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Isolation and characterisation of a fourth hemagglutinin from the red alga, *Gracilaria verrucosa*, from Japan

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Abstract

Isolation and characterisation of marine algal hemagglutinins or lectins are essential for their potential industrial application as specific carbohydrate affinity ligands. The phosphate buffer extract of the red alga, *Gracilaria verrucosa* (Huds.) Papenfuss (Gigartinales, Rhodophyta) from Japan is known to contain three different hemagglutinins. The extract of the alga collected in March 1993 from Kagawa Prefecture, Japan, was purified by ammonium sulphate fractionation, ion exchange and gel filtration chromatography. Using gel filtration, two peaks were obtained (hereafter Peak 1 and Peak 2) which differed in molecular size and hemagglutinating activity against horse erythrocytes. Peak 1 corresponded to the known high molecular weight hemagglutinin, H-GVH. Peak 2 contained large amounts of hexose and sulphate along with a small amount of protein. It had a low molecular weight (gel filtration) similar to that of two of the previously reported *G.verrucosa* hemagglutinins but differed in its electrophoretic behaviour. Peak 2 is therefore a fourth hemagglutinin. Its activity was not inhibited by any of the monosaccharides tested but by the complex glycoproteins such as asialofetuin and fetuin. It had no divalent cation requirement for hemagglutination. The properties of this novel hemagglutinin could prove useful in industrial applications.

List of abbreviations: H-GVH, high molecular weight *Gracilaria verrucosa* hemagglutinin; L-GVH, low molecular weight *G. verrucosa* hemagglutinin; BSA, bovine serum albumin; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Introduction

Hemagglutinins are natural bioactive products, which are the focus of research aimed at developing new industrial tools using their specific carbohydrate binding abilities. In the course of such studies, we have already purified a chick 14 kDa lectin and applied it as an affinity ligand (Kakita et al., 1991). Unfortunately, this lectin had the triple disadvantage of being thermolabile, requiring a reductant such as 2-mercaptoethanol and having low carbohydrate specificity. Therefore, the search for other hemagglutinins with higher stability and carbohydrate specificities, requiring no additives, and available in a steady quality and quantity was undertaken. As marine organisms contain many different bioactive substances to those of terrestrial ones, the marine algae were surveyed for hemagglutinins as affinity ligands.

The phosphate buffer extract of the red seaweed, *Gracilaria verrucosa* has previously been reported to agglutinate rabbit erythrocytes (Hori et al., 1981, 1988a; Chiles & Bird, 1989) and three hemagglutinins have been purified. The first (named GVA-1) is a protein or glycoprotein with low carbohydrate content

(Shiomi et al., 1981), the second is a 49 kDa proteoglycan (Kanoh et al., 1992) and the third (named H-GVH) is a high molecular weight sulphated polysaccharide (Kakita et al., 1997). The first and second lectins have low molecular weights, oligomeric structures, and no disulphide bond. GVA-1 (Mr 41 000) is a tetramer constituted of two subunits (Mr 10 500 and Mr 12 000). The second hemagglutinin is a dimeric proteoglycan (Mr 49 000) consisting of two protomers (Mr 27 000 and Mr 23 000). The hemagglutinating activities of these two lectins disappeared or were reduced after treatment at 100 °C for 30 min. By contrast, the molecular weight of H-GVH is approximately Mr 480 000 and its hemagglutinating activity is heat-stable but periodate-sensitive.

Recently, we found a new low molecular weight hemagglutinin in the phosphate buffer extract of *G. verrucosa* and named it L-GVH. In this paper, we describe its isolation and properties.

Materials and methods

Samples of the red alga, *Gracilaria verrucosa*, were collected from Aji Peninsula, Kagawa Prefecture, Japan, in March 1993. After collection, the algae were washed with water, freeze-dried, and stored at – 20 °C until use. Although the Japanese '*G. verrucosa*' was previously identified as *G. verrucosa* (Huds.) Papenfuss, several researchers objected to the classification and pointed out the necessity for re-examination (Yoshida et al., 1995). For the purpose of this paper, we refer to it as *G. verrucosa*.

