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Bioadhesives: A biotechnological opportunity

Bioadhesivos: Una oportunidad biotecnológica

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Marine mussels secrete the byssus in order to attach to solid surfaces and to survive under the turbulent effects of waves. The adhesive responsable for this attachment is the polyphenolic protein secreted by the phenol gland in the foot of the animal. To purify this adhesive protein from the chilean mussel *Mylilus chilensis,* a modification of previous procedures has been developed. Accordingly, the protein is differentially precipitated with acetone in the presence of 0.25 N HCl. The purified protein is rich in the amino acids lysine, 3,4-dihydroxyphenylalanine, serine, threonine, proline and hydroxyproline. The protein exhibited strong adhesion to glass and other solid supports. Moreover, it has been found that the adhesive protein can mediate the immobilization of β-galactosidase to glass. About 75% of the enzyme activity was immobilized under the experimental conditions described. This is the first study reporting the use of the polyphenolic protein to immobilize enzymes.

In the ocean, adhesion has evolved as a necessary strategy to resist the impact of waves and the bouyant effect of water (Young and Crisp, 1982). Sessile invertebrates such as mussels, oysters and barnacles represent some of the adhesive strategies practiced by some species. Thus, the mussels attach themselves to solid surfaces through a complex array of threads knows as byssus, which allow them to survive in turbulent aquatic environments. In exchange, they can profit directly from the abundance of nutrients and the rate of gas exchange. The byssus is connected to the animal by a rootlike process and to the solid surface by the adhesive plaques (Tamarin *et al.,* 1976; Young and Crisp, 1982). The plaque contains an adhesive protein knowns as polyphenolic protein which is synthesized and secreted by the phenol gland localized in the foot of the animal. The adhesion mediated by the polyphenolic protein is attracting much attention since, unlike most of the synthetic adhesives, it resists the presence of water and it is quite stable to the marine environment. Moreover, the polyphenolic protein exhibits strong adhesion to a variety of surfaces such as glass, plastics, slate and metals (Waite, 1987). Although the reason for this strong adhesion and resistance to water remains unknown, there is compiling information that might be related to the unusual chemical composition of the polyphenolic protein (Waite, 1983a). This is a basic protein with a molecular weight of about 120,000. About 90% of the amino acid residues in the protein of *Mytilus edulis* are lysine, proline, alanine, serine, threonine and tyrosine (Waite, 1983b). Moreover, and perhaps one of the most interesting characteristics of these adhesive proteins is the presence of 3,4-dihydroxyphenylalanine (Dopa) and hydroxyproline in their structure. These residues seem to arise from post-synthetic modifcation of the primary translation product (Waite, 1983b). Evidences for repetitive sequence in the polyphenolic protein was obtained by trypsin digestion. Thus, the protein from *M. edulis* contains about 75 closely related peptides, which are related by the consensus sequence: Ala-Lys-Pro-Ser-Tyr-Pro-Pro-Thr-Tyr-Lys (Waite *et al.,* 1985). In this sequence the tyrosine at positions 5 and 9 are found as Dopa, while the proline residue are found as 3- or 4-hydroxyproline (Waite *et al.,* 1985; Waite, 1986).

This report shows some studies on the polyphenolic protein isolated from the

chilean mussel *Mytilus chilensis*. The protein has been purified and the amino acid composition is similar to the polyphenolic protein reported previously (Waite *et al.*, 1985; Waite, 1986). Furthermore, we have found that the polyphenolic protein from *M. chilensis* is able to mediate the immobilization of β-galactosidase (E.C. 3.2.1.23) to glass.

