blue mussel, together with medical adhesives based on fibrin isolated from human blood, has been described, suggesting applications such as biocompatibility and adherence and curing in moist environments (Strausberg and Link, 1990). The cross-reaction of the adhesive proteins purified from three Chilean mussels with an antiserum produced against the protein of the species was studied. From the immunological examination the adhesive proteins were poor antigens, and exhibited an advantageous property for the potential use of these adhesive proteins in medicine (Saez *et al.*, 1991).

A small cardioactive peptide (SCP), Ala-Pro-Asn-Phe-Leu-Ala-Tyr-Pro-Arg-Leu-NH_2 was isolated from the anterior byssus retractor muscle of the blue mussel *Mytilus edulis*. At 10^{-10} M or higher, *Mytilus* SCP showed a potentiating effect on phasic contraction of the anterior byssus retractor muscle. It was found that Leu-Ala-Tyr-Pro-Arg-Leu-NH_2 was to be the minimum structure required for both potentiating and relaxing activities on the anterior byssus Fujisawa *et al.*, 1993).

A totally synthetic fibrin glue was developed. A water-soluble copolymer com-

posed of N-isopropyl acrylamide and vinyl units, which has a cell-adhesion peptide (Arg-Gly-Asp) in the side chain, bound to platelet receptors on mixing with platelet-rich plasma. When applied to living tissue of a goat, spontaneous precipitation and subsequent platelet aggregation occurred, indicating that the synthetic bioactive polymer, effectively, functions like fibrin glue (Matsuda and Moghaddam, 1993).

Antifouling

Dr Ina's group in Shizuoka University has been investigating promising natural antifouling agents and found that new acylated rhaponitin isolated from *Eucalypus rubida* (Yamashita *et al.*, 1989a), isothiocyanates, nicotinamide from *Mallotus* leaves, acylated kaempferol glucoside isolated from *Quercus dentata*, and nonylphenols were effective attachment repellents against the blue mussel *Mytilus edulis* (Ina *et al.*, 1989a; Yamashita *et al.*, 1989b, 1989c; Takazawa *et al.*, 1990). They developed an improved laboratory screening and bioassay method for antifouling substances using the blue mussel (Ina *et al.*, 1989b), and also determined the antimicrobial activity to better understand the antifouling action of substituted phenolic compounds (Takazawa *et al.*, 1992).

From the tyrosinase inhibitory effect (*Table 4*) described already, the antifouling surfaces were prepared by the silane coupling procedure. A variety of substrates with different surface free energies and modified glass surfaces were used in antifouling experiments toward blue mussel *Mytilus edulis*. *Table 8* summarizes the results of the blue mussel attachment to the Cl-, SH-, amino- and glycidoxy-silane coupled surfaces. Firstly, it is clear that the mussel chooses the high energy surfaces (655–670 feet on slate and glass) in preference to low energy surfaces (220–250 feet on Teflon and polyethylene). Secondly, when the glass surfaces were silane coupled, the NH_2 -, Cl-, SH- and glycidoxy-coupled glass surfaces reduced the attaching numbers of the mussel from 80% to 40–20% and reduced numbers of the attached byssus from 655 feet to 77–79 feet, exhibiting a marked antifouling effect. Among the four silane

Table 8. Attachment of blue mussel on the solid surfaces

Substrate	Total	Attached	Attachment[a]	Byssus[b]		
	A	B	B/A(%)	C	C/A	C/B
Teflon	25	12	48	210	8.4	17.5
Silicon	25	8	32	228	9.1	28.5
OH-silicon	25	17	68	410	16.4	24.1
Aminosilicon	25	9	36	152	6.1	16.9
PMMA	25	10	40	253	10.1	25.3
Nylon	25	11	44	176	7.0	16.0
Glass	25 (150)[c]	20 (118)	80	655 (3930)	26.2	32.8
Aminosilane	25 (50)	10 (20)	40	168 (336)	6.7	16.8
Chlorosilane	25 (50)	10 (19)	40	77 (154)	3.1	7.7
Mercaptosilane	25 (50)	5 (10)	20	123 (245)	4.9	24.6
Glycidoxysilane	25 (50)	6 (12)	24	79 (158)	3.2	13.2
Slate	25	23	92	693	27.7	30.1

[a] The ratio of (attached mussels)/(total mussels).
[b] The number of byssuses of mussels attached on the solid surfaces.
[c] The numbers in parenthesus are the total numbers of mussels and byssuses.

coupled surfaces the Cl-coupled glass exhibited the strongest antifouling effect, showing only 77 feet on it. Thus, the combination between the surface modification and the enzymic kinetics will be promising for the preparation of ecologically clean antifouling materials (Ogawa and Yamamoto, 1995a, b).