Samples of animal blood were obtained from Cosmo-Bio (Tokyo, Japan). Other reagents were of analytical grade.

Purification of the hemagglutinin

The purification experiments were carried out at 4 °C. The dried alga (200 g) was homogenised with 2 L of buffer A [0.02 mol L^{-1} sodium phosphate buffer (pH 7.0), 0.15 mol L^{-1} NaCl] containing 0.005 mol L^{-1} ascorbic acid and 1% polyvinylpyrrolidone. After centrifugation to remove insoluble materials, the fraction precipitated between 35% and 70% saturation with ammonium sulphate was collected. The precipitate was dissolved in buffer A and dialysed against the same buffer. A sample of 62.0 mL was applied to a DEAE-Toyopearl 650M column (Tosoh, Japan, 29 cm × 2.6 cm I.D.) equilibrated with buffer A and eluted by stepwise increase of NaCl concentration up to 0.5 mol L^{-1} in 0.02 mol L^{-1} sodium phosphate buffer (pH 7.0). Flow rate was 2 mL min^{-1}. The eluate was collected in 10 mL fractions, which were examined for agglutination of rabbit erythrocytes. The active fractions were collected, dialysed against buffer A and concentrated to 12.5 mL by ultrafiltration (Grace amicon membrane PM10). A 500-μL volume of the concentrate was applied to a G3000PWxl column (Tosoh, Japan, 300 mm x 7.8 mm I.D.) and eluted with buffer A at a flow rate of 0.5 mL min^{-1} in 0.1 mL fractions. The gel filtration was repeated 24 times to pool sufficient fractions for testing. The active fractions (Fr. 78 and 79) were collected, dialysed against buffer A, and concentrated to 2.2 mL by ultrafiltration. A 100 μL volume of the concentrate was applied to the same column. The eluate was collected in 0.1 mL fractions and, again, this was repeated 20 times. The active fractions were collected, dialysed against buffer A, and concentrated to 0.8 mL by ultrafiltration. Thyroglobulin (Mr 669 000), ferritin (Mr 440 000), BSA (Mr 66 000), and ovalbumin (Mr 43 000) obtained from Pharmacia (Uppsala, Sweden), were used as molecular weight markers for gel filtration.

Determination of the hemagglutinin activity

Hemagglutinating activity was measured according to Hori et al. (1981). One unit of hemagglutinating activity was defined as the reciprocal of the highest dilution of the fraction (50 μL) which gave detectable hemagglutination of a rabbit erythrocyte suspension. Total activity was defined as activity (units) multiplied by the fraction volume (mL). Specific activity was defined as activity (units) divided by the quantity of hexose (mg). Chicken, cow, goose, guinea pig, horse, rabbit, sheep, and rat erythrocytes were used for hemagglutination assays. In erythrocyte specificity experiments, the minimum concentration of hemagglutinin was defined as the lowest hexose concentration (mg mL^{-1}) required to provide hemagglutination.

Sheep erythrocyte suspension treated with pronase (SETP) was prepared and the effects of dialysis, heat, pH, and divalent cations on the activity were measured according to Hori et al. (1986a, 1988a). Monosaccharides, acids, and glycoproteins were obtained from Sigma (St. Louis, MO, U.S.A.). Compounds used for the inhibition assay were: D-glucose, L-fucose, D-mannose, L-ascorbic acid, L-rhamnose, D-arabinose, D-galactose, D-ribose, D-galactosamine,