MATERIALS AND METHODS

Purification of the polyphenolic protein

Mytilus chilensis were obtained from local fishermen out of the coast of Valdivia. The fresh animals were transferred to the laboratory and opened as usual. The foot was excised at the base and frozen in liquid nitrogen. The purification of the polyphenolic protein was carried out according to the procedure described by Waite (1983b) with some modifications. Briefly, 10 g of frozen feet were homogenized in a Waring blender with 100 ml of a medium containing 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 5 mM EGTA, 25 mM EDTA, 1 mM KCN, 1 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide and 20 μg per ml of soybean trypsin inhibitor. The homogenization was carried at 4°C for 5 min at full speed and under a nitrogen atmosphere. The homogenate was centrifuged at 5,000 x g for 10 min and the sediment was rehomogenized with 100 ml of 0.9 N acetic acid solution containing 0.5 mM phenylmethylsulfonyl fluoride and 15 mM 2-mercaptoethanol. This homogenate was centrifuged at 10,000 x g for 15 min at 4°C, and the supernatant was dialyzed against 6,000 ml of cold distilled water and then for 24 h against 6,000 ml of 0.1 M sodium borate pH 8.5 at 4°C. The insoluble proteins were removed by centrifugation at 5,000 x g for 10 min, and the supernatant was dialyzed overnight at 4°C against 6,000 ml of 0.9 N acetic acid. The acidic solution of the polyphenolic protein, in a final volume of about 100 ml, was precipitated with two volumes of acetone in the presence of 0.25 N HCl, 0.1% Triton X-100 and 25 mM 2-mercaptoethanol. The mixture was left overnight at −20°C and the precipitated polyphenolic protein was collected by centrifugation at 5,000 x g for 10 min at 4°C. The final preparation of the protein was dissolved in 2 to 5 ml of 0.9 N acetic acid, and dialyzed against 0.9 N acetic acid for 6 h at 4°C. The final solution of the protein was stored at 4°C.

Protein was determined by the method of Hartree (1972) using bovine serum albumin as standard. Dopa was determined by the procedure described by Waite and Benedict (1984).

Gel electrophoresis

Slab (15 x 15 x 0.15 cm) polyacrylamide gel (10%) electrophoresis of the polyphenolic protein was carried out in 0.9 N acetic acid and 2.5 M urea according to the procedure described by Panyim and Chalkley (1969). After electrophoresis the gel was stained with Coomassie Blue as described previously (Vera *et al.*, 1984).

Amino acid analysis

About 200 μg of th epolyphenolic protein was transferred to a hydrolysis tube and lyophilized. To each tube, 0.5 ml of 6 N HCl containing 5% of phenol was added and the tubes were sealed under vaccum as described before (Vera *et al.*, 1984). The tubes were incubated at 110°C for 20, 40 or 60 h and the hydrolyzate was flash evaporated and dissolved in a small volume of 0.2 N sodium citrate at pH 2.2. The amino acid analysis was carried out according to Spackman *et al.* (1958) in a Beackmann Amino Acid Analyzer Model 120C. The values of serine, threonine, valine and isoleucine were corrected as described before (Vera *et al.*, 1984). The amount of Dopa was also corrected to zero time of hydrolysis.

Immobilization of β-galactosidasa

β-galactosidase from *Kluyveromyces lactis* with a specific activity of 3,000 units per gram was obtained from Gist-Brocades (LX 3000 Maxilat, Industrial Product Division, Holland). Appropriated dilutions of the enzyme were prepared in a solution containing 0.2 M potassium phosphate (pH 6.5), 0.15 mM MnC12 and 0.015 mM EDTA (Wallenfels, 1962).

The immobilization procedure was carried out in disposable borosilicate tubes (Corning, 13 x 100 mm). To each tube, 0.5 ml containing 100 μg of polyphenolic protein in 50 mM sodium phosphate buffer adjusted to pH 7.5 and 5 mM DTT, was added and incubated for 30 min at room temperature. To eliminate the unbound proteins the tubes were washed two times with 3 ml of 5 mM DTT at pH 7.0. The tubes were left upside down to eliminate the excess of buffer and 0.5 ml of the enzime dilution in the enzyme buffer was added and incubated for 60 min at room temperature. The tubes were washed with the enzyme buffer three times as described before and the immobilized enzyme activity was assayed. The assay was carried out at 35°C in a final volume of 1.2 ml of 10 mM o-nitrophenyl-β-D-galactopyranoside (ONPG) in the enzyme buffer (Wallenfeld, 1962). After incubation for 10 min, 0.4 ml of 7.5% sodium carbonate was added and the amount of o-nitrophenol produced was determined by measuring the absorbance at 420 nm. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μmol of ONPG per min at 35°C.