Finally, in our ongoing study, the polymer and surface chemical properties of synthetic mussel and related adhesive proteins and their practical applications as bioadhesive formulations and as antifouling materials, will be explored under aqueous conditions in order to increase our knowledge of marine adhesive proteins.

Epilogue

Natural materials are being increasingly regarded by biologists and material scientists as masterpieces of design, economy, function, and ecology. We do not yet fully understand all the characteristics of the marine adhesive proteins. Numerous studies are planned or in progress to further understand the basic science of marine adhesive proteins. Some groups of adhesive researchers recognize that marine adhesive proteins show great potential for new bioadhesive applications. The author hopes that the coming decade will be epoch-making.

Acknowledgements

The author is grateful to the following people for their collaboration: Ayako Nishida, Akira Nagai, Tomoyuki Takimoto, Kengo Ikeda, Sigeru Yamauchi, Sin-ichi Ohara, Hajime Tanisho, Kousaku Ohkawa. Hideki Tatehata, Takahiro Ogawa, Yuuki Hirata, Michiki Asai, Masato Amaike and Takahiro Yamamoto. He also appreciates the permission from CRC Press Inc to use *Tables 1* and *7*.

References

ADAMSON, A.W. (1976). Principles of protein adsorption. In *Physical Chemistry of Surfaces*, pp. 1–80. John Wiley & Sons, New York.

ALLEN, J.A., COOK, M., JACKSON, D.J. PRESTON, S. AND WORTH, E.M. (1976). Observation on the rate of production and mechanical properties of the byssus threads of *Mytilus edulis*. *Journal of Molluscan Studies* **42**, 279–289.

BAIER, R.E. (1982). Comments on cell adhesion to biomaterial surfaces: conflicts and concerns. *Journal of Biomedical Materials Research* **16**, 173–175.

BENEDICT, C. AND PICCIANO, P. (1989). Adhesives from marine mussels. In *Adhesives from Renewable Resources* (R.W. Hemingway and A.H. Conner, Eds), pp. 465–483. American Chemical Society, Washington DC.

BROWN, C.H. (1949). Protein skeletal materials in invertebrates. *Experimental Cell Research, Supplement* **1**, 351–355.

BROWN, C.H. (1952). Some structural proteins of *Mytilus edulis*. *Quarterly Journal of Microscopical Science* **93**, 487–502.

BURZIO, L.O., GUTIERREZ, E., PARDO, J., FUENTE, E., BRITO, M. AND SAEZ, C. (1990). Bioadhesives: A biotechnological opportunity. *Archivos de Biologia y Medicina Experimentales* **23**, 173–178.

COOK, M. (1970). Composition of mussel and barnacle deposits at the attachment interface. In *Adhesion in Biological Systems* (R.S. Manly, Ed.), pp. 139–150. Academic Press, New York.

CRISP, D.J. (1973). Mechanism of adhesion of fouling organisms. In *Proceedings 3rd International Congress on Marine Corrosion and Fouling*, Northwestern University Press, Evanston IL, pp. 691–699.

CRISP, D., WALKER, G., YOUNG, G. AND YULE, A. (1985). Adhesion and substrate choice in mussels and barnacles. *Journal of Colloid and Interface Science* **104**, 40–50.

DANN, J.R. (1970). Forces involved in the adhesive process. *Journal of Colloid and Interface Science* **32**, 302–331.

DICKIE, R.A., LABANA, S.S. AND BAUER, R.S., Eds (1988). In *Cross-Linked Polymers*. American Chemical Society, Washington, DC.

DOHMOTO, N., VENKATESWARAN, K., NOSE, T., TANAKA, H., MIKI, W. AND MIYACHI, S. (1993). Marine mussel-thread-degrading bacteria and partial purification of proteinases responsible for degradation. *Journal of Marine Biotechnology* **1**, 83–87.

FILPULA, D., LEE, S.-M., LINK, R., STRAUSBERG, S. AND STRAUSBERG, R. (1990). Structural and functional repetition in a marine adhesive protein. *Biotechnology Progress* **6**, 171–177.

FOWKES, F.M. (1964). Attractive forces at interfaces. *Industrial and Engineering Chemistry* **56**, 40–52.