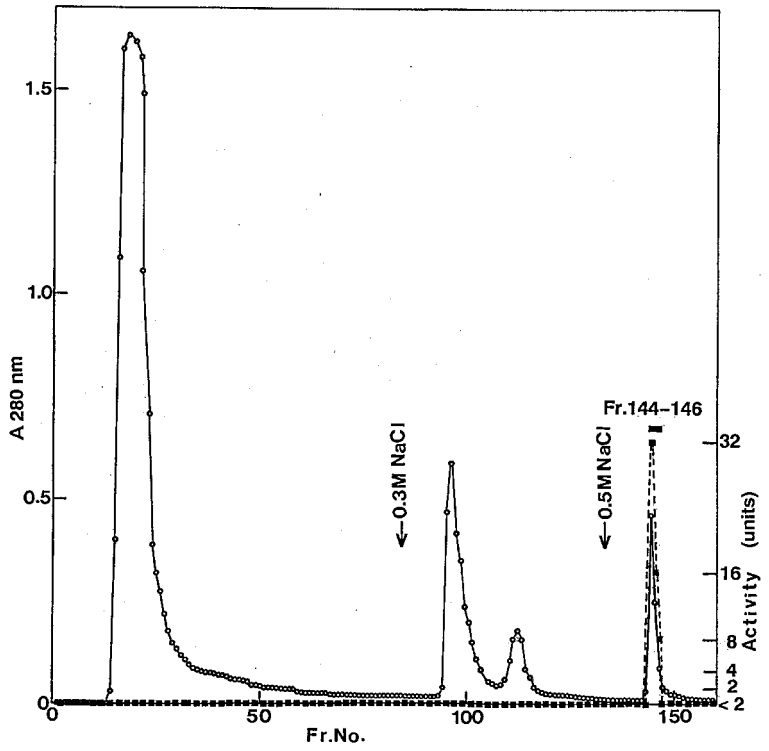

Figure 1. Ion exchange chromatography of the ammonium sulphate fractionated extract on TSKgel DEAE-Toyopearl 650M column. The arrows indicate the positions of stepwise increase of NaCl concentration. Protein elution was monitored by absorbance at 280 nm (○–○). Hemagglutinating activity against rabbit erythrocytes is represented as units (■–■). The fractions (Fr. 144–146) indicated by the solid bar were pooled for further separation.

D-glucosamine, N-acetyl-D-galactosamine, N-acetyl neuraminic acid, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, fetal calf fetuin, fetal calf asialofetuin, bovine asialomucin, bovine mucin, bovine lactoferrin, bovine ribonuclease B, *Saccharomyces cerevisiae* mannan, chicken ovalbumin, chicken egg white trypsin inhibitor, and porcine thyroglobulin. Enzymatic digestions and periodate treatment of L-GVH were carried out according to Lai et al. (1989).

Electrophoresis

SDS-PAGE was carried out by the method of Laemmli (1970). The sample was treated with 1% SDS for 5 min at 100 °C. Staining for protein was performed with a silver staining kit (Daiichi Pure Chemical, Tokyo, Japan) according to Ohsawa & Ebata (1983). The acidic polysaccharide band was stained with Alcian blue (Misevic & Burger, 1986). Acidic polysaccharide density on the stained gel was measured by absorbance at 633 nm using a Shimadzu densitometer. Rabbit myosin (Mr 200 000), *E.coli* β-galactosidase (Mr 116 000), BSA (Mr 66 000), rabbit aldolase (Mr 42 000), bovine carbonic anhydrase (Mr 30 000), and horse myoglobin (Mr 17 000) were obtained from Daiichi Pure Chemical (Tokyo, Japan) and were used as molecular weight markers.

Cellulose acetate membrane electrophoresis (Jookoo, Tokyo, Japan) was carried out according to the method of Wessler (1971). Staining of acidic polysaccharides was performed with 0.5% Alcian blue. Human umbilical cord hyaluronic acid (Seikagaku,Tokyo, Japan) was used as standard for nonsulphated polysaccharide. Shark cartilage chondroitin sulphate C and porcine intestinal mucosa heparin were obtained from Sigma (St.Louis, MO, U.S.A.) and were used as standards for sulphated polysaccharides.

Compositional analysis

Protein was determined according to Lowry et al. (1951) using BSA as standard. Hexose was determined by the phenol-sulphuric acid method using galactose as standard (Dubois et al., 1956). Sulphate

content was determined by the rhodizonate method using sodium sulphate as standard (Terho & Hartiala, 1971). Sample hydrolysis was performed according to Arakawa et al. (1976). The neutral sugar composition of hydrolysates was determined by high performance liquid chromatography (Mikami & Ishida, 1983). Amino sugars were determined using a Shimadzu amino acid analyser equipped with a Shin-pack Amino-Na ion exchange column and an Amino Acid Analysis reagent kit (Shimadzu, Kyoto, Japan).