RESULTS

The purification of the polyphenolic protein from feet of *Mytilus chilensis* is illustrated in Table I. As reported before (Waite, 1983b) this protein was also insoluble in high salt buffer and soluble in diluted acetic acid. Also, the protein remained in solution after dialysis against 0.1 M sodium borate (pH 8.5). The precipitation of the protein from the acetic acid solution was quite efficient. The final specific activity of the purified protein was on the average about 0.18 mg of Dopa per mg of protein, which is higher than the specific activity reported by Waite (1983b) for the protein of *M. edulis*. Furthermore, the final yield of Dopa was similar or better than the one reported before (Waite, 1983b).

TABLE I

Purification of the polyphenolic protein of *Mytilus chilensis*

Step	Total Protein (mg)	Total Dopa (mg)	Dopa/ Protein	Yield (%)
Acetic acid extract	287.0	6.3	0.022	100
Dialysis against borate	70.4	3.7	0.052	59
Precipitation with acetone	14.4	2.6	0.181	41

This purification was carried out with 15 g of frozen tissue which is equivalent to 98 feet of *M. chilensis*.

Polyacrylamide gel electrophoresis of two different preparations of the polyphenolic protein from *M. chilensis* is shown if Fig. 1. A single band of protein was always observed,in contrast with the doublet reported previously for the polyphenolic protein of *M. edulis* (Wayte, 1983b; Waite *et al.*, 1985).

Fig. 1: Polyacrylamide gel electrophoresis of the polyphenolic protein of *M. chilensis*. The electrophoresis was carried out in acid-urea gels as described in Methods. The stained profile with Coonassie Blue of two different preparation of polyphenolic protein is shown (30 μg of protein in each case).

The amino acid composition of the purified polyphenolic protein is shown in Table II. Like the protein isolated from *M. edulis* (Waite *et al.*, 1985) and from *Mytilus californianus* (Waite, 1986b), the polyphenolic protein from *M. chilensis* also contains high proportion of proline, 3- and 4-hydroxyproline, serine, threonine, Dopa and lysine.

Previous studies have shown that the polyphenolic protein from *M. chilensis* bounds quite strongly to glass and plastic (Burzio *et al.*, 1989). Also, we have found that this protein is able to mediate the adsorption of other proteins to the above solid supports (data not shown). Consequently, it was reasonable to ask if the polyphenolic protein did have the capacity to mediate the immobilization of an enzyme to a solid support such as glass. Fig. 2 shows the activity (in nmol of ONP produced per min or mU of enzyme activity) of β-galactosidase immobilized to the wall of a disposable tube. In this example the tubes were precoated with 100 μg of the polyphenolic protein isolated from *M. chilensis* as described in Methods. Under these experimental conditions, the enzyme activity immobilized to the glass tube wall remains linear up to 600 units. Moreover, these results also show that about 75% of the amount of enzyme units incubated in the tube precoated with the adhesive protein, was immobilized.

TABLE II

Amino acid composition of the polyphenolic protein of *Mytilus chilensis*

Amino acid	*M. chilensis*	*M. edulis* (a)	*M. californianus* (b)
		(Residues/1,000)	
3-hydroxyproline	64	30	30
4-hydroxyproline	93	118	137
Aspartate	32	22	25
Threonine	96	113	105
Serine	106	102	107
Glutamate	15	9	10
Proline	75	81	48
Glycine	32	18	37
Alanine	74	81	37
Half-cystine	0	0	0
Valine	16	10	11
Methionine	3	0	0
Isoleucine	15	7	37
Leucine	16	11	9
Dopa	140	138	126
Tyrosine	47	40	74
Phenylalanine	3	1	6
Histidine	2	7	8
Lysine	158	210	180
Arginine	13	2	13

The amino acid composition of *M. edulis* and *M. californianus* were reportes previously (Waite *et al.*, 1985; Waite, 1986b).