FUJISAWA, Y., KUBOTA, I., NOMOTO, K., MINAKATA, H., Y.-KAMATANI, Y., IKEDA, T. AND MUNEOKA, Y. (1993). A Mytilus peptide related to the small cardioactive peptides (SCPs): structure determination and pharmacological characterization. *Comparative Biochemistry and Physiology*, **104C**, 469–475.

HADWIGER, L.A., LOSCHKE, D.C. AND TEASDALE, J.R. (1977). An evaluation of pea histones as disease resistance factors. *Phytopathology* **67**, 755–758.

HATA, T. (1983). Surface energy of polymers. *Kobunshi* **32**, 648–653.

INA, K., TAKAZAWA, R., YAGI, A., ETOH, H. AND SAKATA, K. (1989a). Isothiocyanates as an attaching repellent against the blue mussel *Mytilus edulis*. *Agricultural and Biological Chemistry* **53**, 3323–3325.

INA, K., TAKAZAWA, R., YAGI, A., YAMASHITA, N., ETOH, H. AND SAKATA, K. (1989b). An improved assay method for antifouling substances using the blue mussel, *Mytilus edulis*. *Agricultural and Biological Chemistry* **35**, 3319–3321.

IUPAC-IUB commission on biochemical nomenclature symbols for amino-acid derivatives and peptides recommendations (1972). *Biochemistry* **11**, 1726–1732; *Biopolymers* **11**, 321–327.

KAGIYA, T. (1973). In *Kinetics of Chemical Reactions (in Japanese)*, pp. 166–193. Kagakudojin Press, Kyoto.

KITAZAKI, Y. AND HATA, T. (1972). Extension of the Fowkes equation and the estimation of surface tension of polymer solids. *Journal of the Adhesion Society Japan (in Japanese)*, **8**, 131–141.

KON-YA, K. AND ENDO, M. (1995). Catecholamines as settlement inducers of barnacle larvae. *Journal of Marine Biotechnology*, **2**, 79–81.

LINDNER, E. (1984). The attachment of macrofouling invertebrates. In *Marine biodeterioration: an interdisciplinary study* (J.D. Castlow and R.C. Tipper, Eds), pp. 183–201. Naval Institute Press, Annapolis.

LINDNER, E. AND DOOLEY, C.A. (1973). Chemical bonding in cirriped adhesive. In *Proceedings 3rd International Congress on Marine Corrosion and Fouling*, pp. 653–673. Northwestern University Press, Evanston, IL.

LINDNER, E. AND DOOLEY, C.A. (1976). Studies of the reaction mechanism of the adhesive of barnacles. In *Proceedings 4th International Congress on Marine Corrosion and Fouling*, pp. 333–344. Antibes, France.

MARUMO, K. AND WAITE, J.H. (1986). Optimization of hydroxylation of tyrosine and tyrosine-containing peptides by mushroom tyrosinase. *Biochimica et Biophysica Acta* **872**, 98–103.

MATSUDA, T. AND MOGHADDAM, M. (1993). Molecular design of artificial fibrin glue. *Materials Science & Engineering* **C1**, 37–43.

MATSUO, E.S. AND TANAKA, T. (1992). Patterns in shrinking gels. *Nature* **358**, 482–485.

MAUGH, K.J. (1984). Proteins for the production of bioadhesives. Patent application serial no. 650128.

MORISAWA, S. (1994). Cell signaling mechanisms for sperm motility. *Zoological Science* **11**, 647–662.

NAGAI, A. AND YAMAMOTO, H. (1989). Insolubilizing studies of water-soluble poly(Lys Tyr) by tyrosinase. *Bulletin of the Chemical Society of Japan* **62**, 2410–2412.

NAGY, P.I., PATEL, H.C., DREYER, W. AND HOPFINGER, A.J. (1991). Conformational analysis and aqueous hydration studies of model peptides for the adhesive protein of the mussel, *Mytilus edulis* L. *International Journal of Peptide and Protein Research* **38**, 84–89.

NAKAMURA, T., SHO, S. AND OGURA, Y. (1966). On the purification and properties of mushroom tyrosinase. *Journal of Biochemistry* **59**, 481–486.

NALDRETT, M.J. (1993). The importance of sulphur cross-links and hydrophobic interactions in the polymerization of barnacle cement. *Journal of the Marine Biological Association of the United Kingdom* **73**, 689–702.

OGAWA, T. AND YAMAMOTO, H. (1995a). Preparation of antifouling surfaces toward blue mussels. In *Annual Meeting of 4th Marine Biotechnology*, p. 106B. May 1995 (Tokyo).