Results

Hemagglutinins extracted by phosphate buffer from *G.verrucosa* and precipitated by ammonium sulphate were purified by ion exchange chromatography (Figure 1). Gel filtration of the hemagglutinating active fractions (Figure 2A) resulted in two peaks (hereafter Peak 1 and Peak 2) with different molecular weights. Peak 1 (Fr. 68–69) corresponded to the high molecular weight hemagglutinin reported previously as H-GVH by Kakita et al. (1997). The low molecular weight hemagglutinin (L-GVH) was purified from Peak 2 (Fr. 78–79) by re-chromatography on the same column (Figure 2B). The activity recovery of L-GVH using this purification scheme is summarised in Table 1. The specific activity increased to 41.3 units mg^{-1} hexose. The total activity of L-GVH fraction was 0.2% of that of the crude extract and 6.20 mg hexose were recovered. The molecular weight of L-GVH, estimated by gel filtration to be about Mr 51 000 (Figure 2B), is similar to that of GVA-1 (Shiomi et al., 1981) and that of the 49 kDa proteoglycan reported by Kanoh et al. (1992). However, on SDS-PAGE, L-GVH (20 μL, 0.111 mg protein mL^{-1}, 3.87 mg hexose mL^{-1}) migrated as a diffuse band which stained with Alcian blue (Figure 3A) at an equivalent molecular weight of about Mr 71 000 [indicated by 71 kDa on Figure 3B]. No other band was detected by Coomassie brilliant blue or by silver staining (data not shown). This migration behaviour and the Mr value differed from the other two *G. verrucosa* hemagglutinins. On cellulose acetate membrane electrophoresis, L-GVH migrated as one spot different from that of H-GVH (Figure 4).

Chemical analysis showed that 9.0 mg of L-GVH contained 6.20 mg of hexose, 0.177 mg of protein, and 1.56 mg of sulphate. The major sugar component of L-GVH was galactose and the minor components were fucose, glucose, xylose, and glucosamine. The