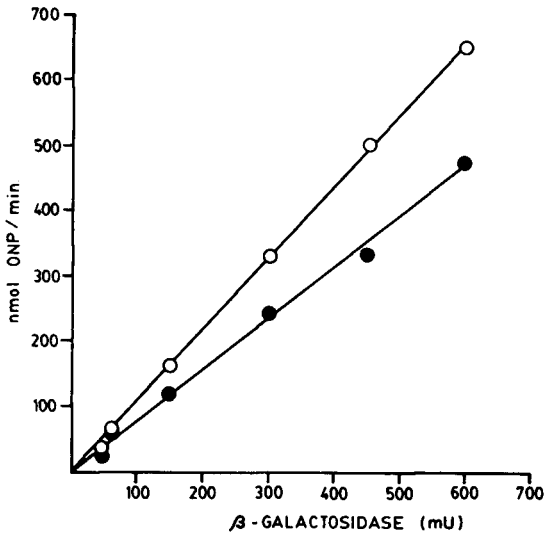

Fig. 2: Activity of β-galactosidase immobilized to glass. The tubes were incubated with 100 μg of polyphenolic protein from *M. chilensis* as described in Methods. The precoated tubes were incubated with the indicated amount of enzyme in mU for 60 min at room temperature and then washed to eliminate the unbound enzyme. The activity was assayed with 10 mM ONPG as indicated in Methods. The activity (nmol of o-nitrophenyl produced per min) of the soluble enzyme (○) as well as the immobilized enzyme (●) is shown.

DISCUSSION

The major modification that we have introduced in the procedure described by Waite (1983b) to purify the polyphenolic was the differential precipitation of the protein with acetone and 0.25 N HCl. Similar results have been achieved with ethanol, and also 0.2 to 0.4 N H_2SO_4 can be used instead of HCl. Under these conditions a final protein with a specific activity of 0.16 to 0.18 mg of Dopa per mg of protein was obtained, which is equal to or higher than the specific activity reported by Waite (1983b). Moreover, the present modification avoided the use of several chromatography steps (Waite, 1983b; Waite *et al.*, 1985) which are time consuming and produced a low recovery of the protein. Indeed, our usual yield was about 40%, higher than the yield of 28% reported by Waite (1983b). Also it is interesting to notice that the content of Dopa or polyphenolic protein in *M. chilensis* was higher than in *M. edulis*. From 450 phenol glands or feet of *M. edulis* about

10 mg of purified polyphenolic protein was obtained, or 25 μg per mussel (Waite, 1983b). In contrast, we obtained about 150 μg of polyphenolic protein per mussel. The reason for the different content of the adhesive protein between these two species is unknown at present time.

As shown in this study, the protein from *M. chilensis* is similar to the adhesive protein from *M. edulis* in regard with size and amino acid composition. An apparent molecular weight of 130,000 (data not shown) was determined for the polyphenolic protein of *M. chilensis* by using a 0.9 N acetic acid polyacrylamide gel (Panyim and Chalkley, 1969) plus 0.1% of cetyltrimethyl-ammonium bromide (Waite, 1983b). This apparent molecular weight is similar to the one reported for the protein of *M. edulis* (Waite, 1983b). However, it differs quite markedly from the molecular weight of 85,000 reported for the polyphenolic protein of *M. californianus* (Waite, 1986b). The amino acid composition is also similar to *M. edulis* (Waite *et al.*, 1985). In regard with the higher content of arginine, the protein from the chilean species is more related to the composition of the polyphenolic protein of *M. californianus* (Wayte, 1986b).

In the literature several strategies to immobilize enzymes have been described. They involve adsorption of the enzymes to solid supports (Messing, 1976), entrapping in gel matrixes (O'Driscoll, 1976), aggregation of enzymes (Broun, 1976) and covalent coupling to different solid supports (Srere and Uyeda, 1976). In this regard, most of these different procedures have been used for the immobilization of β-galactosidase (Greenberg and Mahoney, 1981); Gekas and López-Leiva, 1985). In the present study we have shown for the first time the effectiveness of using the polyphenolic protein from mussel to immobilize β-galactosidase to a glass support. Indeed, about 75% of the total enzyme activity incubated in a glass tube previously coated with the adhesive protein, was immobilized (see Fig. 2). This level of immobilization of β-galactosidase is comparable to or even better than those reported previously using other strategies (Pitcher *et al.*, 1976; Greenberg and Mahoney, 1981). Moreover, we have found that the polyphenolic protein isolated from another chilean mussel (*Choromytilus chorus*) was even more efficient to immobilize the same enzyme to glass support (data not shown). The results presented in this study open new possibilities for using these adhesive proteins, as well as new means for the immobilization of enzymes for the design of bioreactors and enzyme electrodes or biosensors (Guilbault, 1976).

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