OGAWA, T. AND YAMAMOTO, H. (1995b). Effect of the molecular weight of poly(L-lysine) for the wettability characteristics. *Journal of the Adhesion Society of Japan (in Japanese)* **31**, 233–236.

OHARA, S., OHKAWA, K. AND YAMAMOTO, H. (1993). Work of adhesion of the adhesive proteins in aqueous solution on the various solid surfaces. *Journal of the Adhesion Society of Japan (in Japanese)*. **29**, 345–352.

OLIVIERI, M.P., BAIER, R.E. AND LOOMIS, R.E. (1992). Surface properties of mussel adhesive protein component films. *Biomaterials* **13**, 1000–1008.

OLIVIERI, M.P., LOOMIS, R.E., MEYER, A. AND BAIER, R.E. (1990). Surface characterization of mussel adhesive protein films. *Journal of Adhesion Science and Technology* **4**, 197–204.

PARDO, J., GUTIERREZ, E., SAEZ, C., BRITO, M. AND BURZIO, L.O. (1990). Purification of adhesive proteins from mussels. *Protein Expression and Purification* **1**, 147–150.

RUSENKO, K., DONACHY, J. AND WHEELER, A. (1991). Purification and characterization of a shell matrix phosphoprotein from the American oyster. In *Surface reactive peptides and polymers* (C.S. Sikes and A.P. Wheeler, Eds), pp. 107–124. American Chemical Society, Washington DC.

RZEPECKI, L., HANSEN, K. AND WAITE, J.H. (1992). Characterization of a cystine-rich polyphenolic protein family from the blue mussel *Mytilus edulis*. *Biological Bulletin* **183**, 123–137.

RZEPECKI, L., CHIN, S.-S., WAITE, J.H. AND LAVIN, M.F. (1991). Molecular diversity of marine glues: polyphenolic proteins from five mussel species. *Molecular Marine Biology and Biotechnology* **1**, 78–88.

SAEZ, C., PARDO, J., GUTIERREZ, E., BRITO, M. AND BURZIO, L.O. (1991). Immunological studies of the polyphenolic proteins of mussels. *Comparative Biochemistry and Physiology* **98B**, 569–572.

SI, Y. AND OKUNO, M. (1993). Multiple activation of mouse sperm motility. *Molecular Reproduction and Development* **36**, 89–95.

SMEATHERS, J.E. AND VINCENT, J.F.V. (1979). Mechanical properties of mussel byssus threads. *Journal of Molluscan Studies* **45**, 219–230.

STRAUSBERG, R. AND LINK, R. (1990). Protein-based medical adhesives. *Trends in Biotechnology* **8**, 53–57.

STRAUSBERG, R., ANDERSON, D., FILPULA, D., FINKELMAN, M., LINK, R., MCCANDLISS, R., ORNDORFF, S., STRAUSBERG, S. AND WEI, T. (1989). Development of a microbial system for production of mussel adhesive protein. In *Adhesives from renewable resources* (R.W. Hemingway and A.H. Conner, Eds), pp. 453–464. American Chemical Society, Washington, DC.

TAKAZAWA, R., ETOH, H., YAGI, A., SAKATA, K. AND INA, K. (1990). Nonylphenols as promising antifouling agents found by a simple bioassay method using the blue mussel, *Mytilus edulis*. *Agricultural and Biological Chemistry* **54**, 1607–1610.

TAKAZAWA, R., OGURA, C., WATANABE, N., ETOH, H., SAKATA, K. AND INA, K. (1992). An efficient laboratory matching and screening method for antifouling substances using the blue mussel, *Mytilus edulis*, and an antimicrobial assay. *Bioscience, Biotechnology, and Biochemistry* **56**, 1208–1211.

TAMARIN, A., LEWIS, P. AND ASKEY, J. (1976). The structure and formation of the byssus attachment plaque in *Mytilus*. *Journal of Morphology* **149**, 199–220.

TATEHATA, H. AND YAMAMOTO, H. (1993). Synthesis of two mussel adhesive proteins with deca-repeating peptide sequences and their oxidative properties. In *274h Annual Meeting of Union of Chemistry-Related Societies in Chubu Area, Japan*, p. 245.

TEGTMEYER, K. AND RITTSCHOF, D. (1989). Synthetic peptide analogs to barnacle settlement pheromone. *Peptides* **9**, 1403–1406.