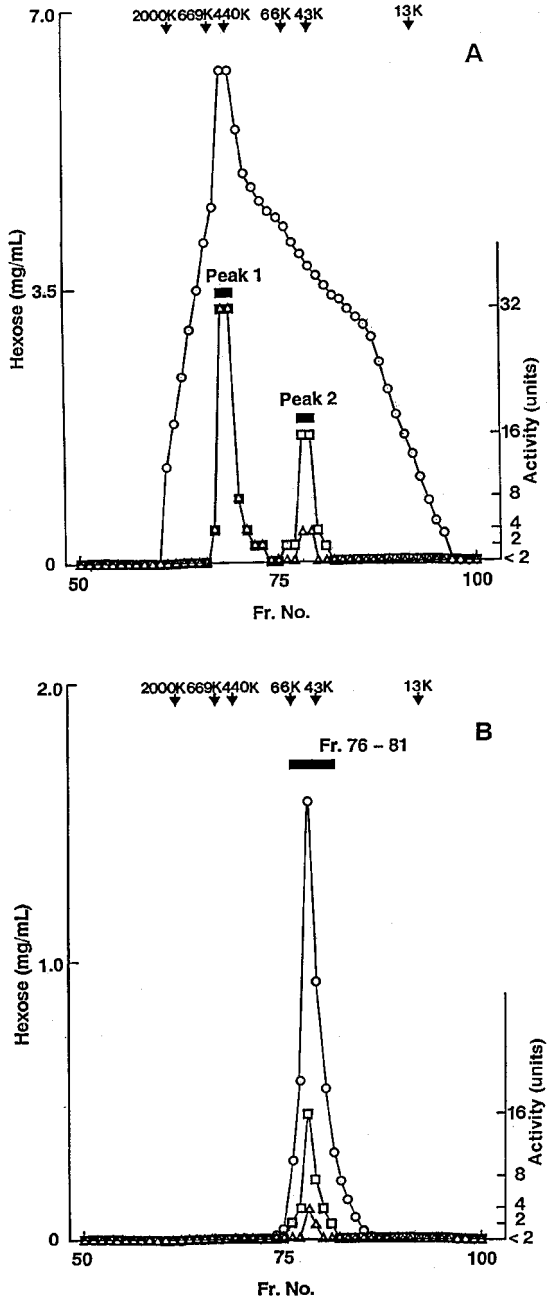

Figure 2. Gel filtration chromatographs of the active fractions on TSK gel G3000PWxl column. (A). Gel filtration chromatography of the active fraction from a TSKgel DEAE-Toyopearl 650M column. Hexose elution was determined by colorimetry (○–○) and the hemagglutinating activities against rabbit erythrocytes (Δ–Δ) and SETP (□–□) are represented as units. Peak 2 (Fr. 78–79) indicated by the solid bar were pooled for re-chromatography. (B). Re-chromatography of the Peak 2 fraction from TSKgel G3000PWxl column. The fractions (Fr. 76–81) indicated by the solid bar were pooled and concentrated. Molecular weight standards were: blue dextran (Mr > 2 000 000); thyroglobulin (Mr 669 000); ferritin (Mr 440 000); BSA (Mr 66 000); ovalbumin (Mr 43 000); ribonuclease A (Mr 13700).

Table 1. Activity recovery of L-GVH during purification (ASppt: ammonium sulphate precipitation; RC: re-chromatography of peak 2 on G3000PWxl)

Fraction	Protein (mg)	Hexose (mg)	Total activity (units)	Specific activity (units mg^{-1})	Activity yield (%)
Crude extract	1802	9182	109900	12.0	100.0
ASppt	303	1707	39680	23.5	36.1
DEAE-Toyopearl	13.8	245	8000	37.5	7.3
G3000PWx1					
Peak 1	0.874	30.1	3072	102.2	2.8
Peak 2	0.623	14.6	352	24.1	0.3
G3000PWx1 (RC)	0.177	6.20	256	41.3	0.2

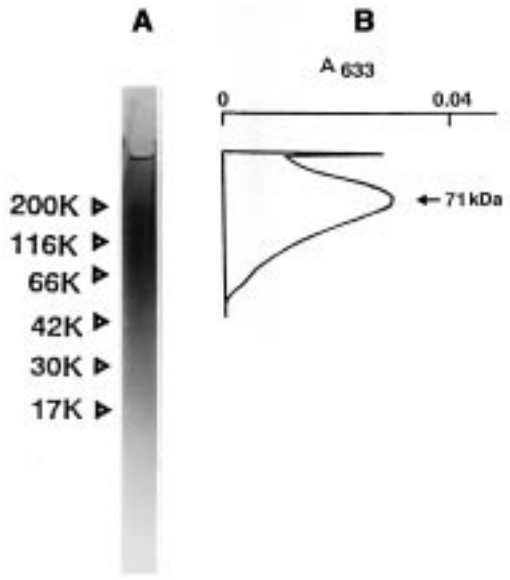

Figure 3. Sodium dodecyl sulphate electrophoresis of L-GVH on a 5–20% linear polyacrylamide gel. (A). Sodium dodecyl sulphate electrophoresis of L-GVH. Alcian blue staining was used. The open arrows indicate the position of the following molecular markers: rabbit muscle myosin (Mr 200 000), *E.coli* β-galactosidase (Mr 116 000), BSA (Mr 66 000), rabbit muscle aldolase (Mr 42 000), bovine erythrocyte carbonic anhydrase (Mr 30 000), and horse muscle myoglobin (Mr 17 000). (B). Densitometry of sodium dodecyl sulphate electrophoresis gel.

galactose content represented 97.8% of the total sugar content.