WAITE, J.H. (1983). Evidence for a repeating 3,4-dihydroxyphenyl-alanine- and hydroxyproline-containing decapeptide in the adhesive protein of the mussel,*Mytilus edulis* L. *Journal of Biological Chemistry* **258**, 2911–2915.

WAITE, J.H. (1985). Catechol oxidase in the byssus of the common mussel *Mytilus edulis*. *Journal of the Marine Biological Association of the United Kingdom* **65**, 359–371.

WAITE, J.H. (1986). Decapeptides produced from bioadhesive polyphenolic proteins. US Patent 4585585.

WAITE, J.H. (1987). Nature's underwater adhesive specialist. *International Journal of Adhesion and Adhesives* **7**, 9–14.

WAITE, J.H. (1990). The phylogeny and chemical diversity of quinone-tanned glue and varnishes. *Comparative Biochemistry and Physiology*, **97B**, 19–29.

WAITE, J.H. (1992a). The Dopa ephemera: a recurrent motif in invertebrates. *Biological Bulletin* **183**, 178–184.

WAITE, J.H. (1992b). The formation of mussel byssus: anatomy of a natural manufacturing process. In *Results and problems in cell differentiation 19, Biopolymers Chapter 2* (S.T. Case, Ed.), pp. 27–54. Springer, Berlin.

WAITE, J.H. AND ANDERSON, S.O. (1978). 3,4-Dihydroxyphenylalanine in an insoluble shell protein of *Mytilus edulis*. *Biochimica et Biophysica Acta* **541**, 107–114.

WAITE, J.H. AND TANZER, M.L. (1980). The adhesive of *Mytilus* byssus: a protein containing L-Dopa. *Biochemical and Biophysical Research Communications* **96**, 1554–1561.

WAITE, J.H. AND TANZER, M.L. (1981). Specific colorimetric detection of *o*-diphenols and 3,4-dihydroxyphenylalanine-containing peptides. *Analytical Biochemistry* **111**, 131–136.

WAITE, J.H., HOUSELEY, T.J. AND TANZER, M.L. (1985). Peptide repeats in a mussel glue protein: theme and variations. *Biochemistry* **24**, 5010–5014.

WALKER, G. (1972). The biochemical composition of the cement of two barnacle species, *Balanus hameri* and *Balanus crenatus*. *Journal of the marine biological Association United Kingdom* **52**, 429–435.

WILLIAMS, T., MARUMO, K., WAITE, J.H. AND HENKENS, R.W. (1989). Marine glue protein has an open conformation. *Archives of Biochemistry and Biophysics* **269**, 415–422.

YAMAMOTO, H. (1987a). Synthesis and adhesive studies of marine polypeptides. *Journal of the Chemical Society, Perkin Transactions* **1**, 613–618.

YAMAMOTO, H. (1987b). Adhesive studies of synthetic polypeptides: a model for marine adhesive proteins. *Journal of Adhesion Science and Technology* **1**, 177–183.

YAMAMOTO, H. (1994). Adhesive proteins of marine animals. *Fisheries Research (in Japanese)* **13**, 66–74.

YAMAMOTO, H. (1995). Synthetic marine adhesive proteins. In *The Polymeric Materials Encyclopedia* (J.C. Salamone, Ed.), CRC Press, Florida.

YAMAMOTO, H. AND AMAIKE, M. (1995). Biodegradable characteristics of cross-linked biopolymer hydrogels. *Proceedings of 4th Japan International Society for the Advancement of Material & Process Engineering*, 589–594.

YAMAMOTO, H. AND HAYAKAWA, T. (1976). Sense of helix of poly-0,0'-dicarbobenzoxy L-DOPA. *Macromolecules* **9**, 532–534.

YAMAMOTO, H. AND HAYAKAWA, T. (1977). Synthesis and conformational study of poly(L-β-3,4-dihydroxyphenyl-α-alanine). *Polymer* **18**, 979–983.

YAMAMOTO, H. AND HAYAKAWA, T. (1982). Synthesis of sequential polypeptide containing L-β-3,4-dihydroxyphenyl-α-alanine (DOPA) and L-lysine. *Biopolymers* **21**, 1137–1151.

YAMAMOTO, H. AND HIRATA, Y. (1995a). Gel properties as a hydrogel of crosslinked poly(L-ornithine) using organic crosslinking agents. *Polymer Gels and Networks* **3**, 71–84.

YAMAMOTO, H. AND HIRATA, Y. (1995b). Biodegradation of the crosslinked cationic polyamino acid hydrogels by proteolytic enzymes. *Macromolecules* **28**, 6701-6704.

YAMAMOTO, H. AND NAGAI, A. (1992). Polypeptide models of the arthropodin protein of the

barnacle *Balanus balanoides*. *Marine Chemistry* **37**, 131–143.

YAMAMOTO, H. AND OHKAWA, K. (1993). Synthesis of adhesive protein from the vitellaria of the liver fluke *Fasciola hepatica*. *Amino Acids* **5**, 71–75.

YAMAMOTO, H. AND TAKIMOTO, T. (1991). Synthesis and conformational study of cuticle collagen models and application as a bioadhesive. *Journal of Materials Chemistry* **1**, 947–954.

YAMAMOTO, H. AND TANISHO, H. (1993). Gel formation and its properties as hydrogel of crosslinked lysine polypeptides using organic crosslinking agents. *Materials Science and Engineering* **1**, 45–51.

YAMAMOTO, H. AND TATEHATA, H. (1995). Oxidative reaction mechanism by the use of tyrosinase toward synthetic mytilid bivalves marine adhesive protein precursors. *Journal of Marine Biotechnology* **2**, 95–100.

YAMAMOTO, H., IKEDA, K. AND OHKAWA, K. (1993). Study on the attachment and the adhesive proteins of the pearl oyster *Pinctada fucata*. *Marine Fouling (in Japanese)* **10**, 1–10.

YAMAMOTO, H., OGAWA, T. AND NISHIDA, A. (1995). Molecular weight dependence of wettability and molecular adsorption of poly(L-lysine) at the air-water interface. *Journal of Colloid and Interface Science* **176**, 105–110.

YAMAMOTO, H., OGAWA, T. AND OHKAWA, K. (1995). Wettability and adhesion of synthetic marine adhesive proteins and related model compounds. *Journal of Colloid and Interface Science* **176**, 111–116.

YAMAMOTO, H., YAMAUCHI, S. AND OHARA, S. (1993). Synthesis and adhesive studies of marine adhesive proteins of the Chilean mussel *Aulacomya ater*. *Biomimetics* **1**, 219–238.

YAMAMOTO, H., TANISHO, H., OHARA, S. AND NISHIDA, A. (1992). Crosslinking and gel formation of water-soluble lysine polypeptides. An insolubilization model reaction for adhesive proteins. *International Journal of Biological Macromolecules* **14**, 66–72.

YAMAMOTO, H., OHARA, S., TANISHO, H., OHKAWA, K. AND NISHIDA, A. (1993). Work of adhesion of synthetic polypeptides containing L-lysine. *Journal of Colloid and Interface Science* **156**, 515–517.

YAMAMOTO, H., KUNO, S., NAGAI, A., NISHIDA, A., YAMAUCHI, S. AND IKEDA, K. (1990). Insolubilizing and adhesive studies of water-soluble synthetic model proteins. *International Journal of Biological Macromolecules* **12**, 305–310.

YAMAMOTO, H., OGAWA, T., OHARA, S., NISHIDA, A., HIRATA, Y., TATEHATA, H. AND OHKAWA, K. (1994). Adhesion and wettability of marine adhesive proteins in aqueous systems. In *International Adhesion Symposium in Japan, Abstract Book*, pp. 93–94, Yokohama.

YAMASHITA, N., SAKATA, K., INA, H. AND INA, K. (1989a). Isolation of nicotinamide from *Mallotus* leaves as an attaching repellent against the blue mussel, *Mytilus edulis*. *Agricultural and Biological Chemistry* **53**, 3351–3352.

YAMASHITA, N., ETOH, H., SAKATA, K., INA, H. AND INA, K. (1989b). New acylated rhaponticin isolated from Eucalyptus rubida as a repellent against the blue mussel *Mytilus edulis*. *Agricultural and Biological Chemistry* **53**, 2827–2829.

YAMASHITA, N., ETOH, H., SAKATA, K., YAGI, A., INA, H. AND INA, K. (1989c). An acylated kaempferol glucoside isolated from *Quercus dentata* as a repellent against the blue mussel *Mytilus edulis*. *Agricultural and Biological Chemistry* **53**, 1383–1385.

YOUNG, G.A. AND CRISP, D.J. (1982). Marine animals and adhesion. In *Adhesion 6* (K.W. Allen, Ed.), pp. 19–39. Applied Science Publishers, London.

ZISMAN, W.A. (1964). In *Contact Angle, Wettability, and Adhesion* (F.M. Fowkes, Ed.), pp. 1–51. American Chemical Society, Washington, DC.