As shown in Table 2 the erythrocyte specificity of L-GVH was lower than that of H-GVH. Horse and rabbit erythrocytes and SETP were more strongly agglutinated by L-GVH than the other erythrocytes tested. Chicken erythrocytes were not agglutinated by a concentration of 7.74 mg of L-GVH hexose per mL. The erythrocyte specificity of L-GVH was different from those of the other three *G. verrucosa* hemagglutinins reported to date. The divalent cations such as 0.01 mol L^{-1} Ca^{2+}, Mg^{2+}, or Mn^{2+} did not affect agglutination (data not shown). The activity was stable at 100 °C for 30 min and between pH 5–10. It was not affected by chondroitinase ABC or pronase, but disappeared after periodate treatment.

The results of the inhibition of L-GVH activity by monosaccharides and glycoproteins showed that its activity was not inhibited by any of the monosaccharides tested at a concentration of 0.25 mol L^{-1}. Similar phenomena have been described for marine algal hemagglutinins but not from any terrestrial organism (Kamiya et al., 1980; Shiomi et al., 1981; Ferreiros & Criado, 1983; Rogers & Topliss, 1983; Hori et al., 1986a,b,c, 1987, 1988b; Okamoto et al., 1990; Kanoh et al., 1992; Kakita et al., 1997). The results of the inhibition assay also showed that its activity was inhibited by three kinds of complex type glycoproteins. The

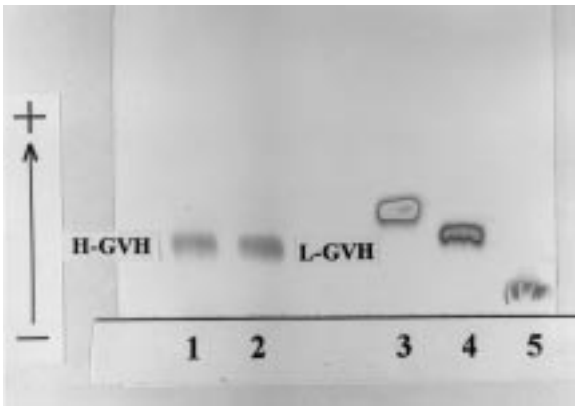

Figure 4. Electrophoresis of L-GVH on cellulose acetate membrane. Applied samples: (1) H-GVH (1 μL, 0.051 mg protein mL^{-1}, 3.89 mg hexose mL^{-1}), (2) L-GVH (1 μL, 0.111 mg protein mL^{-1}, 3.87 mg hexose mL^{-1}), (3) heparin (2 μL, 2 mg mL^{-1}), (4) chondroitin sulphate C (2 μL, 2 mg mL^{-1}), (5) hyaluronic acid (3 μL, 3 mg mL^{-1}). Alcian blue staining was used.

Table 2. Erythrocyte specificities of H-GVH and L-GVH (MC: minimum concentration; SETP: sheep erythrocyte suspension treated with pronase)

Erythrocyte	MC (mg hexose mL^{-1})	
	H-GVH	L-GVH
Chicken	> 7.78	> 7.74
Cow	> 7.78	1.93
Goose	> 7.78	1.93
Guinea pig	3.891	1.93
Horse	> 7.78	0.96
Rabbit	0.243	0.96
Sheep	> 7.78	1.93
Rat	> 7.78	3.87
SETP	0.243	0.242

minimum concentrations of these glycoproteins required to inhibit its activity were: asialofetuin, 2.48 μg mL^{-1}; fetuin, 37.5 μg mL^{-1}; thyroglobulin, 20.1 μg mL^{-1}. Its activity was not inhibited by any other glycoproteins tested at a concentration of 2 mg glycoproteins per mL. Among the glycoproteins tested, asialofetuin was the most effective inhibitor of L-GVH activity.

Discussion

The two low molecular weight *G. verrucosa* hemagglutinins reported previously have been shown to migrate as compact bands in SDS-PAGE analysis (Shiomi et al., 1981; Kanoh et al., 1992). Although the chromatographic molecular weight of L-GVH differed from that of H-GVH, it was similar to those of the two other *G. verrucosa* hemagglutinins, yet it produced a diffuse band in SDS-PAGE analysis (Figure 3A). In addition, L-GVH had a molecular weight of 71 000 by SDS-PAGE, its activity was heat-stable, periodate-sensitive. It was active against sheep erythrocytes but inactive against chicken erythrocytes. From these results, L-GVH represents a fourth hemagglutinin. Takahashi and Katagiri (1987) showed that the hemagglutinating activity of the phosphate buffer extract of *G. verrucosa* changes with seasons. Shiomi et al. (1981) purified GVA-1 from algae harvested in November 1980 from Chiba Prefecture (central east Japan). Kanoh et al. (1992) found the 49 kDa proteoglycan in algae harvested in July–August 1989 from Kagawa Prefecture (Inland Sea in southwest Japan). Kakita et al. (1997) purified H-GVH from algae harvested in June 1992 and L-GVH from algae collected in March 1993 both from Kagawa Prefecture. These findings suggest that the four *G. verrucosa* hemagglutinins differed according to the season of harvest. Thus, the difference between L-GVH and the other three *G. verrucosa* hemagglutinins may reflect seasonal variation. Hori et al. (1993) showed that novel hemagglutinins can be extracted from algae treated with pronase. That also suggests that some marine algae may possess multiple hemagglutinins.

By SDS-PAGE analysis, L-GVH produced a diffuse band, which stained with Alcian blue whereas no other band was detected using other stains. Noro et al. (1983) reported on a sulphated proteolycan that produces a diffuse band on SDS-PAGE and suggested

that its broadness reflected an heterogeneous glycosylation. The migration of L-GVH as one spot on cellulose acetate membrane electrophoresis suggests that the broadness of the diffuse band observed by SDS-PAGE probably arises, not from impurities, but from electrophoretic micro-heterogeneity (Figure 4). We infer that L-GVH is not composed of a single molecular species but a group of micro-heterogeneous molecules having hemagglutinating activity. These molecules would differ in the composition or length of the sulphated polysaccharide chains (e.g. sulphated galactan) linked to a proteoglycan.

Coombe et al. (1987) suggested that marine sponge cell aggregation is caused by ionic and hydrogen interactions between sulphated polysaccharides and cell surface receptors (baseplate), and the specificity of aggregation is determined by the orientation of the sulphate groups on the polysaccharide. Our results suggest that L-GVH is not a lectin but a sulphated polysaccharide hemagglutinin, the sulphated polysaccharide moiety of which seems to be essential for hemagglutination. The chemical analysis showed that L-GVH differs from H-GVH in the molar ratio of sulphate to hexose (L-GVH, 0.46; H-GVH, 0.76). Wessler (1971) showed that the electrophoretic mobility of polysaccharide on a cellulose acetate membrane in 0.1 mol L^{-1} hydrochloric acid is proportional to the sulphate content of the polysaccharide. Because L-GVH differed from H-GVH in the migration distance on the cellulose acetate membrane, it is likely that L-GVH and H-GVH differ in their sulphate content (Figure 4). Although the hemagglutinating activities of L-GVH and H-GVH were similarly heat-stable and periodate-sensitive, the two activities differed in erythrocyte specificities. From these results, we infer that their sulphate moiety is related to their hemagglutinating property. The results from inhibition assays suggest that L-GVH has an affinity to the desialylated oligosaccharide sugar chain of complex type glycoproteins such as asialofetuin. The heat-stability and high carbohydrate specificity will prove useful in applying L-GVH as an analytical and diagnostic tool.

In a preliminary evaluation of L-GVH as an affinity ligand, we found that it met three of the five requirements (high stability, high carbohydrate specificity, and no need for any additive) but ranked only poor to fair on the other two (steady supply and steady quality). From 200 g of dried alga, we purified only 6.20 mg of L-GVH (Table 1). Thus, as for other hemagglutinins obtained from algae, *G. verrucosa* has a low L-GVH content. Furthermore, the seasonal variation of hemagglutinin types from *G. verrucosa* ,discussed above, is another limitation for having a constant quality and quantity of L-GVH.

Thus, and as with other marine algae, *G. verrucosa* is a suitable source of various industrially useful hemagglutinins with some limitations that remain to be solved.

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